STUDIES ON EUKARYOTIC DNA SUPERSTRUCTURE

DISSERTATION

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By

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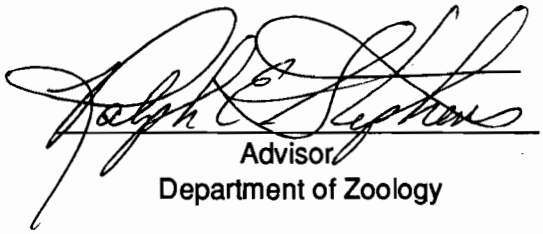

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

PREFACE

This Dissertation is concerned with the problem of DNA superstructure in eukaryotes. Since the term "DNA superstructure can be interpreted in a variety of ways I feel compelled to explain the "rules of the game" relative to the work which is being presented here. The primary sequence of bases in a single DNA strand represents the first level of order. The double helix represents secondary structure. DNA superstructure refers to any conformation of the DNA beyond the level of the double helix. The formation of nucleosomes from histones and the double helix constitute the tertiary structure of DNA. Removal of histones from the DNA by salt extraction or other means reveals supercoiling in the DNA double helix. The fourth level of eukaryotic DNA structure or the second level of DNA superstructure is the DNA domain. A DNA domain is an independently supercoiled loop of DNA such that destruction of supercoiling within the loop or destruction of the loop itself has no effect on other DNA domains under a specified set of conditions. It is at this level (quaternary structure) that the debate on DNA superstructure becomes widely divergent. Higher levels of DNA structure have traditionally been explained in terms of supercoiling (solenoids) of lower levels of supercoiling (Bak and Zeuthen, 1978; Sedat and Manuelidis, 1978). There is little doubt that several orders of supercoiling must exist within the eukaryotic chromosome in order to compact the DNA into a manipulatable form during mitois and meioisis. However, I feel that DNA superstructure can be viewed in a context other than supercoiling; one which may shed some light on both the evolutionary development of the eukaryotic chromosome and changes in eukaryotic chromosome structure which take place during such processes as cellular differentiation, aging, and carcinogenesis. In this regard, models of chromosome structure involving chains of circles (Stahl, 1962) or radial loops (Paulson and Laemmli, 1977) are more appropriate. The levels of DNA superstructure to be presented here are as follows: clusters of DNA domains (fifth level), sub-chromatids (sixth level), chromatids (seventh level), chromosomes (eighth

level), and the eukaryotic genome (ninth level). Those levels of DNA structure which are suggested to exist above the level of the DNA domain will be discussed more fully in Appendix D which deals with theoretical models.

It will be apparent throughout the introduction of this work that a large amount of time and effort were expended in the development of techniques which could be utilized for investigating eukaryotic DNA superstructure in the manner as described in this Dissertation. Some experiments were attempted only one time and abandoned. This "shotgun" approach to the development of methodologies though time consuming was felt to be necessary because it appeared to me that existing methodologies were not addressing the problems of DNA superstructure that I felt should and could be answered. As a result, many of the experiments performed here could be considered as pilot studies leading to still other pilot studies. Those which yielded useful data were explored more fully while others were either modified or discarded.

At the beginning of my tenure in this laboratory my efforts were devoted to three basic areas: alkaline elution, nucleoid sedimentation analysis in neutral sucrose gradients, and gel electrophoresis of nucleoids in dilute gels. DNA damage by X-ray or gamma ray irradiation can be detected by alkaline elution (Kohn and Grimek-Ewig, 1973) or nucleoid sedimentation analyses (Cook and Brazell, 1975). Because of difficulties in reproducibility, results obtained by the nucleoid technique are not as widely accepted as those derived from alkaline elution even though the assay is more sensitive to X-ray damage. The focus of these initial studies was to corroborate the results obtained from these two techniques. The electrophoresis of nucleoids was attempted in the hope of improving the nucleoid technique by reducing sample variability. Most of the work in the first two areas of study is not directly applicable to the final investigations that comprise the body of work reported here. However, it did provide a framework for future research endeavors. Attempts to electrophorese nucleoids led to the "discovery" that most or all of the DNA from intact cells is incapable of migrating in an electric field, even through agarose as dilute as 0.15%. During the course of alkaline elution of DNA through polycarbonate filters it was observed that a small residue of DNA resisted elution even when the filters were extensively irradiated with X-rays. These two independent observations have led to the development of the bulk of the research presented in this Dissertation.

CHAPTER I

INTRODUCTION

Theoretical Assumptions

The following is a list of theoretical assumptions which I have formulated throughout the course of this research. They are modifications of older assumptions which were used initially in directing the course of these investigations. They include an integrated network of original concepts proposed by other investigators, modifications of such concepts by this author, and original concepts which I have proposed. Evidence for at least some of them will be discussed throughout the Dissertation. Theoretical models together with appropriate evidence and citations will be presented in Appendix D.

1. The first true cell (progenote) was generated from a series of homologous viral replicon fusions coupled with transpositions, both of which increased the size of the genome. In both processes the viral replicons paired and fused together at their origins of replication. These origins of replication were preserved during homologous fusions but they were partially or completely deleted out during transpositions. Two alternative outcomes were possible depending upon which process was dominant: 1) the generation of a replicon with multiple origins of replication and 2) the generation of a replicon with one origin of replication. The pairing up and connection of single origin replicons to a multiple origin replicon by flanking covalent RNA crosslinks generated a tetrameric base paired region containing paired origins of replication. This combination of crosslinked replicons and multiple origins of replication (a replicon cluster) constituted the chromosome of the progenote.

2. Origins of replication within replicon clusters can initiate DNA synthesis only when they are permanently attached to a membrane anchorage component such as a prokaryotic mesosome or the inner membrane of the nuclear envelope. All replicon clusters contain at least one membrane-bound origin of replication (master origin) which initiates DNA synthesis.

3. The evolution of the prokaryotic chromosome from the progenote is characterized by the fusion of all but one of the single origin replicons through the deletion of their origins. The inner multiple origin replicon did not fuse with these replicons and became the DNA attachment sites for the outer fusion replicon. The initiation of replication at the master origin involves both the inner replicon and the outer fusion replicon, the former completing a round of replication far more quickly than the latter because of its smaller size.

4. Mesokaryotes and eukaryotes evolved from the progenote by a series of cellular symbioses. Prokaryotic and mesokaryotic chromosomes have similar DNA super-structures and modes or replication. The evolution of the eukaryotic chromosome from the progenote is denoted by the presence of one or more membrane-bound origins of replication within a single replicon cluster. The initiation of DNA synthesis occurs at the master origin of replication which in turn initiates secondary rounds of replication in the membrane-bound slave origins of replication.

5. The replicon clusters of eukaryotes (unlike prokaryotes and mesokaryotes) have fused together to form a chromosome composed of a linear array of complex, interconnecting circles which are capable of extensive DNA rearrangements during cellular differentiation. If intact DNA from a eukaryotic chromosome could be separated from all non-covalently bound materials in a manner non-destructive to the DNA, the linear array of DNA circles would remain intact because of the presence of 2' RNA covalent bonds flanking the tetrameric region. Therefore, any intact DNA isolated from a eukaryotic chromosome in such a manner would not exist as a simple, linear molecule.

6. During cellular differentiation, DNA domains may be fused, deleted, amplified, or transposed. Deletions and fusions result in the destruction of the tetrameric region at the DNA domain attachment sites of origins of replication and the inactivation of the ARR crosslinks by methylation at the 2' positions of the ribose sugars.

Statement of the Problem

The problem under discussion in this Dissertation is as follows: What is the role of permanent nuclear matrix DNA attachment sites in the overall organization of eukaryotic DNA superstructure?

Initial Hypotheses

1. In the absence of non-covalently bound material, intact eukaryotic DNA superstructure exists in a complex, non-linear form.

2. This non-linear DNA superstructure is established by permanent nuclear matrix DNA domain attachment sites.

3. The molecules and bonds responsible for establishing this superstructure are covalent crosslinks which are associated with RNA that is resistant to RNAse A and alkali.

4. The RNAse A/alkali resistant RNA associated with the non-matrix DNA is chemically different from its nuclear matrix counterpart, suggesting that functional differences exist between the two classes of RNA.

Methods of Testing

The following is a list of the methods of testing which have been used to support the previously stated hypotheses.

1. Agarose gel retention studies of intact DNA and associated molecules.

2. Filter and agarose gel retention studies of nuclear matrix and non-matrix DNA's and associated molecules.

3. Characterization of DNA associated molecules (RNA) by alkali hydrolysis etc.

4. Double label studies to compare the relative proportions of matrix alkali resistant "RNA" (ARR) to non-matrix ARR.

5. Double label studies to compare qualitative differences in matrix and non-matrix ARR's.

6. Transmission electron microscopic analysis of purified matrix and non-matrix DNA samples coupled with physical, chemical, and enzymatic treatments.

Historical Overview

Introduction

The problem of DNA superstructure in both prokaryotes and eukaryotes has been approached from at least four principal vantage points: 1) Direct observation of chromosomes by light or electron microscopy. 2) Studies on independently super-coiled DNA domains within the chromosome. 3) Studies comparing differences in

matrix and non-matrix DNA and other related components. 4) Genetic analyses involving losses, gains, and rearrangements of large pieces of DNA which behave as autonomous units.

In this laboratory the problem of DNA superstructure has been traditionally approached by studying independently supercoiled DNA domains within the eukaryotic nucleus. Typically, this involves the use of neutral sucrose gradients which are used to analyze two basic parameters: 1) the total amount of supercoiling present within the nucleus after salt extraction, and 2) the total number and size of DNA domains (Cook and Brazell, 1975). The theory behind these techniques and the methods employed are more fully explained in Appendix A. In my early work I utilized the nucleoid technique to gain information about the effects of urea and salt extraction and proteinase K on the integrity of supercoiling in nucleoid DNA domains. The results and conclusions drawn from this work are presented in Chapter III.

The Eukaryotic Chromosome May Not Contain a Simple Linear Piece of DNA

My initial studies with agarose gel electrophoresis in 1984 involved lysing X-ray irradiated cells in agarose wells to generate nucleoids and subjecting them to electrophoresis under various conditions. Some of the results obtained from these early experiments are described in Chapter III and in Appendix C. The most salient point of interest obtained from the gel studies was the fact that, depending on cell type, some or all of the DNA within the nucleoids was resistant to electrophoresis. Similar observations involving gel encapsulated cells have also been reported in the literature (Cook, 1984; Schwartz and Cantor, 1984; Van der Ploeg et al, 1984). It occurred to me that this resistance to electrophoresis may be the direct result of DNA superstructure. However, another possibility was that the enormous size of the DNA molecules was preventing them from electrophoresing. As more literature became available it became clear that even huge pieces of linear DNA are capable of electrophoresis. In fact, linear DNA's beyond the 50 kilobase range could migrate more rapidly during electrophoresis than their smaller counterparts! (Schwartz and Cantor, 1984). These observations added impetus to the idea that the resistance of nucleoid DNA to electrophoresis was not due to the enormous size of linear DNA molecules but

rather due to some form of superstructure. Furthermore, this superstructure was resistant not only to high salt extraction (Cook and Brazell, 1975) but also to more harsh treatments like extraction in the ionic detergent lithium dodecyl sulfate (Cook, 1984). Treatments with ionic detergents or urea are known to destroy the chromosome scaffolding complex which is composed largely of proteins (Adolph et al, 1977; Adolph et al, 1977). Therefore, the resistance of nucleoid superstructure to electrophoresis after such treatments was probably due to covalent instead of ionic or hydrogen bonds. Whether the superstructure was the result of DNA topological constraints such as catenated circles and/or non-DNA linkers was not established.

The most direct evidence available that the eukaryotic chromosome may not contain a simple, continuous thread of DNA was available from electron micrographs of chromosomes treated with alkali and urea in which circular networks of "DNA" were observed (Comings and Okada, 1973; Sorsa, 1973; Sorsa and Castrodeza, 1973). Therefore, I decided to observe matrix and non-matrix DNA's directly under the electron microscope. The results of these studies led to the discovery of not only linear fragments of DNA and circles but also of circular networks which had the appearance of DNA molecules. These networks were probed with DNAse I, RNAse A, phospholipase C, and proteinase K as well as with heat and alkali. The results suggested that the circular networks were sensitive to DNAse I and to "phospholipase C" but not to proteinase K. Heating with or without proteinase K treatment had no apparent effect on the junctions between circular elements. The results obtained from RNAse A and alkali treatments were too poor to evaluate. I speculated that the junctions between the circles within the networks were covalent in nature and represented a small fraction of the DNA superstructure responsible for whole cell and matrix DNA retention during alkaline elution and/or gel electroelution (Chapter VIII). If such were the case, then, the superstructure of the DNA within the eukaryotic chromosome would be far more complex than could be accounted for by a simple, continuous, linear strand.

A Novel Assay Procedure for Measuring DNA Superstructure

As the experiments progressed, extraction of cells or nuclei in agarose wells was replaced by a gel encapsulation technique in which the cells and/or nuclei were mixed with a low temperature melting agarose at a temperature compatible with cell

viability. The cell-agarose mix was pipetted into agarose wells and chilled to allow gelation. These cells within these gel inserts could then be extracted in high salt or SDS and electrophoresed, leaving behind DNA which is essentially devoid of any non-covalently bound impurities. Similar efforts have been reported elsewhere (Cook, 1984; Schwartz and Cantor, 1984; Van der Ploeg et al, 1984). The gel inserts can be removed from the agarose wells without physically shearing the DNA as would occur in a free-flowing liquid environment. They can be incubated in various kinds of enzyme solutions or subjected to other forms of treatment to determine the effects of these treatments on the ability of the DNA to be retained within the gel insert following electrophoresis. As a result of these experiments a new assay procedure for studying DNA superstructure had evolved to test the ability of the DNA to resist electrophoresis. Therefore, the term electrophoresis was replaced with electroelution to emphasize the fact that it was the lack of DNA migration that was important in these studies.

Could Non-DNA Linkers be Responsible for DNA Superstructure at the Level of Nuclear Matrix DNA Domain Attachment Sites?

In both prokaryotes and eukaryotes, DNA is held together as independently supercoiled DNA domains. In prokaryotes RNA appears to be involved in this process (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972; Pettijohn and Hecht, 1973; Worcel et al, 1973; Drlica and Worcel, 1975; Kavenoff and Ryder, 1976; Hecht et al, 1977). The role of RNA in the establishment of independently supercoiled DNA domains in eukaryotes is less clear (Cook and Brazell, 1976; Adolph et al, 1977; Cook and Brazell, 1978; Cook, 1984).

Proteins have been demonstrated to establish supercoiling in DNA domains (Cook, 1984). This has also been supported in our laboratory by other methods described in Chapter III. Topoisomerase II DNA cleavage sites have been cloned which are located near the DNA attachment sites (Cockerill and Garrad, 1986). Furthermore, *in situ* hybridization studies have indicated that topoisomerase II is located in the nuclear matrix (Berrios et al, 1985). It is also associated with mitotic chromosomes (Earnshaw et al, 1985) at the base of DNA loops (Earnshaw and Heck, 1985). In addition, it is known that eukaryotic topoisomerase II enzymes can form covalents links with DNA (Liu et al, 1983; Sander and Hsieh, 1983; Osheroff and Zechiedrich, 1987).

However, it does not necessarily follow that DNA domain anchorage sites are established by proteins just because supercoiling is sensitive to proteases and topoisomerase sites are near the DNA domain attachment sites. It is possible to relax the supercoiling in DNA domains without the concommittent destruction of the DNA domain attachment site as has been shown in prokaryotic studies (Worcel and Burgi, 1972; Pettijohn and Hecht, 1973).

Evidence for the involvement of phospholipids with DNA domain attachment sites is also available (Maraldi et al, 1984). Such evidence is less compelling because of results generated in this laboratory which strongly suggest that phospholipase C contains an endonuclease contaminant (Chapter VIII). Therefore, the exact nature of DNA domain attachment sites in eukaryotes still remains a mystery.

If it can be assumed that the establishment of eukaryotic DNA domains occurs within the nuclear matrix region, then, the DNA domain attachment sites must punctuate the DNA at regular intervals. Any perturbations in the DNA which occur at regular intervals most probably do so within the nuclear matrix and may be the result of DNA domain attachment sites. Three such perturbations which occur at periodic intervals include alkali sensitive linkers, (Filippidis and Meneghini, 1977), regions recalcitrant to DNA cloning (Marx, 1985), and A+T-rich regions (Moreau et al, 1981; Moreau et al, 1982). In all three cases the periodicity is within the size range of replicons. Other evidence indicates that specific DNA attachment sites for chromatin loops within the nuclear scaffold are within A+T-rich regions (Mirkovitvch et al, 1984). There is some controversy about the role of the nuclear matrix during DNA replication (Djondjurov et al, 1986). However, the initiation of DNA synthesis appears to begin at the level of the nuclear matrix. Therefore, the origins of replication may be anchored there (McCready et al, 1980; Vogelstein et al, 1980; Wanka, 1982; Aelen et al, 1983; Smith, 1984; Bekers et al, 1986; Dijkwel et al, 1986; Jackson and Cook, 1986; Carri et al, 1986; Razin et al, 1986). Even a modest literature review reveals that origins of replication are A+T-rich (Moore, 1979; Chan et al, 1981; Stinchcomb et al, 1981; Tschumper et al, 1981; Zyskind et al, 1981). Origins of replication may also contain ARR (Birkenmeyer et al, 1987). These observations suggest that the DNA domains may be replicons which are held together at their origins of replication within the nuclear matrix at sites that are

alkali sensitive, resistant to cloning, A+T-rich, and partially composed of ARR. Therefore, a 1:1 correlation between DNA domains and replicons should exist, but instead there may be as much as a 4:1 ratio of replicons to DNA domains (McCready et al, 1980). One possible explanation for this apparent discrepancy is that the current techniques employed to measure average DNA domain sizes result in an underestimation of the number because many of the domains are (due to their small size) beyond the limits of detection.

While the molecule which most readily comes to mind as a potential candidate for an alkali sensitive linker is RNA, protein was still another likely prospect. From gel studies (Chapter IV) it was hypothesized that matrix DNA may be responsible for the DNA retention of whole nuclear DNA. Based upon these results a series of filter experiments was performed in which matrix or matrix-enriched DNA samples were subjected to alkaline elution through one set of filters and non-matrix DNA was eluted through another set (Chapter V). These studies suggested that matrix DNA, RNA, and protein were all preferentially retained on the filters as opposed to non-matrix DNA, RNA, and protein. Based on these observations a pilot electroelution study was performed using whole nuclear DNA labeled with [^{14}C]uridine. Nuclei were mixed with agarose and allowed to gel into inserts. The inserts were incubated with either proteinase K, and/or RNAse A, and/or phospholipase C, and/or DNAse I to determine the effects of these enzymes in various combinations on the ability of the gel inserts to retain the RNA label (Chapter IV). The results suggested that the DNA superstructure responsible for retention of the label by the gel insert was unaffected by proteinase K and RNAse A. Therefore, either the superstructure was held together by non-protein and non-RNA bonds or the proteins and/or the RNA were inaccessible to hydrolysis by these enzymes. Later results suggested that protein may play a role in gel retention of matrix DNA. The conclusions drawn from these studies suggested that removal of such proteins most probably disrupted supercoiling by generating a single strand nick within the DNA at the protein linker which did not disrupt the DNA superstructure responsible for gel retention during electroelution (Chapter V). One possible candidate for such a protein would be Topoisomerase II which has been found near DNA domain attachment sites as previously described.

The role of RNA in DNA retention was as yet unclear. What was the nature of the alkali resistant uridine labeled material that remained tenaciously bound along with the DNA and protein on filters following alkaline elution? Was it the same material that remained strongly bound after the SDS extraction and electroelution of whole nuclei? Was it RNA or DNA which had taken up the RNA label? These questions were addressed in a series of experiments involving gel electroelution of whole cell DNA. Cells were exposed for 24 hours to medium containing 50 μg/ml of each of the four deoxynucleosides required for DNA synthesis in order to suppress any uptake of uridine radiolabel into DNA via such routes as the ribonucleotide reductase pathway. Following this 24 hour incubation, radiolabel was added to the medium and the cells were allowed to grow for an additional 24 hours. The cells were centrifuged and resuspended in low serum medium containing the four deoxynucleosides without radiolabel for 24 hours prior to harvesting. This last step allowed time to "chase out" unincorporated label. Most of the RNA label which was involved in the production of transient Okazaki RNA primers would be removed because of the inhibition of DNA synthesis both by low serum and by the presence of the exogenous deoxynucleosides. Even under these stringent conditions RNA label could still be recovered from the gel inserts. The material was referred to as DNA-associated "RNA" (DAR). The next step was to isolate the DAR and characterize it. The gel inserts were incubated exhaustively in DNAse I, the incubation buffer was precipitated in either ethanol or trichloroacetic acid, and the resulting pellets rinsed, and found to contain DAR. The DAR isolated by ethanol precipitation was subjected to alkali hydrolysis, neutralized, ethanol precipitated again, and rinsed twice to remove any contaminating supernatant label. It was discovered that the DAR contained a sub-component of ARR. Furthermore, the ARR was recovered much more efficiently than its counterpart DNA which suggested that the ARR was not merely DNA which had incorporated uridine radiolabel. This was supported by still later investigations involving mass spectrometery which indicated that the uridine content of mouse cell ARR was about 100 times higher than that observed in total cellular DNA from herring sperm. The ARR was further characterized with alkaline phosphatase to determine if it's resistance to alkali was due to free phosphate groups on the 2' position of the ribose moiety (Wallace and Edmonds, 1983). No more than 7% of the resistance of ARR to alkali could be explained in

terms of phosphate groups which were susceptible to alkaline phosphatase. Some other kind of bonding such as methyl groups or diphosphate bridges were responsible for the alkali resistance (Chapter VI).

The ARR was used as a "probe" to determine if it had any correlation to DNA domain attachment sites in the nuclear matrix. This was accomplished by isolating purified matrix and non-matrix DNA samples, exhaustively digesting them in DNAse I, and subjecting them to a protocol leading to the isolation of ARR. The results indicated that about 20% of the ARR was associated with less than 1.5% of the DNA or the matrix DNA. This predilection of ARR for the nuclear matrix suggested that there may be differences in matrix and non-matrix ARR's. I hypothesized that matrix ARR was alkali-resistant because of diphosphate bridges which contributed to the superstructure of the DNA and that non-matrix ARR derived its alkali resistance from methylation at the 2' position at the ribose. I further hypothesized that during cellular differentation, methylation inactivated DNA domain attachment sites during DNA loop fusions or deletions. In the former case the inactivated sites were released into the non-matrix and in the latter case they remained associated with the matrix. Experiments were performed in which the matrix and non-matrix samples had been double labeled for both methyl groups and for ARR. Results indicated that the non-matrix ARR may be more than twice as methylated as matrix ARR; thereby supporting the conclusion that there may be chemical differences between matrix and non-matrix ARR's (Chapter VII). Other experiments were performed to determine if ARR content in the matrix and non-matrix regions was developmentally regulated but the data obtained was inconclusive. In other investigations it was found that ARR could be isolated in significant amounts from both single and double stranded fractions during hydroxylapatite batch elutions. Both fractions of the ARR demonstrated at least a six fold predilection towards the matrix DNA (data not shown). Attempts to isolate ARR from hydroxylapatite after DNAse I digestion and alkali hydrolysis were not successful although counts could be recovered from some of the samples not exposed to alkali. Only one attempt was made to determine the molecular weight of ARR by polyacrylamide gel electrophoresis. The counts recovered were too close to background to evaluate.

CHAPTER II

MATERIALS AND METHODS

General Cell Maintenance

Isolation and Subculturing

Chinese Hamster Ovary (CHO) cells were grown as monolayers in filter sterilized Ham's F12 nutrient mixture with L-glutamine (F12) medium (GIBCO). The medium was supplemented with 10% fetal bovine serum (K. C. Biologicals) and will be referred to as F12-10 medium. The cells were kept in log phase in tissue culture flasks at 37°C under a humidified atmosphere of 5% CO_2. Cells in T-75 flasks were subcultured at confluency by first rinsing the monolayer twice with 5 mls of Phosphate Buffered Saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2). This rinse is necessary to remove serum which inactivates trypsin. The cells were exposed to 1 to 1.5 mls per T-75 flask of a 0.01% solution of trypsin (Worthington) made up in PBS containing 1 μg of Phenol Red pH indicator and adjusted to pH 7.8. They were incubated on a plate warmer at 37°C and periodically examined under an inverted phase contrast microscope to monitor the progress of cell detachment which was generally completed in 5 minutes or less. Detachment was usually facilitated by thumping the flask gently against the side of the work bench. Detached cells assumed a spherical shape and floated freely in the solution. Cells were counted with a Coulter Counter and diluted as necessary for subculturing.

Human lymphocytes used in early studies were isolated from either freshly drawn blood or from buffy coats obtained from the Red Cross. Five mls of blood or buffy coat were mixed with 20 mls of PBS and underlayered with 25-30 mls of Ficoll-Paque (Pharmacia). The samples were centrifuged at room temperature for 30 minutes at 1900 RPM in a Sorvall desk top centrifuge. The lymphocytes, seen as a white turbid

interface between the Ficoll-Paque and aqueous layers, were gently collected with a 1ml pipette, mixed with PBS in a 15 ml centrifuge tube and centrifuged for 10 minutes at 900 RPM. The cell pellet was rinsed again in PBS and centrifuged as before. The cells were resuspended in filter sterilized Eagles MEM (Earles) with 2X nonessential amino acids, 1.5X essential amino acids, and 1.5X vitamins (GIBCO). The medium was supplemented with 10% fetal bovine serum and referred to as B-10 medium. The cells were incubated as described for CHO cells.

Lymphocytes used in the studies described in Chapter VII were prepared in a different manner. Eight mls of freshly drawn blood was diluted in a 50 ml centrifuge tube in 25 mls of RPMI 1640 medium (GIBCO) containing 0.5% fetal bovine serum (K. C. Biologicals). This was underlayered with 15 mls of Ficoll-paque. The samples were centrifuged for 30 minutes at 2000 RPM. The isolated lymphocyte layer was removed and centrifuged without dilution for 5 minutes at 1000 RPM. The cell pellet was resuspended in 10 mls of medium and centrifuged as before. The resulting pellet was resuspended and the cells were diluted in RPMI 1640 containing 10% fetal bovine serum, 50 units/ml of penicillin 50 µg/ml of streptomycin (Sigma), and 3% phytohemaglutinin-M (GIBCO) (to stimulate cell division). They were incubated for 24 hours at 37°C, 5% CO_2, and maximum humidity.

Mouse L-1210 cells were maintained in log phase as a suspension culture in B-10 medium at 37°C under a humidified atmosphere of 5% CO_2. Cells were counted using a hemocytometer, and cell viability was determined by trypan blue exclusion .

Human foreskin fibroblasts and human fetal fibroblasts were provided by Mr. Paul Devore through the Cell Culture Laboratory Service.

Freezing and Thawing of Cells

Cells were resuspended in their respective medium containing 10% dimethyl-sulfoxide and aliquoted into screw cap cryovials. Each cryovial contained about 1 to 3 million cells. The cryovials were frozen slowly under liquid nitrogen vapors and stored in liquid nitrogen. Cells were recovered from the vials by rapidly thawing the vial contents under hot water. The cells were resuspended in medium and centrifuged

at about 800 RPM in a Sorvall table top centrifuge for about 5 minutes to remove dimethylsulfoxide, resuspended in medium, and subcultured.

Sample Preparations

Radiolabeling Cells

Cells were generally radiolabeled in log phase with either 1 μCi/ml of tritiated radiolabel or 0.1 μCi/ml of radiolabel containing Carbon 14. Radiolabeled compounds used include [methyl-^{14}C]thymidine (0.25 mCi/1.3 mg), [methyl-^{3}H]thymidine (6.7 ci/mmol), [2-^{14}C] uridine, (55.9 mCi/mmol,), L-[1-^{14}C]leucine (55 mCi/mmol), and [^{3}H]methyl-methionine,(200 mCi/mmol). The [^{3}H]methyl-methionine was purchased from Dupont. All other radiolabeled compounds were purchased from New England Nuclear. The cells were subjected to one of four sets of incubation conditions prior to harvesting: 1) continued incubation in high serum, (10% FBS) medium with radiolabel, 2) change from high serum medium with radiolabel to low serum (0.5% FBS) medium with no radiolabel, 3) change from high serum medium with radiolabel and 50 μg/ml each of deoxyadenosine, deoxyguanosine, deoxycytosine, and deoxythymidine (dN) to low serum medium with no radiolabel and dN, 4) change from high serum medium with radiolabel and 50 μg/ml each of adenosine and guanosine (rN) to low serum medium with no radiolabel and rN. The purpose for adding deoxynucleosides was to inhibit the conversion of radiolabeled uridine into thymidine by ribonucleotide reductase (Thelander and Reichard, 1979). The purpose for adding ribonucleosides was to inhibit the non-specific uptake of radiolabeled methyl groups into purines by *de novo* biosynthetic pathways (Weinberg and Penman, 1968; Maden and Salim, 1974).

In most cases cells were aliquoted, centrifuged, and the pellets resuspended in fresh medium at a concentration of 30 million cells/ml. To each 100 mm petri plate was added 4.4 mls of medium containing 50 U/ml of penicillin and 50 μg/ml of streptomycin (Sigma) with or without deoxynucleosides or ribonucleosides and 100 μl of the concentrated cell suspension. Each plate was incubated for about 24 hours to allow the cells time to recover from the subculturing procedure prior to radiolabeling

and also to allow them time to be preincubated in any added deoxynucleosides or ribonucleosides. At the end of this period, 0.5 mls of medium containing 0.5 μCi of the appropriate carbon-14 radiolabel was added to the plates depending upon the experiment being performed. For double labeling experiments 5 μCi of tritiated thymidine or methionine was added along with 0.5 μCi of [^{14}C]uridine label. The solutions were mixed and the cells allowed to incubate an additional 24 hours. At the end of this period cells were counted, harvested, pelleted, and resuspended for immediate use as in procedure 1 or resuspended in low serum medium as described under procedures 2, 3, and 4 at a concentration of 200,000 cells/ml and incubated in an upright position for 24 hours in a T-75 tissue culture flask. At the end of this time the cells were counted and pelleted at 4^{o}C in preparation for the appropriate assay procedures.

Irradiation of Cells With X-Rays

Cells were irradiated with either a Norelco MG150 kV Constant Potential X-ray unit (water-cooled) or a Profex-Ray unit. The former unit was calibrated with a Victoreen chamber and the latter by lithium fluoride actinometry. Cells in PBS were usually irradiated with 100 rads of X-rays in a small plastic dish. They were kept on ice to inhibit DNA repair prior to the nucleoid analysis.

Nuclei Purification

The protocol used in purifying nuclei was a modification of earlier procedures (Basler et al, 1981). Several modifications were tried, the least successful of which involved the use of buffers containing EDTA instead of $MgCl_2$. Apparently the $MgCl_2$ serves as a nuclear pellet binder during centrifugation which allowed for a more efficient recovery. The procedure adopted as the method of choice was as follows: Fresh, ice cold cell pellets were resuspended in cold TM buffer (10mM Tris, 5mM $MgCl_2$, pH 7.4) containing 1% triton-X 100, nonionic detergent. The cells were triturated and allowed to sit for 5 minutes on ice, then, brought up to 0.25 M sucrose by the addition of 2.2 M sucrose TM buffer. In earlier studies the cells were triturated directly in 0.25 M sucrose TM buffer but it was felt that by adding the sucrose later,

the decreased tonicity of the solution would facilitate in cell lysis. The crude nuclear preparation was centrifuged in the cold in a table top Sorvall at 1000 X g for 15 minutes in 4 ml polyallomer tubes. The supernatant was decanted and the pellet was resuspended in 2.2 M sucrose TM buffer at a concentration of 3 million nuclei per ml. If the volume of the buffer in the tubes was too low, it was overlayered with 0.25M sucrose TM until the meniscus was near the top of the tube. These tubes were placed into Beckman SW 28 or SW 56 rotors and centrifuged at 80,000 X g for 30 minutes at 0°C. The supernatants were decanted and the purified nuclear pellets were resuspended in about 2 ml volumes of 0.25 M sucrose TM and centrifuged in the cold at 1000 X g for 15 minutes in the Sorvall. The supernatants were decanted and the pellets were kept on ice prior to resuspension in the appropriate assay buffers.

Preparation of Crude Nuclear Matrix and Non-matrix Fractions

The production of matrix and non-matrix DNA by DNAse I digestion was by a method modified from previously described procedures (Basler et al,1981). Freshly purified nuclear pellets in polyallomer tubes were digested in 40 units of DNAse I/mg of DNA sample in 10mM Tris, 5mM $MgCl_2$, (pH 7.4) for 5 minutes at 30°C at a concentration of 200,000 nuclei/5 µl. It has been reported that these incubation parameters yield matrix DNA fragments with an average size range of 250-300 base pairs after phenol extraction (Basler et al, 1981). The reaction was terminated by the addition of one volume of cold 10mM Tris, 20 mM EDTA, (pH 8.0). After mixing, 2 volumes of ice cold 10mM Tris, 4M NaCl, 10mM EDTA, (pH 8.0) was added, mixed, the contents transferred to a 1.5 ml microfuge tube, and allowed to sit on ice for 5 minutes to extract the non-matrix DNA from the matrix DNA. In one experiment hydrodynamic shearing was substituted for DNAse I digestion. The nuclei were incubated in 2M Nacl-TE buffer for 5 minutes on ice and then mechanically sheared for one minute by a rapid series of intermittent vortexing. After salt extraction the microfuge tube was placed inside a 50 cc centrifuge carrier tube and centrifuged at 1000 X g for 10 minutes at 4°C. The supernatant was decanted into a second microfuge tube and stored on ice. One volume of 10mM Tris, 2M NaCl, 10mM EDTA, (pH 8.0) was added to the pellet which was triturated to mix it with the buffer, incubated on ice for 5 minutes, and centrifuged as previously described. The first and second supernatants were

pooled and the pellet was saved as the matrix DNA fraction. In all experiments except for the one described in Table 26 the supernatants were warmed to 37°C and underlayered with 100 µl of 0.8% Sea Prep low melting temperature agarose (FMC Corp.) in 10mM Tris, 2M NaCl, 10mM EDTA, 20% sucrose, (pH 8.0). The tube was centrifuged at room temperature for 15 minutes at 1000 X g in a Sorvall desk top centrifuge to pull any remaining matrix DNA down into the Sea Prep layer and the tube was removed from the centrifuge and chilled on ice 15 minutes to gel the agarose. After gelling, the supernatant was decanted and the Sea Prep gel was rinsed and added back to the matrix fraction.

Preparation of Purified Nuclear Matrix
DNA and Non-matrix DNA Fractions

The matrix and non-matrix samples were adjusted to 1% SDS and either dialyzed overnight or diluted in TE buffer to yield a final salt concentration of 0.1M NaCl. At this point the samples were made up to 10% in formamide and subjected to batch hydroxylapatite chromatography (Kanter and Schwartz, 1979). Hydroxylapatite was boiled briefly in 0.01 M potassium phosphate buffer, pH 7.0 in 15 cc centrifuge tubes, then centrifuged 15 seconds at 600 X g (later changed to 1 minute at 1000 X g to insure that the hydroxylapatite fines were completely removed). The supernatant was discarded and the tubes were transferred to a 60°C water bath. Five mls of sample were added to each tube of hydroxylapatite, vortexed briefly, and incubated at 60°C for 15 minutes. Every five minutes the tubes were removed, vortexed briefly, and returned to the water bath. At the end of this time period the tubes were centrifuged as before and the supernatants removed. At this point 5 mls of 0.01M potassium phosphate buffer containing 20% formamide was added to each tube, the tubes were vortexed, incubated for 10 minutes, and centrifuged. The supernatants were removed and the process was repeated. The process was repeated twice more using 0.5M potassium phosphate buffer with 20% formamide to remove all single and double stranded nucleic acids which at this point should be relatively free of all protein and other non-nucleic acid components. In the finalized protocol the two 0.5M potassium phosphate supernatants were pooled and dialyzed overnight against 50 volumes of distilled water. They were dialysed at 37°C to avoid precipitation. The dialysis was

repeated twice at room temperature using 10mM Tris, 0.1M NaCl, (pH 7.4). The dialysates were removed, volumes measured, and 0.1 volumes of 10mM Tris, 0.1M NaCl, 0.1M $MgCl_2$, (pH 7.4) were added to bring the final magnesium concentration to 10 millimoles in preparation for ethanol precipitation.

Assay Procedures

Nucleoid Sedimentation Assays

Isokinetic 15% to 30% neutral sucrose gradients were prepared with solutions of 2M NaCl, 10mM Tris, 10mM EDTA, (pH 8.0) containing either 15% or 30% sucrose. These two solutions were continuously mixed together with a gradient maker and added to the bottom of 4 ml polyallomer gradient tubes for a total volume of about 3.5 mls per tube. Modifications from this formula included the addition of proteinase K or 4M urea. Proteinase K was added in concentrations varying from about 20 to 40 µg/ml. In some cases the enzyme was added to only one sucrose solution (15% sucrose) and in others it was added to both of them. The ability of proteinase K to digest proteins under these conditions was demonstrated by incubating 10 µg of bovine serum albumin with or without 4 µg of the enzyme followed by agarose gel electrophoresis and staining of the gel with amido black protein stain. Protein bands were visible only from those wells which had not been exposed to the enzyme.

Only human lymphocytes were used in the nucleoid studies described here. They were irradiated with 50 to 100 rads of X-rays in shallow dishes and kept cold to inhibit DNA repair. The basic formula used for lysing solution was 13.3mM Tris, 13.3mM EDTA, and 2.6M NaCl, pH 8.0. Just before use, 0.5% of triton-X 100 was added to the lysing solution and 150 ul aliquots of this solution were carefully layered on top of the sucrose gradients at room temperature. Fifty µl of PBS containing 200,000 lymphocytes was carefully layered on top of the lysing solution layer and the cells were allowed to incubate for 15 to 20 minutes prior to centrifugation to allow proper lysing and nucleoid formation (see Appendix A). The nucleoids were centrifuged in a Beckman Model L5-50 ultracentrifuge at 20°C for a specified time and speed. In the case of the urea gradient the ultracentrifugation was carried out in a

Beckman SW 56 rotor for 90 minutes at 40,000 RPM. Gradients containing proteinase K but no urea were centrifuged in a Beckman SW 28 rotor for times ranging from 60 to 120 minutes and at a final speed of 20,000 RPM. In the last two experiments the initial speed was kept at 5,000 RPM for 60 minutes to allow time for the nucleoids to enter the proteinase K solution and be digested by it with a minimum of migration into the gradient. After 60 minutes the speed was increased to 20,000 RPM for 90 minutes.

Gradient tubes were simultaneously fractionated and recorded using a meniscus follower (Buchler Auto Densi-Flow II), a peristaltic pump (Brinkmann), a flow cell fluorometer (Fluoro-Tec), and a chart recorder (Isco). Three ml aliquots of a 10 µg/ml stock solution of the DNA binding fluorescent dye 4', 6-diamidino-2-phenyl-indole (DAPI) from Sigma was diluted in 97 mls of distilled water and continuously pumped and mixed with the gradient solution during the fractionation process just prior to the fluorometric assay. The location of the nucleoids within the gradient was recorded as peaks by the chart recorder (Lipetz et al, 1982; Lipetz et al, 1982; Stephens and Lipetz, 1983).

Alkaline Elution Assays

The alkaline elution assay was originally designed to detect single-stranded DNA damage (Kohn and Grimek-Ewig, 1973). The assays I used were modified from procedures originally employed in this laboratory by Dr. Nirendra Singh during the alkaline elution of X-ray irradiated DNA from whole cells. The basic procedure involved slowly injecting an alkaline solution through a filter unit containing a 0.22 micron polycarbonate filter which had been previously loaded with sample. However, in my assays the samples were crude preparations of nuclear matrix or non-matrix DNA instead of whole cells. In the first set of experiments only a portion (5 mls) of the supernatant (non-matrix fraction) was added to their respective filters. There was trouble with clumping of the nuclear matrix pellets which precluded the use of triplicate assays. (Later it was discovered that the pellets could be solubilized by incubating them in NaOH for 5 minutes at 37^{o}C.) Therefore, four single filter assays were performed as described in the results in which 40 million cell equivalents were applied per filter set of matrix and non-matrix sample. The two filter set samples

were derived from mouse L-1210 cells radiolabeled with either [^{14}C]thymidine and [^{3}H]uridine or [^{3}H]thymidine and [^{14}C]leucine. The cells were radiolabeled continously for 30 hours with no change of medium prior to harvesting. The concentration of radiolabel in the medium was twice that used in later assays (0.2 μCi/ml and 2.0 μCi/ml of [^{3}H] and [^{14}C]radiolabels respectively). After loading the supernatants, the filters were slowly injected with 5 mls of NaOH solution (pH 13.5), the units were sealed at the bottom, filled with NaOH solution and allowed to incubate at 37^{o}C for one hour. The matrix pellets were mixed with 1.5 mls of NaOH and allowed to incubate for one hour at 37^{o}C. It was during this incubation period that the discovery was made that the pellets solubilized after 5 minutes in NaOH at 37^{o}C. At the end of this time the matrix fractions were injected into their respective filters followed by two injections of NaOH in 5 ml volumes. A second 5 ml injection of NaOH was also passed through the supernatant filters. The filters units were emptied, filters and elution solutions were neutralized with HCl, mixed with 10 mls of Aquasol (New England Nuclear), and counted by liquid scintillation.

In the second experiment the filter sets were assayed as duplicates with 22 million cell equivalents used per set. All of the supernatant samples were injected through the filters. The matrix pellets were solubilized for 5 minutes in 0.3M KOH at room temperature prior to aliquoting. An additional filter unit was used as a blank and was loaded with 0.5 mls of the B-10 medium from which the cells had been isolated. Five mls of 0.3M KOH were injected slowly through the units which were sealed as before, filled with KOH solution, and incubated for 16 hours at 37^{o}C. At the end of this time the units were emptied and injected twice with 5 mls of KOH. This was followed by two 5 ml injections of TE buffer (10mM Tris, 10mM EDTA, pH 7.8). The units were resealed and filled with the buffer with one of each duplicate set containing 10 mg/ml of RNAse A. Prior to use, the RNAse A had been heated to 100^{o}C for 15 minutes in TE buffer to inactivate DNAse I and then slowly cooled to room temperature (Maniatis, 1982). The filters were incubated for one hour at 37^{o}C, drained, injected twice with 5 mls of PBS and disassembled. Solutions and filters were neutralized and counted as before.

Serum Arrest Assays

To determine the effectiveness of the serum arrest, 3 X 10^6 cells were aliquoted and mixed with [^{3}H]thymidine at a final concentration of 1.0 µCi /ml, incubated for 30 minutes (37 ^oC) and then precipitated with cold trichloroacetic acid. The first sample (zero time control) was taken at the initiation of serum arrest. Two more samples were taken at 24 hours and 48 hours respectively. Samples were allowed to precipitate for 5 hours or more (4 ^oC) and collected on Whatman GF/B glass filters with cold 10% trichloroacetic acid followed by a rinse in cold 95% ethanol. The filters were placed into glass scintillation vials along with 10 mls of Aquasol and counted as triplicate samples.

Gel Electrophoresis/Electroelution Assays

A considerable number of parameters were manipulated during the early development of nucleoid gel electrophoretic techniques. Electrophoretic times varied between 35 and 180 minutes but 70 volts per gel was generally used. Cell types investigated included human lymphocytes, CHO cells, human foreskin dermal fibroblasts (FS1 cells), and human fetal dermal fibroblasts. In the earliest studies the cells were placed directly into slab gel wells at concentrations of 200,000 cells or less and overlayered with varying kinds of lysing solutions. After varying incubation periods the lysed cells were subjected to electrophoresis. In later studies the cells or nuclei were mixed with a low temperature melting agarose called Sea Prep (FMC Corp.) which was made up in a buffer with sucrose (usually 5%). The agarose was pipetted in 10 to 50 µl aliquots under buffer into gel wells made from a cork borer. The petri plates containing the gel were placed on ice for 10 to 15 minutes to gel the Sea Prep and the gel encapsulated cells (gel inserts) were removed by hand suction with a large bore, bent tip, glass pipette. The cells within the gel inserts could be lysed outside the electrophoretic gel or subjected to other kinds of treatment without the concommittent physical shearing of the DNA which would otherwise result once the cells had been lysed and the proteins removed from the DNA. After cell lysis or other treatments the gel inserts could be inserted into electrophoretic gel wells and electrophoresed.

In later studies Sea Plaque agarose was substituted for Sea Prep because of its superior gel strength. Gel inserts with volumes as high as 250 µl were made in

agarose embedded pipette sections. This greatly enhanced reproducibility. Cell concentrations were increased to 3 million cells per insert in order to detect radiolabeled DAR's and ARR's. This later electroelution technique is illustrated in the flow chart shown in Figure 33 and in the photographs in Figures 34 through 39 in Appendix C. In the early gel electroelution studies the inserts were formed inside 100 to 150 µl sections of 1 ml tuberculin syringes which were sealed on one side with high melting temperature agarose (Figure 4). These gel insert "capsules" were sealed on both ends with high melting temperature agarose and used for melting and regelling gel inserts as described in the next paragraph. These capsules were placed in between two 4 ml sections of 10 ml plastic pipettes which were coupled together with a rubber collar. In later studies the inserts were formed inside a 1 ml section of 10 ml plastic pipette. The inserts could be removed, extracted in SDS buffer, equilibrated in electroelution buffer, and reinserted back into a section of 10 ml pipette. This pipette section was connected by a rubber collar to another section, rubber support collars were placed on one or both ends of the two tube gels, and the entire complex was submerged in buffer in an electrophoretic tray and electroeluted. Once electroelution was completed the gel inserts were easily removed for additional manipulations. The tube gels were removed for melting and counting using a makeshift plunger composed of a rubber tip from a 3 ml syringe plunger which was attached to the end of a cotton swab. This could be used to eject the tube gels from the 10 ml pipette sections.

The technique of DNA "trapping" in agarose has been used to selectively separate open circular DNA from supercoiled circular DNA and linear DNA (Dean et al, 1973; Schindler et al, 1982). The feasibility of using this technique to "visualize" circular DNA within eukaryotic DNA was investigated using one of two procedures. In the first procedure the low melting temperature Sea Plaque gel inserts were extracted with 1% SDS-TE buffer by briefly electrophoresing it into the inserts followed by a quiescent 30 minute incubation and subsequent 3 hour electrophoresis. The inserts were placed into a gel insert "capsule" (see Figure 4) which was sealed on both ends with high melting temperature agarose and the capsule was placed into a buffer bath ($70^{o}C$) for 5 minutes to melt the SDS-extracted inserts. At the end of this time the inserts were either regelled on ice, or kept at $37^{o}C$ to prevent regelling. The "non-trapped" DNA would be associated with those inserts which had been never been melted, or melted and

never regelled. The "trapped" DNA would be associated with inserts which had been melted and regelled. They were then electroeluted at 37°C to compare differences in retention of radiolabel (Table 7). Variations in this technique are described in Tables 4 and 9. The second procedure involved overlayering the liquid inserts onto a gel containing 1% SDS-TE buffer at 37°C. After one hour of quiescent incubation the inserts were either gelled on ice or kept liquid at 37°C. Other inserts were gelled and then placed over the gel containing the 1% SDS-TE buffer and incubated under the same conditions as the liquid inserts. The "non-trapped" DNA would be associated with those inserts which had been gelled prior to exposure to SDS or with those which were never gelled. The "trapped" DNA would be associated with those inserts which were exposed to the SDS prior to gelling and then gelled (Table 8).

Buffers used in these experiments varied somewhat in terms of salt concentration and pH. Only the most frequently used forms will be described here. They can be categorized in terms of three basic types: 1) extraction buffers (10mM Tris, 10mM EDTA, 1% SDS, pH 8.0), 2) electroelution buffers, (40mM Tris, 10mM EDTA or {5mM $MgCl_2$}, pH 8.0), 3) and enzyme incubation buffers, (10mM Tris, 5mM EDTA, pH 7.8 {for proteinase K,}) or (10mM Tris, 5 or 10mM $MgCl_2$, pH 7.4 {for DNAse I, RNAse A, and phospholipase C}). The extraction buffers were used to denature endogenous nucleases and remove most of the protein and much of the RNA from the DNA prior to electroelution. Electroelution buffers were used to make agarose gels and electroelute gel inserts. Enzyme incubation buffers were used to incubate gel inserts in various enzyme solutions before or after various electroelution steps.

Gels were overlayered for 5 minutes with 3 µg/ml of ethidium bromide, rinsed several times with water, and photographed on top of a long wave ultraviolet light box usually for 30 or 45 seconds. Pictures were taken with Polaroid Land cameras using either Polaroid Type 667 or 57 black and white films. Gel inserts were usually sealed into their wells with agarose but in some cases they were removed and stained separately with ethidium bromide prior to photography.

In Chapter IV the ability of nuclear DNA to retain RNA radiolabel following SDS extraction and electroelution was tested after incubation of gel inserts in various solutions with and without enzymes followed by additional electroelutions. The nuclei

of cells radiolabeled with [^{14}C]uridine were incorporated into gel inserts, extracted overnight in SDS, and subjected to an alternating series of incubations and electroelutions. The incubations involved sham treatments or exposure to proteinase K, RNAse A, phospholipase C, and DNAse I. All enzymes were obtained from Sigma Chemical Company. The procedure was as follows: Gel inserts were extracted overnight. They were then removed from the buffer and electroeluted for 3 hours at 70 volts (4^{o}C) in a slab gel containing Tris-EDTA. All of the gel inserts were removed from the slab gel and incubated for 8 hours (37^{o}C) in Tris-EDTA (10mM Tris, 5mM EDTA, pH 7.8). In all cases but one the buffer contained 40 units/ml units of proteinase K. Following this incubation the gel inserts were electroeluted in a second slab gel containing Tris-magnesium overnight at 25 volts (4^{o}C). They were removed and incubated for an additional 8 hours at 37^{o}C in Tris-magnesium enzyme incubation buffer. In all cases but one the buffer contained either 40 units/ml of RNAse A, phospholipase C, or a combination of both. One gel insert continued to receive no enzyme treatment of any kind. Following this incubation the gel inserts were electroeluted for 3 hours at 70 volts (4^{o}C) in a second Tris-magnesium gel. They were removed and incubated for an additional 8 hours (37^{o}C) in Tris-magnesium incubation buffer. One gel insert was incubated in 40 units/ml of DNAse I. This insert had received treatments with proteinase K, RNAse A and phospholipase C. The inserts were then electroeluted in a fresh Tris-EDTA gel for 7.5 hours at 70 volts (4^{o}C). The inserts were removed, stained with 3 μg/ml of ethidium bromide, and electroeluted for an additional 1.5 hours. The inserts and slab gels were photographed, melted, and counted in aquasol. The results of this preliminary experiment are presented in Table 3 and the protocol is presented as a flow chart in Figure 3.

In Chapter VI Nuclear DNA was tested for the presence of RNA oligomers following SDS extraction, gel electroelution, RNAse A digestion, and a second gel electroelution. Gel inserts containing cells were extracted overnight at room temperature in TE buffer containing 1% SDS. They were electroeluted for 3 hours at 40 volts and room temperature in Tris-magnesium agarose tube gels and incubated in Tris-magnesium incubation buffer for 16 hours (37^{o}C) in the presence or absence of RNAse A (20 units/ml). The inserts were electroeluted for 3 more hours at 40 volts and room temperature in fresh Tris-magnesium tube gels and then incubated in the presence or

absence of DNAse I followed by ethanol and trichloroacetic precipitation procedures as described in the next section (see Figure 15).

DAR, Pre-ARR, ARR Assays

Precipitation methodologies involving ethanol and trichloroacetic acid were modified from Maniatis et al, 1982. The most commonly used buffer formulae included 10mM Tris, 10mM EDTA or (5 or 10mM $MgCl_2$), 0.1M NaCl, pH 7.4 for ethanol precipitation and (10mM Tris, 1mM EDTA, 0.1M NaCl, pH 8.0) for trichloracetic acid precipitation. Some of the buffer formulae were also derived from Maniatis et al, 1982. These buffers were used to precipitate DNA and associated molecules (RNA and protein) extracted from gel inserts following gel insert extraction, electroelution, and enzymatic digestions. In those experiments involving trichloroacetic acid or ethanol precipitation of DAR and ARR the gel inserts were incubated for about 24 hours at 37^{o}C in 4-5 mls of Tris-magnesium containing 20 units/ml of DNAse I. In later experiments 0.1M NaCl was added to the buffer to facilitate precipitation. In one experiment the gel inserts were melted and used to compare trichloroacetic acid and ethanol precipitation. However, it was discovered that following DNAse I digestion most of the DAR label was extracted out of the gel insert, so in future experiments the gel insert was simply counted and the gel insert extract was used for the isolation of DAR via ethanol precipitation. Since it was uncertain whether the DAR could be precipitated with ethanol, some of the samples were precipitated in trichloroacetic acid containing Tris, NaCl, and EDTA in the presence of 250 µg/ml of carrier DNA. Later it was discovered that ethanol precipitation of DAR in Tris, NaCl, and magnesium could be accomplished even in the absence of carrier DNA and the trichloroacetic acid precipitation procedure was discontinued since recovery of enzymatically appropriate substrate from the pellets was necessary for future experiments. Trichloroacetic acid and ethanol precipitations were performed overnight at 4^{o}C and -50^{o}C respectively. Samples were precipitated in approximately 2.5 volumes of ethanol to facilitate RNA precipitation followed by ultracentrifugation (0^{o}C) for 30 minutes at 106,600 X G (Maniatis et al, 1982).

The supernatants were decanted and the tubes inverted and drained. When the pellet needed to be rinsed free of buffer for alkali hydrolysis the tubes were refilled

with 1 ml of cold ethanol: 0.1M NaCl, 10mM $MgCl_2$ ({2.6}:1, v:v), vortexed briefly, filled with an additional 2.5 mls of alcohol, centrifuged, and subjected to two alcohol rinses to insure that the final pellet was relatively devoid of supernatant label. The pellets were drained as before and then placed into a vaccuum dessicator and evacuated for 10 minutes at room temperature to remove the last traces of alcohol. They were redissolved in 0.5 mls of 10mM Tris, 10mM EDTA, pH 8.0 and transferred into liquid scintillation vials together with 10 mls of aquasol. When counts were fairly high, samples were counted for 5 minutes; otherwise they were counted for 50 minutes.

Dried radiolabeled matrix and non-matrix ethanol precipitates were dissolved in 0.9 volumes of 10mM Tris, 0.1M NaCl, (pH 7.4) by vortexing and incubating at 37°C for 5 minutes followed by a second vortexing. Next, 0.1 volumes of TNM buffer (10mM Tris, 0.1M NaCl, 0.1M $MgCl_2$, pH 7.4) was added to bring the magnesium concentration up to 10 millimoles. The samples were divided into 0.5 ml aliquots, one of which was counted to determine total CPM. The other aliquots were placed into separate 4 ml polyallomer tubes along with 53 U/ml DNAse I, incubated at 37°C for about 24 hours, and then ethanol precipitated. Except for one set of samples described in Table 26 the precipitated pellets were dried, resolubilized, and exposed to a second treatment of DNAse I. They were then ethanol precipitated in preparation for alkali hydrolysis.

Alkali hydrolysis was performed according to standard procedures (Bock, 1968). One-half ml of either pre-neutralized KOH with buffer (control) or 0.1M KOH was added to dried buffer-free ethanol precipitated pellets which were vortexed and incubated (37°C) for 5 minutes. They were vortexed again and boiled for 20 minutes. The tubes were chilled on ice and 0.25 mls of 0.2M HCl was added to neutralize KOH samples while 0.25 mls of 0.1M KCl was added to the controls. All tubes were vortexed after the addition of HCl or KCl, buffered by the addition of 0.25 mls of 40mM Tris, 0.1M KCl, 40mM $MgCl_2$, pH 8.0, and ethanol precipitated.

Samples of ARR exposed to alkaline phosphatase were treated as follows: DNAse I extracts were ethanol precipitated in Tris, NaCl, magnesium with 250 µg/ml carrier DNA. After the samples were hydrolyzed and neutralized the solution was buffered by the addition of 0.25 mls of pre-alkaline phosphatase solution (0.4M Tris, 0.1M KCl,

0.04M $MgCl_2$, 4mM $ZnSO_4$, pH 8.0) followed by the addition of alkaline phosphatase (Bethesda Research Laboratories) added in a 10 µl volume of alkaline phosphatase buffer (0.1M Tris, 0.1M KCl, 0.01M $MgCl_2$, 1mM $ZnSO_4$, pH 8.0) or in the case of control tubes, the same volume of alkaline phosphatase buffer without the enzyme. The alkaline phosphatase buffer was based upon earlier procedures (Reid and Wilson, 1970). The alkaline phosphatase reaction solution was made by diluting 250 U/µl of alkaline phosphatase in the appropriate volume of alkaline phosphatase buffer to yield 25 U or 50 U of alkaline phosphatase per 10 µl of alkaline phosphatase buffer. Ten µl aliquots of the enzyme were added to the sample tubes, mixed by vortexing briefly, and incubated along with sham treated controls for 1 or 3 hours at 37°C. The samples were ethanol precipitated and the pellets rinsed and dried. The pellets were again subjected to alkali hydrolysis or sham treated as above to see if the sensitivity of the alkaline phosphatase treated pellets to alkali had been increased above the non-enzyme treated controls.

Electron Microscopy

Most of the methods used in these procedures were adopted from the electron microscopy laboratory manual used by the Department of Microbiology at Ohio State University. Other procedures and techniques were provided by Mr. Terry McBride who is the electron microscopy technician for the Department of Pathology at Ohio State University.

The copper grids used in electron microscopy were covered with a film of parlodion which had been air-dried on the surface of water or on microscope slides from a solution of parlodion in amyl acetate. The original water surface drying technique involved the use of a buchner funnel containing a piece of Whatman filter paper on which had been placed grids with the shiny (smooth) side up. The funnel was carefully filled with distilled water to avoid displacing the grids and a drop of parlodion solution was dropped onto the surface and allowed to air dry. This film was removed with a glass rod along with surface debris. A fresh drop of parlodion was placed onto the clean surface and allowed to dry as before. Then, the water was drained slowly allowing the parlodion film to be deposited onto the surface of the grids. The filter was removed and

dried in an oven at 65°C. After drying, the grids were used directly for picking up DNA spreads or they were coated with 200 angstroms of carbon in a Denton vaccuum evaporator (courtesy of Mr. Terry McBride, Department of Pathology). Samples on carbon coated grids which gave the best results were missing the parloidon coating.

The samples used in these experiments were all purified nuclear matrix and non-matrix DNA preparations which had not been exhaustively digested in DNAse I. Each 4 ml polyallomer tube contained ethanol precipitated DNA pellets most of which were derived from 3 million cell equivalents. These pellets were dissolved in 10 µl of the appropriate buffer and either used as is for DNA spreads or treated with heat and/or enzymes prior to DNA spreading. Sample treatment protocols involved incubation of the DNA with various enzymes with or without a subsequent exposure to conditions favorable for denaturation. The conditions used for denaturation included heating 3 µl of sample to 100°C for one minute in DNA buffer followed by chilling on ice or heating of 3 µl samples at 80°C for one minute in DNA spreading solution which contained 40% formamide and cytochrome C followed by chilling on ice. Proteinase K incubation solutions and conditions were modified from conventional procedures (Maniatis, 1982). Enzyme incubation conditions included three hours at 37°C and 0.5 hours at room temperature in 200 µg/ml of proteinase K, three hours at 37°C in 40 units/ml of either RNAse A or phospholipase C, 15 seconds at room temperature in 0.1 or 1.0 units/ml of DNAse I, or 60 to 120 seconds at room temperature in 10 unit/ml of DNAse I. Samples heated to 100°C had either not been exposed to enzymes or had been exposed to proteinase K. Samples heated to 80°C in DNA spreading solution had either not been exposed to enzymes or had been exposed to DNAse I.

DNA samples were dissolved in 10 µl of either DNA dilution buffer (1.5M NaCl, 0.15M Na_3 citrate, pH 7.0) if they were to be exposed to no enzymes or to proteinase K. Otherwise, they were dissolved in TN (10mM Tris, 0.1M NaCl, pH 7.4). Samples exposed to RNAse A or phospholipase C were adjusted to 5mM $MgCl_2$. In addition, the phospholipase C buffer was also adjusted to 0.1mM $ZnSO_4$ in accordance with instructions from Sigma Chemical Company which suppied the enzyme. Samples exposed to DNAse I were adjusted to 0.5mM $MnCl_2$. Manganese was substituted for magnesium because DNAse I appears to generate more blunt ended cuts in the presence

of the former divalent cation. Unfortunately, the presence of NaCl together with manganense probably negated this effect (Melgar and Goldthwait, 1968). The DNA spreading solution was composed of 3 µl of DNA sample, 2 µl of carbonate buffer (0.4 mls H_20, 40 µl of 1M Na_2CO_3, 20 µl of 0.2M EDTA)), 1 µl of 0.1% cytochrome C in H_20, and 4 µl of formamide. A 35 mm petri dish was filled with distilled water and the surface was rubbed over with a teflon bar to remove dust. A copper grid was picked up with jewelers forceps and inverted in preparation for dipping into the DNA solution. The water surface was dusted with talc to visualize the DNA film and a 3 µl drop of DNA spreading solution was dropped into the middle of the talc film. The copper grid was quickly touched to the surface of the DNA film to pick up a small droplet of solution. If the DNA was to be stained in 5% aqueous uranyl acetate the grid was placed (DNA side down) onto a drop of uranyl acetate so that the grid floated on the surface of the drop. It was allowed to stain for 90 seconds and then removed with the forceps and allowed to float (DNA side down) on the surface of a drop of 90% ethanol for 90 seconds to dehydrate the sample. If the sample was not stained, then, it was simply dehydrated in the alcohol. The grid was removed from the surface of the alcohol solution and placed onto the edge of a piece of double-sided scotch tape (DNA side up) and allowed to air dry.

Stained samples were either viewed as is by transmission electron microscopy or stained and unstained samples were shadowed. Two kinds of shadowing protocols were used: 1) rotary shadowing in which the grids rotated as they were being coated at angles of 6 to 8 degrees under vaccuum with a platinum-pallidium alloy and 2) low angle shadowing in which the grids remained stationary as they were being coated under vaccuum with a pallidium-gold alloy at an angle of about 5 degrees. The rotary shadowing was performed by Mr. John Hanson in the Department of Microbiology using a Hitachi vaccuum evaporator and the low angle shadowing was performed by Mr. Terry McBride using a Denton vaccuum evaporator.

Microscopy was performed by Mr. McBride with a Hitachi HU-12 transmission electron microscope at 50 kilovolts. The original electron micrographs were provided by Mr. McBride and all the photographs used in the plates were prepared by Mr. Art Weeks and Mr. Nima Shafaieh in the Photographic Laboratory of the Department of Pathology.

CHAPTER III

SUPERCOILING AND X-RAY TITRATION STUDIES

Introduction

Overview

A prokaryotic chromosome can be isolated in a condensed state in which it responds to DNAse I and RNAse A damage in a manner that strongly suggests the presence of independently supercoiled DNA domains (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972; Pettijohn and Hecht, 1973; Worcel et al, 1973; Kavenoff and Ryder, 1976; Hecht et al, 1977). When cells are lysed by the non-ionic detergent triton X-100 in the presence of high concentrations of salt (2M NaCl) the DNA is stripped of proteins resulting in a release of giant loops of DNA from the nuclear matrix region which surround the remnants of the nuclear envelope (Cook and Brazell, 1975; Cook et al, 1976; Cook and Brazell, 1976). Such a structure is called a nucleoid (Cook and Brazell, 1976; Cook et al, 1976). Unfortunately, this term has also been applied to an intact prokaryotic chromosome (Pettijohn and Hecht, 1973; Drlica and Worcel, 1975; Hecht et al, 1977). These eukaryotic nucleoids respond to low doses of X-rays or gamma rays as if they are composed of independently supercoiled DNA domains (Cook et al, 1976; Warren and Cook, 1978; Cook and Brazell, 1978; Lipetz et al, 1982; Stephens and Lipetz, 1983). This is similar to how prokaryotic condensed chromosomes respond to limited DNAse I treatment (Worcel and Burgi, 1972; Pettijohn and Hecht, 1973). In our laboratory and others this phenomenon has been measured by the use of the "nucleoid sedimentation technique" (Stephens and Lipetz,

1983; Lipetz et al, 1982; Lipetz et al, 1982). This technique usually involves the ultracentifugation of nucleoids through an isokinetic neutral sucrose gradient containing a high concentration of salt. The rate at which the nucleoids migrate during the course of ultracentrifugation is a function of the degree of supercoiling present. See Appendix A for additional information on the nucleoid technique.

Nucleoid studies can be categorized in two ways: 1) Those which measure changes in average supercoiling by ethidium bromide titration. 2) Those which measure DNA domain sizes and numbers by X-ray or gamma ray titration (Cook et al, 1976; Cook and Brazell, 1976). In the first case the average relative supercoiling is usually measured by ethidium bromide titration. Ethidium bromide is a planar molecule which can intercalate between the bases in double stranded DNA. The extent of intercalation is a function of the ethidium bromide concentration. As the ethidium bromide concentration is increased, negatively supercoiled DNA is progressively relaxed. The concentration of ethidium bromide which reduces supercoiling to zero is referred to as the titration point for supercoiling. Further increases in the concentration of ethidium bromide beyond the titration point results in the generation of positive supercoiling which increases with further increases in the concentration of ethidium bromide. These changes in supercoiling can be measured by sedimenting nucleoids in sucrose gradients containing varying concentrations of ethidium bromide. The minimum migration point is the titration point for supercoiling. A shift in the titration point to the left (top of the gradient) indicates a reduction in average supercoiling, i.e., less ethidium is required to reach the titration point. A shift to the right (bottom of the gradient) indicates that the average supercoiling has increased (see Figure 17).

In X-ray or gamma ray titration studies the cells are irradiated just prior to nucleoid formation and then subjected to ultracentrifugation as nucleoids. Irradiation with low doses of X-rays or gamma rays generates primarily single strand nicks. Only one single strand nick per DNA domain is required to completely relax a domain. It is important that no DNA repair occurs during the period following irradiation because such repair will prevent the relaxation of supercoiling upon nucleoid formation. As the intensity or length of time of irradiation is increased, the supercoiling becomes progressively more relaxed, similar to ethidium bromide titration. However, unlike ethidium bromide titration there is no recovery from zero supercoiling. As the length

or intensity of the irradiation is increased beyond this point, the proportion of damage resulting from double stranded breaks increases which results in the breaking up of the DNA and a loss of linearity between X-ray or gamma ray dose and loss in nucleoid migration. Relative differences in the total number or size of DNA domains can be compared between two cell types using X-ray or gamma ray titration studies (see Figure 18). The total number of DNA domains is inversely related to the size of the domains assuming no loss or gain in DNA content. Decreases in nucleoid migration can be plotted against increasing doses of X-rays. A set of slopes of -1 and -2 for two sets of cells indicates that the DNA domain sizes for the second set of cells is twice as large or twice as sensitive to X-rays as those in the first set of cells. Assuming no changes in DNA concentration, then the second set of cells contains DNA domains that are twice as large but half the number of those in the first set of cells.

Supercoiling studies can also be accomplished by agarose gel electrophoresis. This is routinely done with small supercoiled circular DNA's such as SV-40 virus to determine what percentage of the molecules are supercoiled, open circular (nicked), or linear. In high strength agarose gels with less than 0.5% agarose the supercoiled form migrates faster than the linear form which in turn migrates faster than the open circular form (Boehringer Mannheim Biochemicals, 1985). Ethidium bromide titration of supercoiled plasmids can also be performed by agarose gel electrophoresis (Maniatis et al, 1982).

Nucleoid Studies

In the course of my early investigations the nucleoid technique was used for X-ray titration studies of human lymphocytes and for X-ray and ethidium bromide titration studies of Chinese Hamster Ovary (CHO) cells. Much of this early work is only peripherally related to the theme of this Dissertation and will not be discussed here. The remainder of the work focuses on the hypothesis that a DNA superstructure exists within a nucleoid which is impervious to treatments that disrupt non-covalent interactions of DNA with other molecules and to enzymatic treatments which disrupt non-DNA covalent bonds. Typical treatments which disrupt non-covalent interactions with DNA would include treatments in 4M urea and 0.1% sodium dodecyl sulfate (SDS) ionic detergent. Isolated chromosome scaffolds exposed to these reagents are rapidly

dissociated (Adolph et al, 1977; Adolph et al, 1977) as are scaffolds within intact histone-depleted chromosomes (Laemmli et al, 1977; Paulson and Laemmli, 1977). One question to be addressed by these observations was the effect these reagents have on DNA superstructure. One of the simplest and quickest methods for doing this in our laboratory was to assay their effect on supercoiling within nucleoids. This could be accomplished by sedimenting nucleoids in 2M NaCl-neutral sucrose gradients containing 4M urea with and without an exposure to a given dose of X-rays. Urea was preferable to SDS because the latter produced too much background fluorescence interference with the DNA signal during the course of flow cell analysis. Nucleoids were also exposed to proteinase K to determine its effect on sedimentation rates with and without X-ray treatment. During the course of these experiments in 1984 a paper was published which indicated that supercoiling was retained in cells which had been electroeluted after treatment in 1% lithium dodecyl sulfate. Furthermore, the supercoiling could be relaxed with proteinase K treatment (Cook, 1984). The results obtained from my work with urea- salt sucrose gradients confirms that some degree of supercoiling is retained in nucleoids following exposure to urea. The results obtained from sucrose gradients containing proteinase K indicated that sedimentation is affected by the enzyme. Whether losses in migration rate during ultracentrifugation are the result of losses in supercoiling was not determined.

Agarose Gel Electrophoresis

The work presented here relates to early X-ray titration studies which were performed on with CHO cells lysed directly in agarose wells (data not shown) and later studies involving proteinase K digestion of gel encapsulated mouse L-1210 nuclei which had been extracted in SDS buffer. It was hypothesized that the bands of material emerging from intact nucleoids during electrophoresis may actually be supercoiled DNA subcomponents behaving as miniaturized versions of the original nucleoid structure. Therefore, their migration rate should be decreased upon exposure to doses of X-rays which produce mostly single strand breaks, e.g., 100 rads or less. The use of intact CHO cells and 2M NaCl-TX lysing buffer was an unfortunate choice for the X-ray experiment because the bands generated in this system are cytoplasmic in origin and probably do not reflect any sub-nucleoid structure (see Table 2 and Appendix C).

Results

Human Lymphocyte Nucleoid Response to X-Rays in 4M Urea or Proteinase K

In nucleoid migration experiments the migration rate of human lymphocyte nucleoids within each tube is normalized against the entire length of the tube and the data is presented as ratios. A ratio of 1.0 indicates that the nucleoids sedimented to the bottom of the gradient. Figure 1 depicts the results of the urea experiment in graphical form. Table 1 presents the results of the proteinase K studies in numerical form. Figure 2 is a graphical representation of one set of the results shown in Table 1, data row 4. Chart recordings for the data presented in Figures 1 and 2 can be found in Plates XVII, XVIII, and XIX. The results of the 4M urea experiment clearly indicate that human lymphocyte nucleoids can respond to X-rays after denaturation in urea. The migration ratio was reduced to about one-half its original value after X-ray irradiation (Figure 1) which suggests that supercoiling is present.

Evidence that DNA supercoiling is retained in the presence of lithium dodecyl sulfate has been shown elsewhere (Cook, 1984). Eukaryotic DNA is composed of independently supercoiled DNA domains which are probably established by DNA domain attachment sites within the nuclear matrix. If so, then, these attachment sites are resistant to strong denaturants such as urea and ionic detergents. Such resistance suggests that these attachment sites are the result of covalent interactions instead of hydrogen and/or ionic bonds.

The results of the proteinase K studies indicate that digestion by the enzyme can affect the migration rate of human lymphocyte nucleoids in the presence or absence of X-ray irradiation (Table 1). The extent of this effect varied considerably between experiments. In Figure 2 the effect of proteinase K was more discernable in the nucleoids which had been exposed to X-rays than in the unirradiated group. However, just the reverse situation was noted in Table 1, data row 5. This data does not indicate whether the reduction in migration after proteinase K treatment is directly due to losses in supercoiling. This would have to be confirmed with ethidium bromide titration. However, losses in supercoiling after proteinase K treatment of eukaryotic cells extracted in lithium dodecyl sulfate have been reported elsewhere (Cook, 1984).

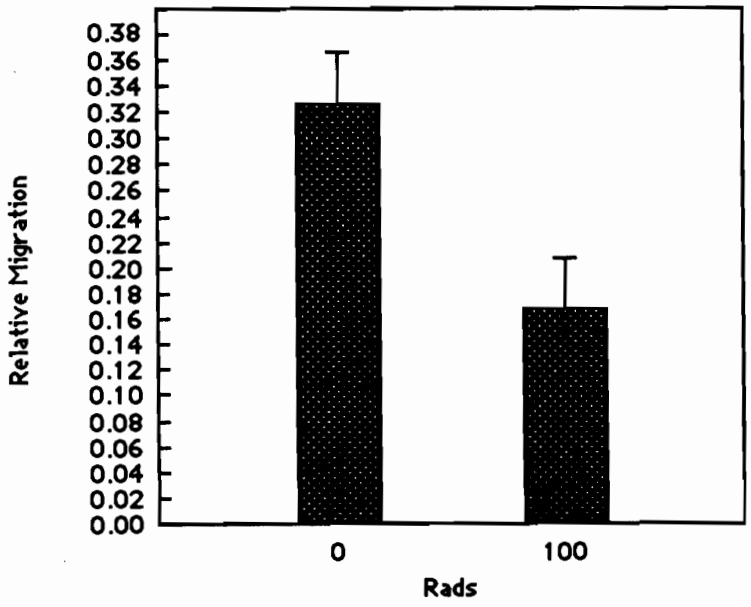


Figure 1

The Effect of X-Ray Irradiation on the Percent Migration of Human Lymphocyte Nucleoids in Neutral Sucrose Gradients Containing 2M NaCl and 4M Urea

Bar graphs represent averages and standard error of triplicate values. See Plate XVII for the nucleoid chart recording.

Effect of X-Rays and Proteinase K on Band Migration Rates During Gel Electrophoresis

The CHO cell cytoplasmic bands responded to X-rays at least 50% of the time and in the majority of cases the response resulted in a decrease in the migration rate of the bands (data not shown). This suggests that the cytoplasmic bands probably contain a supercoiled form of DNA which is being relaxed by the X-rays. Of more importance, however, is the response of the mouse cell nuclear bands to proteinase K because these nuclear bands are probably generated by a subnucleoid structure whose properties reflect those of the intact nucleoid. The reduction in the migration rate of these bands in response to proteinase K suggests that the bands are composed of supercoiled DNA

Table 1

The Effect of Proteinase K Digestion on Human Lymphocyte Nucleoid Sedimentation in the Presence or Absence of X-Ray Irradiation

Human lymphocytes were suspended in PBS on ice and divided into two aliquots. One of the aliquots received either 50 or 100 rads of X-ray radiation and the other aliquot received no radiation. Each aliquot was divided into two smaller aliquots. One set was added to gradients containing no proteinase K enzyme and the other set was added to gradients containing the enzyme. All aliquots were run in triplicate. The migration rate in each tube was normalized against the entire length of the gradient. The data points in the table include triplicate samples which were averaged and the standard error determined. *Includes samples in which one data point was not usable (duplicate averages). Experiments were not averaged together because of differences in centrifugation parameters and proteinase K concentrations.

Average Relative Migration Rate of Nucleoids				
	No proteinase K		Proteinase K	
Rads	No X-rays	X-rays	No X-rays	X-rays
100	0.49 ± 0.04	0.35 ± 0.02	0.31 ± 0.02	0.30 ± 0.06*
100	0.43 ± 0.05	0.22 ± 0.03	0.41 ± 0.05	0.19 ± 0.03
50	0.61 ± 0.08	0.54 ± 0.04	0.51 ± 0.03	0.47 ± 0.05
100	0.52 ± 0.05	0.30 ± 0.02	0.50 ± 0.09	0.21 ± 0.02*
100	0.51 ± 0.02	0.27 ± 0.04	0.40 ± 0.07	0.29 ± 0.05
100	----	0.67 ± 0.01*	0.86 ± 0.02	0.57 ± 0.06

which can be relaxed by proteinase K digestion (Plate XXIIIb). Such a finding supports what has been demonstrated by Cook in 1984 and in the nucleoid sedimentation studies discussed earlier in this chapter.

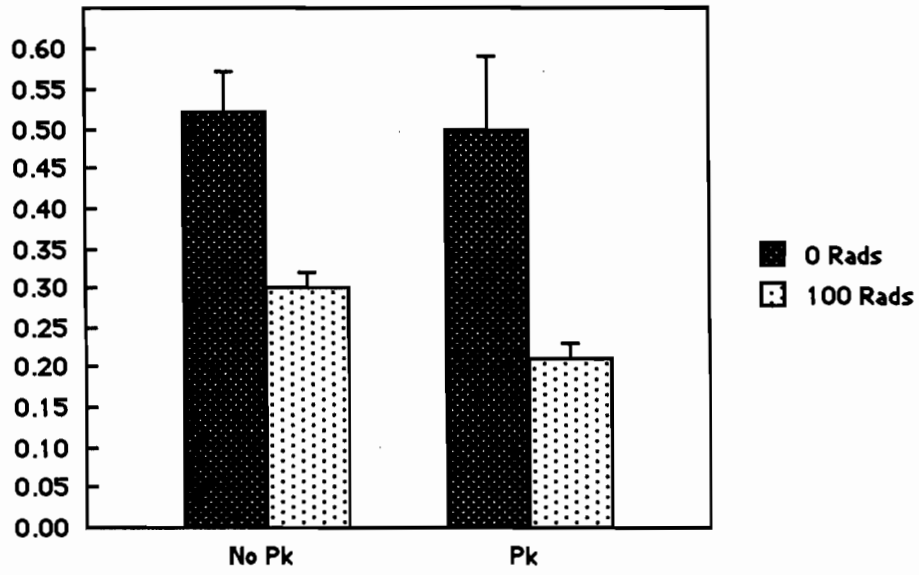


Figure 2

The Effect of Proteinase K Digestion on Human Lymphocyte Nucleoid Sedimentation in the Presence or Absence of 100 Rads of X-Ray Irradiation

See Table 1, data row 4 for numerical data. The migration rate in each tube was normalized against the entire length of the gradient. Bar graphs represent averages and standard error of triplicate values. See Plates XVIII and XIX for the nucleoid chart recordings.

Discussion

The results of the nucleoid sedimentation experiments support a previously published report (Cook, 1984) that nucleoid superstructure is resistant to strong denaturants such as LiDS but that the supercoiling is sensitive to digestion in proteinase K. Sensitivity of supercoiled DNA to proteinase K is further supported by the results obtained with mouse L-1210 nuclei following SDS extraction, proteinase K digestion, and electrophoresis. The fact that the bands generated from these mouse nuclei are in fact DNA was confirmed in later experiments by radiolabeling with either [^{3}H]thymidine or [^{14}C]thymidine.

CHAPTER IV

Intact DNA Retention Studies

Introduction

In the earliest agarose gel electrophoresis experiments cells or nuclei were lysed in agarose wells with conventional nucleoid lysing solution containing high concentrations of salt and 0.1% to 1% of triton-X 100 non-ionic detergent in a Tris-EDTA buffer. Later these cells and nuclei were mixed with low temperature melting agaroses like Sea Prep or Sea Plaque so they could be physically manipulated for incubation in various solutions before and/or after a series of electrophoretic runs with minimal physical damage to the DNA. Most of the early work was performed with very dilute agarose gels (0.15%) in order to offer the least possible impedance to the migration of the nucleoids or their subcomponents. Studies included X-ray irradiations, incubations in SDS, 4M urea, proteinase K, RNAse A, and rabbit serum; the latter of which contains a phosphotriesterase activity (Aldridge, 1953). Interest in phosphotriesters stemmed from a hypothesis that DNA superstructure may be established by phosphotriester bonds (Figure 41). Photographs of many of these gels can be found in Appendix C. From this early work it was evident that during the course of electrophoresis of lysed cells much of the DNA was retained within the gel wells; even in gels at agarose concentrations of only 0.15%. In the case of human lymphocytes all of the DNA detectable by ethidium bromide was resistant to electrophoresis. This phenomenon has been oberved in other studies as well (Cook, 1984). Since the DNA within a single eukaryotic chromosome is considered to be a simple linear strand it would have to be concluded that such retention was due to the immense size of the linear DNA strands. However, recent papers have indicated that whole chromosomes of yeast and trypansomes are capable of electrophoresis (Schwartz and Cantor, 1984; Van der Ploeg et al, 1984). During the course of those procedures it was assumed that the size of the linear DNA molecules was irrelevant in

terms of the complete inhibition of migration of the molecules. In fact, linear DNA molecules larger than 50 kilobases could actually migrate faster than smaller DNA molecules (Schwartz and Cantor, 1984). If this is so, then, DNA from cells which have been lysed in SDS and subjected to proteinase K extraction should be reasonably pure enough to undergo electrophoresis, leaving nothing behind in the wells. However, such is not the case as has been discovered in this laboratory and others (Cook, 1984; Schwartz and Cantor, 1984; Van der Ploeg et al, 1984). Therefore, simple linearity of DNA molecules coupled with large sizes cannot by itself account for DNA retention within agarose wells during electrophoresis. It can only be concluded that the DNA exists in a superstructure form which topologically constrains the molecules during the course of electrophoresis. This superstructure is destroyed during the course of conventional DNA isolation procedures but it is largely or completely retained when DNA is isolated within gel inserts which prevent hydrodynamic shearing of the DNA. This superstructure is resistant to SDS extraction, proteinase K, and RNAse A. Therefore, the topological constraints appeared to be neither protein nor RNA. However, the retention of uridine radiolabel by gel inserts following treatments with both these enzymes was not reduced below that seen in sham-incubated controls, even after a period of electroelution which exceeded 24 hours (Table 3). The retention of RNA radiolabel by gel inserts following such extensive treatments required further study. The work presented in the latter part of this chapter includes the development of the submerged horizontal tube gel electroelution assay as a means for studying intact DNA retention in gel inserts. The procedure was used to study the ability of radiolabeled DNA, RNA, and protein to be retained by the gel inserts during the course of SDS treatments and electrophoresis. The word electroelution was substituted for electrophoresis to emphasize the fact that the retained macromolecules are being studied and not the bands generated during electrophoresis. All radiolabel which was released from the inserts into the agarose gel was pooled and referred to as non-bound label. Submerged horizontal tube gel electroelution was developed as a substitute for conventional slab gel electroelution for three reasons: It simplified the quantitation of non-bound radiolabel, minimizes label dilution, and minimized cross-contamination between individual gel inserts and between their electroeluted labels. The non-bound label was electroeluted into agarose within pipette sections of known volume. Vertical

tube gel electroelution was tried as well but the technique was too unreliable because of leakage problems at the point where tube gels were coupled.

Results

Affect of Lysing Solution Composition on Band Generation During Gel Electrophoresis

The results obtained during the early developmental work on gel electrophoresis of intact nucleoids has been complicated by the fact that many parameters were changed during the course of these experiments including electrophoretic time, temperature, composition of electrophoretic and lysing buffers, pH, and cell type. However, of all these parameters, the ones producing the most noticable effect on the generation of ethidium bromide stained bands during electrophoresis were cell type, use of whole cells versus nuclei, changes in the composition of the lysing solution, and alkali denaturation with or without renaturation (Table 2). When cells were lysed in the presence of 2M NaCl, Tris-EDTA buffer with triton-X 100 (2M NaCl-TX) 3 of 4 cell types exposed to this solution generated bands upon electrophoresis, i.e., CHO cells, foreskin fibroblasts, and fetal fibroblasts. Human lymphocytes produced no such band in the presence of this lysing solution. When CHO nuclei were purified and then lysed in the solution the bands were no longer present, suggesting that in the case of CHO cells they were cytoplasmic in origin. If the CHO cell nuclei were exposed to proteinase K after lysis in 2M NaCl-TX and then electrophoresed, bands would appear. These new bands must be nuclear in origin. If the lysing solution was changed to 1% SDS in Tris-EDTA buffer, bands could be generated from both CHO nuclei and lymphocytes. Lymphocyte bands could also be generated in the presence of 4M urea buffer (4M urea in Tris-EDTA buffer). The results of these experiments indicated that use of 2M NaCl-TX as a lysing solution in the absence of proteinase K resulted in either no band release or the release of bands of cytoplasmic origin. Nuclear bands were released in the presence of 2M NaCl-TX only after proteinase K treatment or from 1% SDS or 4M urea lysing buffers. Bands could be released from CHO cell nuclei in the presence of alkali but this release was prevented if the DNA was renatured prior to electrophoresis (see Appendix C, Plates XX and XXIIa-c).

Table 2

The Effect of Various Lysing Solutions on the Release of Electrophoretic Bands From Whole Eukaryotic Cells or Their Isolated Nuclei

In the earliest experiments cells or their isolated nuclei were incubated with the lysing solution directly in the electrophoretic gel wells. This procedure was modified as early as 11/7/84 (Appendix C) so that the cells were mixed with lysing solution and agarose together to form gel inserts. The procedure was again modified to its present form in which the cells or nuclei are embedded in gel inserts which are incubated in various solutions,then, inserted into the gel wells just prior to electrophoresis. Electrophoretic gels varied in strength but most of the early gels contained only 0.15% agarose to facilitate band release. [a](N) indicates wells containing nuclei. [b]Proteinase K was incorporated into the lysing solution. [c]The gel insert was incubated in proteinase K, electrophoresed, incubated in RNAse A, and electrophoresed again. [d]Gel inserts were incubated in alkali (pH 13.5) and then neutralized prior to electrophoresis. Cell types studied include Chinese Hamster Ovary (CHO) cells, human peripheral lymphocytes, human foreskin fibroblasts, human fetal fibroblasts, and mouse L-1210 cells. Lysing solutions used were made up in Tris-EDTA buffers of varying molarities and pH. These molarities did not exceed 13 millimoles and were usually adjusted to pH 8.0. Lysing solutions contained one of the following: 2M NaCl with 0.1% to 1% triton-X 100 (2M NaCl-TX), 1% SDS, or 4M urea. Electrophoretic bands which were rendered visible after electrophoresis with ethdium bromide are noted in Column 3. For additional information and photographs of results see Appendix C, Plates XX and XXII-XIV.

Cell Type	Lysing Solution	Band Generated
CHO	2M NaCl-TX	Yes
CHO (N)[a]	2M NaCl-TX	No
CHO (N)	2M NaCl-TX-P[b]	Yes
CHO	Alkali	Yes
CHO (N)	Alkali	?
Lymphocyte	2M NaCl-TX	No
Fibroblast	2M NaCl-TX	Yes
Fetal Fibroblast	2M NaCl-TX	Yes

Cell Type	Lysing Solution	Band Generated
CHO	1% SDS	Yes
CHO (N)	1% SDS	Yes
CHO (N)	1% SDS-PR[c]	Yes
CHO	Alk/Neu[d]	No
CHO (N)	Alk/Neu	No
Lymphocyte	1% SDS	Yes
Lymphocyte	4M Urea	Yes
Mouse (N)	1% SDS	Yes

The Effects of Enzymes on DNA Associated Material Radiolabeled With Carbon 14 Uridine

A pilot experiment was carried out to determine the effect of various enzyme treatments on the ability of gel inserts to retain [^{14}C]uridine label after SDS extraction and electroelution. This study pre-dated the use of added deoxynucleosides and quantitative tube gel techniques. Cells were incubated in high serum medium containing radiolabel for 24 hours followed by incubation in low serum medium for 24 hours without radiolabel. Nuclei were purified from these cells and incorporated into gel inserts, and SDS-extracted overnight. The extracted inserts were subjected to a series of slab gel electroelutions followed by their removal from gel wells and incubation in various buffers at 37^{o}C for 8 hours with or without 40 units/ml of enzymes (Figure 3). The results (Table 3) suggested that treatment with proteinase K and RNAse A caused no discernable decrease in the ability of the gel inserts to retain the radiolabeled material. Inserts incubated with these two enzymes retained 1784 and 2095 CPM as compared to 1066 CPM retained by a sham incubated insert. In the presence of phospholipase C retained counts for 2 separate inserts were reduced to 230 and 875 CPM which represent 2 and 9 fold reduction as compared to the above inserts. When the insert was exposed to DNAse I the counts dropped to within background levels. Even though the data was erratic the results strongly suggested that some form of RNA was resistant to SDS extraction, RNAse A treatment, and electroelution. Resistance of some cellular RNA's to RNAse A and RNAse T_1 has been previously demonstrated (Cook and Brazell, 1978). The loss of label following DNAse I treatment suggested that the RNA was intimately associated with "high molecular weight" DNA which may have been responsible for its retention within the gel insert. The partial sensitivity of the label to phospholipase C suggested that either membrane components are playing a role in the retention of the RNA label or the enzyme preparation contained DNAse I activity.

Vertical Tube Gel Electroelution

Only five gel inserts made of low melting temperature Sea Plaque agarose were used in this experiment. They contained cells which had been irradiated with 100 rads of X-rays to generate single stranded nicks. The gel inserts were then extracted in 1%

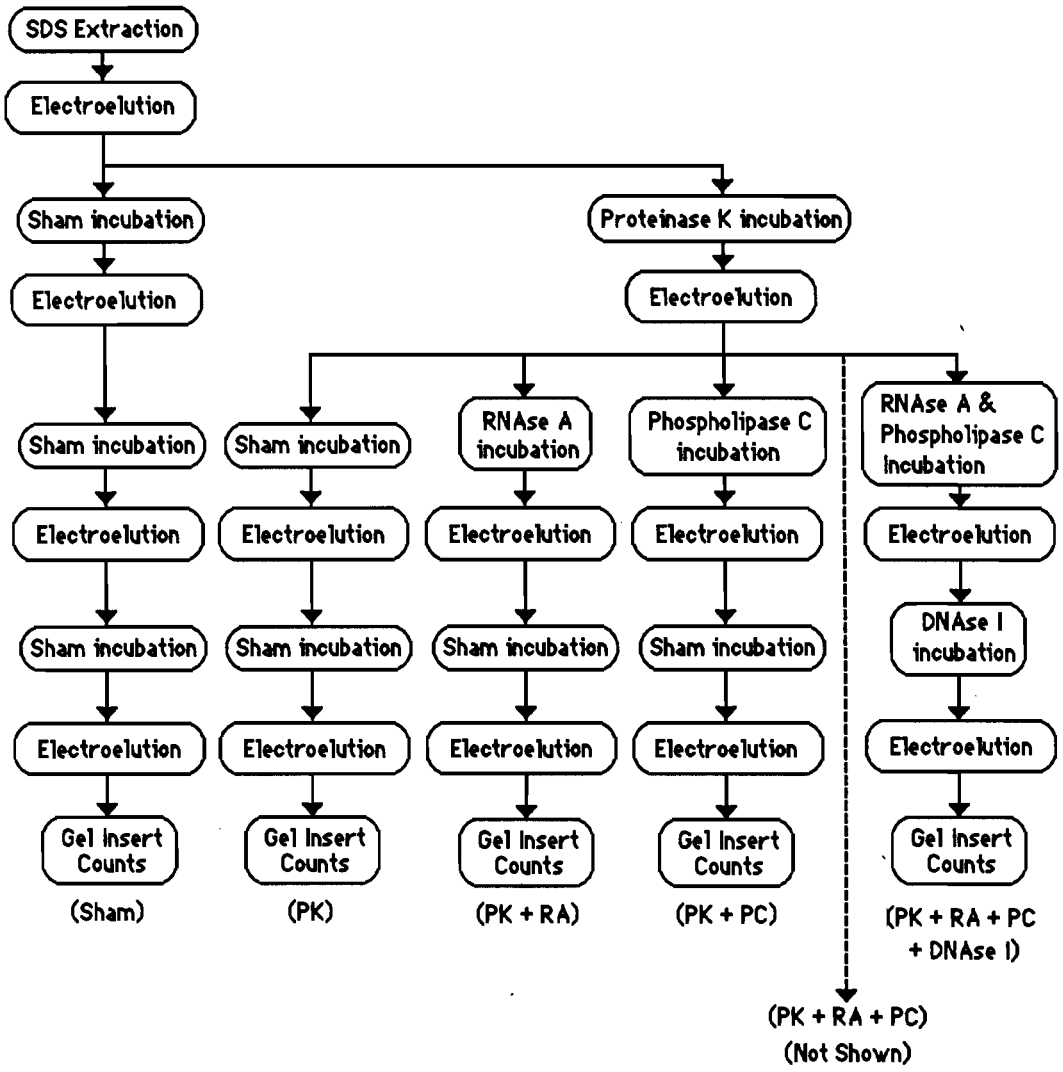


Figure 3

Flow Chart Depicting the Successive Enzymatic Treatment of Electroeluted Gel Inserts

The results of this experiment are shown in Table 3. Additional information is available in Chapter II and in Plate XXIV.

Table 3

Total [^{14}C]Uridine Radiolabel Per Gel Insert After Successive Enzyme Treatments Followed by Electroelution

Mouse L-1210 cells were incubated for 24 hours in high serum medium containing [^{14}C]Uridine radiolabel followed by incubation in low serum medium for 24 hours without radiolabel. Nuclei were purified from these cells and used to make gel inserts. The inserts were subjected to overnight extraction in 1% SDS buffer followed by a series of four electroelutions and three incubations in appropriate buffers with or without enzymes (Figure 3 and Plate XXIV). Each electroelution was followed by an incubation step with the exception of the last electroelution. The sham incubated gel insert received no enzyme treatments. All other inserts were incubated in proteinase K. The second incubation step includes: second row, no enzyme; third row, RNAse A only; fourth row, phospholipase C only; and fifth and sixth rows, both RNAse A and phospholipase C. The third incubation step involved the following kinds of treatment: no enzyme, rows 1 through 5; and DNAse I, row 6.

Treatment	CPM/Gel Insert	Effect Relative to Control
Sham incubation	1066	---
Proteinase K (PK)	1784	No loss
PK then RNAse A (RA)	2095	No loss
PK then phospholipase C (PC)	744	Partial loss
PK then RA and PC	875, 230	Partial loss
PK then RA and PC, then DNAse	0	Complete loss

SDS. The SDS was removed by soaking the inserts in Tris-magnesium chloride buffer. They were sealed within a gel capsule (see Figure 4) with high temperature melting LE agarose and melted in a 70^{o}C buffer bath. The high temperature melting agarose surrounding the molten gel insert prevented it from leaking out of the capsule and into the buffer bath. The capsules were removed and chilled on ice to regell the inserts. The capsules were sandwiched between upper and lower tube gels which were coupled together with a rubber collar. The upper gel was plugged into an upper buffer chamber and the lower gel was inserted into a lower buffer chamber, thereby establishing electrical continuity. DNAse I or DNA polymerase I enzymes were contained within tube gels. This is the only data presented from a vertical tube gel assay because the technique was too inconsistent due to leakage at the point of tube gel coupling or the upper buffer chamber or because of complete gel loss. Gravity combined with the "pressure" of electroelution were apparently responsible for the loss of tube gels.

Figure 4

Preparation of Gel Inserts for Melting and Regelling Prior to Agarose Gel Electroelution

Figure 4a illustrates the low melting temperature agarose gel insert (1) containing SDS extracted cells. A high melting temperature gel plug (2) is placed into one end of a 1 cc syringe 50 µl pipette section (3) followed by the insert and a second gel plug. In (b) this gel insert capsule (4) is sealed on both ends with high melting temperature agarose (5). In (c) the capsules are submerged into a buffer bath (6) in a glass petri dish (7) containing a thermometer (8). The bath has been heated to 70^{o}C by a hot plate (9). After 5 minutes of incubation the low melting temperature agarose melts and mixes with the extracted cellular DNA. In (d) the gel capsules have been removed from the buffer bath and placed on ice (10) to regell the inserts. Such a process should result in the "trapping" of circular DNA structures (Table 7). In (e) the gel capsule has been inserted between two agarose tube gels (11) which are coupled together by a rubber collar (12). The ends of both tube gels contain an empty cavity (13) for the gel capsule. In (f) the tube gel apparatus is assembled and ready for gel electroelution.

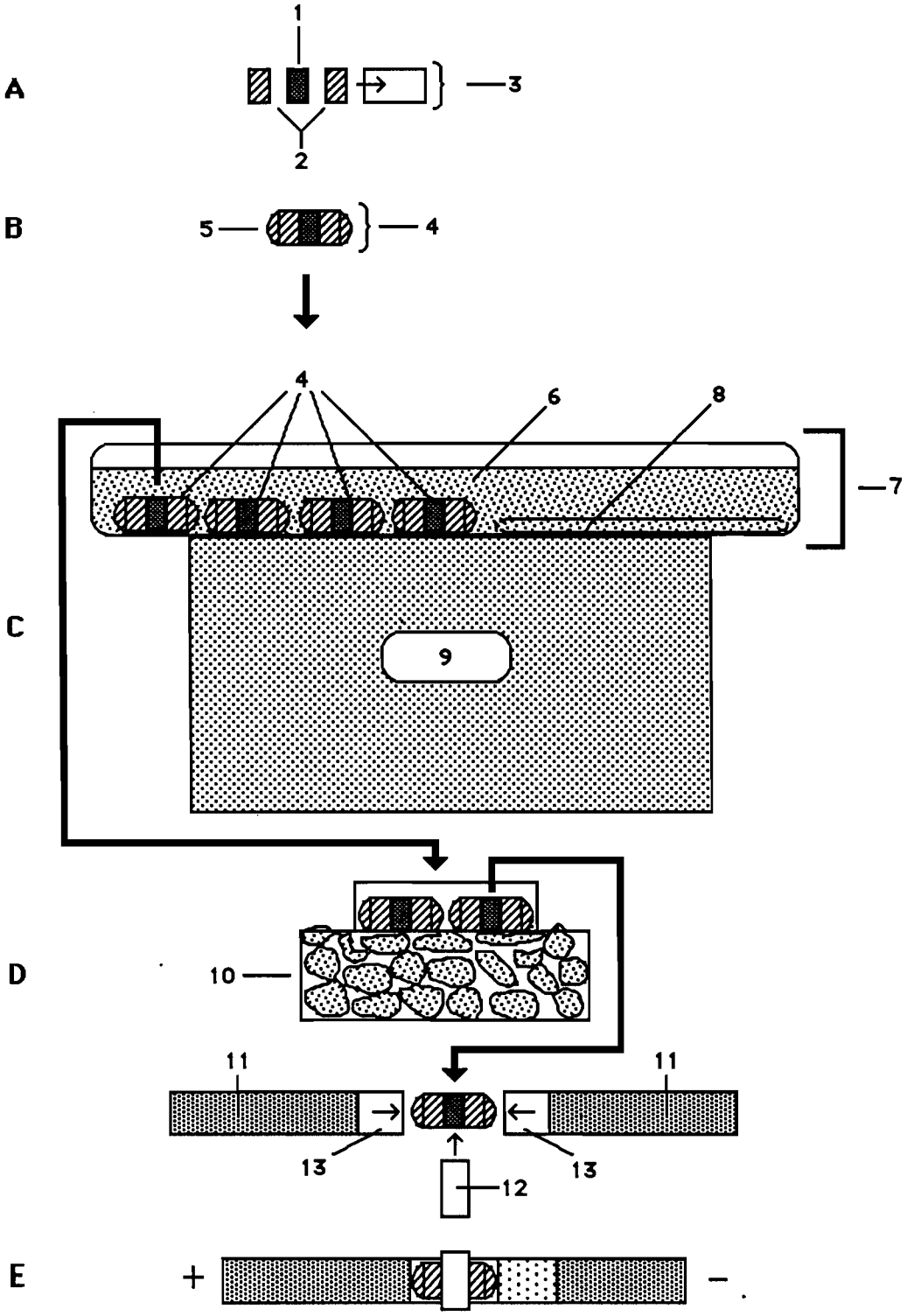


Figure 4

The purpose of the experiment was to determine if this technique could be used to evaluate the effect of these two enzymes on the ability of melted and regelled inserts to retain DNA radiolabel following exposure to the enzymes and electroelution. The DNAse I was used as a positive control for measuring the effects of DNA polymerase I on label retention. The gel inserts were melted and regelled in order to trap any circular DNA within the gel matrix. However, large linear DNA's can also be trapped by the agarose as well (Dean et al, 1973; Schindler et al, 1982). It was hoped that the exonuclease activities of DNA polymerase I would be able to selectively destroy the retention of the large linear DNA's, leaving behind partially gapped circular DNA forms. In this way a method would be available for determining if non-linear DNA superstructure was responsible for some of the DNA retention by gel inserts. I later concluded that this reasoning was fallacious and that any loss of DNA retention due to exonuclease activities would not result in the selective purification of circular over linear DNA forms.

The results indicated that the technique is capable of measuring differences in the ability of gel inserts to retain DNA label following exposure to enzymes and electroelution (Table 4). In the absence of enzyme treatments the relative retention of DNA label by the insert in row 2 as compared to the insert in row 1 which was not electroeluted is 0.145, i.e., 14.5% of the DNA was retained by the gel insert after electroelution in the absence of any enzyme treatments. Treatment with DNA polymerase I caused no effect on the retention of DNA label by the gel insert in row 3 whereas treatment with DNAse I caused the retention to drop from 0.145 to 0.004. Therefore, either the exonuclease activities of the DNA polymerase I had been inactivated prior to exposure of the insert to the enzyme or the incubation parameters were such that the DNA had not been digested enough to significantly affect the retention of the DNA by the insert.

<u>The Affect of SDS Extraction on the Retention of Radiolabeled Material by Gel Inserts as Measured by the Submersible Horizontal Tube Gel Electroelution Assay</u>

In this study and all subsequent ones the technique used for studying label retention by gel inserts after electroelution was the submersible horizontal tube gel electroelution assay. In this procedure the gel inserts (naked or within a capsule) were placed between two tube gels as described before with the exception that the tube gels are submerged in a horizontal position within an electroeluting bath (see Figures 33 through 39). This eliminated leakage and loss of

Table 4

A Feasibility Study on Tube Gel Electroelution of Melted and Regelled Gel Inserts Exposed to DNAse I and DNA Polymerase I.

The purpose of this experiment was to determine the feasibility of using tube gel electroelution as an assay system for determining the effects of different kinds of treatments on the ability of gel inserts to retain DNA and associated moleules during the course of electroelution. In this particular experiment gel inserts made from low-melting temperature Sea Plaque agarose contained mouse L-1210 cells radiolabeled with [^{14}C]thymidine which had been previously extracted in 1% SDS and frozen. The inserts were irradiated with 100 rads of X-rays to provide single stranded nicks as substrates for the 5' and 3' exonuclease activities of DNA polymerase I. They were equilibrated in heating buffer, placed inside pipette sections, and sealed on both ends with high melting temperature agarose as described in "Materials and Methods" and as shown in Figure 4. The resulting gel insert capsule was removed, submerged in Tris buffer containing 0.1 M NaCl, and subjected to 70^{o}C to melt the gel insert. The high temperature melting agarose prevented leaking of the liquid gel insert from the capsule into the buffer. The capsules were removed and placed on ice to regel the inserts which were removed and treated as follows: The insert in row 1 was melted and counted in aquasol without further treatment. The counts obtained from this insert were considered as total counts and all other inserts were normalized against it and the results displayed in column 3. All other gel inserts were placed inside a buffer chamber overlying a vertical tube gel. A second upper tube gel was connected to the lower tube gel with a rubber collar, sealing the system. Gel inserts were exposed to DNAse I and DNA polymerase I by electroeluting the enzymes out of a 0.5 ml gel layer within one of the tubes and into the inserts in Tris-magnesium chloride buffer. The control gel insert was sham treated. After a one hour incubation period at room temperature the inserts were electroeluted, removed, melted, and counted.

Treatment	CPM	Relative Retention
No Electroelution	1643	-----
Control	238	0.145
25 U/ml DNA Pol I	240	0.146
20 U/ml DNAse I	7	0.004

gels due to gravitational effects and also insured that the gel inserts did not dry out because of heating effects which can result in air bubbles which interfere with the current flow. Each data point represents sets (usually triplicates) which were averaged and from which standard errors were derived. In the early studies considerable variations could occur between the total label content of different gel inserts within the same set because of problems with gel insert formation. It was discovered that much of this variation was due to leakage of the liquid gel inserts out of

their containment wells before they gelled (a problem which was corrected in later studies). This variability was minimized by normalizing the retention of each gel insert against the total counts electroeluted from that particular insert. The ratios obtained for each insert of a set were then used to determine the average values of gel insert retention and standard errors.

A study was performed to test the ability of this new horizontal tube gel technique to determine the effects of SDS treatment on the ability of gel inserts to retain DNA, RNA, and protein radiolabels. One set of gel inserts was exposed to SDS by briefly electrophoresing the detergent into the inserts, turning off the current, and incubating the inserts in the detergent. The other set of inserts was sham treated. After the incubation period both sets were electroeluted and the inserts and tube gels removed and counted by liquid scintillation.

As can be seen in Table 5 treatment with SDS affected the ability of the inserts to retain all three forms of label. Ratios were reduced as follows: thymidine was reduced from 0.53 to 0.34, uridine from 0.28 to 0.24, and leucine from 0.58 to 0.06. Uridine label was least affected by SDS treatment followed by thymidine and leucine labels. This is illustrated more clearly in Figure 5. When the relative label retention data from Table 5 is further analyzed the results indicate that SDS treatment caused the additional removal of label in the following amounts: thymidine, 19%, uridine 4%, and leucine, 52% (Table 6). The combined effects of SDS treatment coupled with electroelution resulted in a loss of 66% of the DNA label, 76% of the RNA label, and 94% of the protein label.

The Affect of Melting and Gelling on the
Ability of Gel Inserts to Retain DNA Label

In the experiment described in Table 4 the gel inserts were melted and regelled in capsules prior to electroelution. The rationale behind this procedure is that when linear and circular DNA's of comparable size are mixed with molten agarose and gelled, "trapping" occurs which results in the preferential retention of the circular DNA molecules over the linear forms (Dean et al, 1973; Schindler et al, 1982). This assay can be used to distinguish between linear and circular DNA forms as long as the size ranges are comparable. This was the reason why an attempt was made to preferentially break down large linear DNA molecules (as opposed to DNA circles) by X-ray

Table 5

The Affect of SDS Extraction on the Ability of Gel Inserts to Retain DNA, RNA, and Protein Radiolabeled Materials During Horizontal Tube Gel Electroelution

In this and all subsequent studies all tube gel electroelutions were performed in a horizontal position submerged in electroelution buffer. In this experiment mouse L-1210 cells were radiolabeled with either [^{14}C]thymidine, [^{14}C]uridine, or [^{14}C]leucine for 24 hours in high serum and then incubated in low serum for 24 hours without radiolabel. They were exposed to SDS by briefly electrophoresing it into the inserts from the adjacent tube gel. Control tubes were sham treated. After a 30 minute incubation (in the absence of electrophoresis) the gel inserts were electrophoresed for 3 hours in Tris-EDTA buffer and the capsules removed from the tube gels. The gel inserts were removed from the capsules, melted, and counted in aquasol. Tube gels were melted and 0.5 ml aliquots counted in aquasol. The relative retention of radiolabel by gel inserts was determined by dividing the counts obtained for each gel insert by the total counts obtained for that gel insert and its respective tube gels. Only the anode tube gels were used for DNA and RNA counts whereas both anode and cathode tube gels were used to determine protein counts. The average relative retention of radiolabel by gel inserts is indicated with standard errors.

Treatment	Thymidine	Uridine	Leucine
Sham	0.53 ± 0.10	0.28 ± 0.05	0.58 ± 0.05
SDS	0.34 ± 0.02	0.24 ± 0.02	0.06 ± 0.05

nicking followed by exonuclease digestion. It was an attempt to substantiate the hypothesis that DNA retention by gel inserts following electroelution is due to the presence of complex circular forms of DNA and not due to large linear DNA molecules. The purpose of the experiments performed here was to determine if DNA trapping could be detected by horizontal tube gel electroelution. When cells are trapped in agarose and lysed with lithium dodecyl sulfate, empty pockets can be seen by electron microscopy which appear to be voids left by the lysed cells (Cook, 1984). I assumed that such pockets contained DNA which was in an "untrapped state", i.e., the DNA which was released following cell lysis existed in an aqueous environment devoid of gel matrix because of the pocket created by the intact cell during gel formation. Therefore, I reasoned that in order to trap this DNA, the gel insert must be extracted in SDS in a

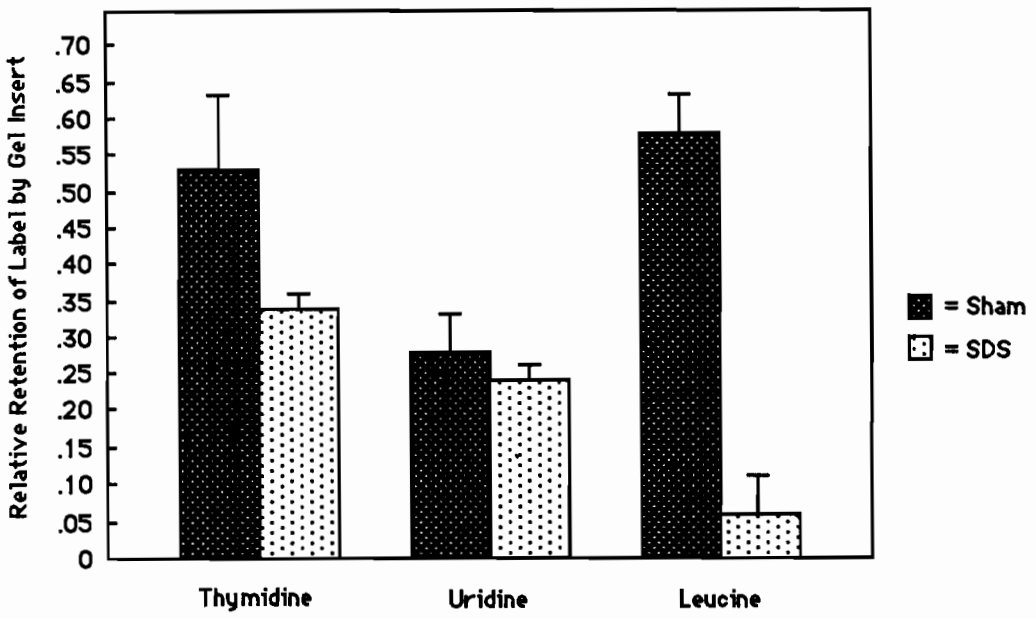


Figure 5

Graph of Data From Table 5

Histograms depict average relative retention of label by gel inserts together with standard error bars.

liquid state or extracted and then melted and regelled. Two sets of experiments were performed to test this hypothesis. The two main differences between the two experiments was the use of Tris-EDTA (TE) versus Tris-magnesium chloride buffers and the method of extraction in SDS. The results for gel inserts electroeluted in TE and TM buffers are described in Tables 7 and 8, respectively. In both experiments three sets of gel inserts were used: These included non-melted, melted, and melted and regelled (regelled) inserts (Table 7) and non-melted, liquid, and gelled inserts (Table 8). Melted inserts were exposed to SDS prior to melting whereas liquid inserts are exposed to SDS prior to gelling. Regelled inserts have been exposed to SDS prior to melting, and are then melted, and regelled. Gelled inserts are liquid inserts which have been gelled. In the experiment described in Table 8 the gel inserts were made up of either whole cells or nuclei in order to determine if any differences in retention could be noted between label retention of gel inserts made of whole cells

Table 6

Percent Retention of Radiolabeled Materials From Gel Inserts In the Presence or Absence of SDS Using Data From Table 5

Data row 2 was subtracted from data row 1 in Table 5 and multiplied by 100% to obtain the data presented here in data row 2. Data row 2 from Table 5 was multiplied by 100% and subtracted from 100% to obtain data row 3 below. In Table 6 data row 2 was subtracted from data row 3 to obtain data row 1.

% Loss Due to:	Thymidine	Uridine	Leucine
Electroelution	47	72	42
SDS Extraction	19	4	52
Both	66	76	94

versus nuclei. Whole cells versus nuclei were used to see if cytoplasmic DNA's such as mitochondrial DNA were significantly influencing DNA retention by gel inserts.

The results obtained from the TE experiment indicated that gel insert retention of DNA label was reduced from 0.38 to as low as 0.30 in inserts which were either melted or melted and regelled as compared to unmelted controls (Table 7). In contrast, non-melted gel inserts containing lysed cells electroeluted in TM buffer demonstrated a much higher retention than their counterparts which were electroeluted in TE buffer. Also, there was less difference in the retention ratios of non-melted, liquid, and gelled inserts (Table 8). Very little difference was noted in the retention ratios of nuclei label electroeluted in TM buffer. Apparently the magnesium functioned as a DNA "binder", crosslinking DNA strands via divalent electrostatic bridges and facilitating DNA retention during gel electroelution. The retention of protein label by inserts in TE buffer was similar to that described in Table 5, or 0.05 and 0.06, respectively.

Final Parameter Determinations for Subsequent Horizontal Tube Gel Electroelutions

The data obtained from the horizontal tube gel experiments previously described were compiled together with additional data from other experiments and the results are

Table 7

Retention of Radiolabeled Materials in Non-melted, Melted, and Melted and Regelled Gel Inserts Following Electroelution in TE Buffer

Mouse L-1210 cells in this experiment were radiolabeled with [^{14}C]thymidine or [^{14}C]leucine. The gel insert capsules were initially treated similar to those described in Table 5 except that twice the volume of SDS gel was used. After exposure to SDS the capsules were removed and two of three sets were heated to 70°C in Tris buffer containing 0.1 M NaCl. After heating, one set of capsules was chilled on ice to regel the inserts and the other set was kept at 37°C to prevent regelling. All capsules were electroeluted in Tris-EDTA buffer at 37°C to prevent regelling of the melted gel inserts. The average relative retention of radiolabel by gel inserts is indicated with standard errors.

Treatment	Thymidine	Leucine
Non-melted	0.38 ± 0.06	0.05 ± 0.004
Melted	0.30 ± 0.05	----
Regelled	0.33 ± 0.02	----

shown in Table 9. As can be seen, a considerable number of parameters were altered including the use of whole cells or isolated nuclei, method of SDS extraction, physical state of the inserts, i.e., (liquid versus gel), electroelution buffer, time, voltages, temperature, and exposure (or not) to RNAse A. Therefore, this table represents a compilation of many different kinds of experiments which were conducted during the developmental stages of the horizontal tube gel electroelution technique. The methodology of choice for future experiments was the one which removed most of the non-DNA material with a minimum loss of DNA label. In Experiment 2 three hours of electroelution removed 76% and 94% of the RNA and protein label respectively while removing 66% of the DNA label. In Experiment 6 only 59% of the DNA label was removed after 3 hours of electroelution together with 84% and 86% of the RNA and protein label respectively. In Experiment 7 three hours of electroelution removed 91% and 97% of the uridine and leucine labels respectively while losing only 30% of the DNA label. After 6 hours of electroelution without incubation in RNAse A (Experiment 8) the DNA, RNA, and protein label removed was

Table 8

DNA Radiolabel Retention in Non-melted, Liquid, and Gelled Gel Inserts Following Electroelution in TM Buffer

Mouse L-1210 cells in this experiment were radiolabeled with [^{14}C]thymidine. Liquid gel inserts containing whole cells or isolated nuclei were overlayered onto a 1% SDS agarose plug at 37^{o}C and allowed to incubate in the presence of the SDS for one hour. Some of these liquid inserts were gelled on ice and referred to as gelled inserts. The others were kept liquid and referred to as liquid inserts. Non-melted inserts were gelled prior to exposure to SDS plugs. All of the inserts were coupled to tube gels and electroeluted at 37^{o}C to prevent gelling of melted inserts. After electroelution, the melted inserts were chilled, gelled, and removed with the other inserts. All inserts were melted and counted. The average relative retention of radiolabel by the inserts is indicated with standard errors.

Treatment	Cells	Nuclei
Non-melted	0.84 ± 0.02	0.93 ± 0.001
Liquid	0.91 ± 0.01	0l95 ± 0.04
Gelled	0.92 ± 0.001	0.94 ± 0.01

53%, 95% and 95% respectively. The combination of SDS extraction of inserts in TE buffer with agitation for 3 hours or overnight coupled with electroelution in TM buffer appeared to give the best DNA retention coupled with minimum RNA and protein retention (Experiments 7 and 8). It also had a distinct advantage in that EDTA was removed during electroelution, allowing for subsequent incubations in EDTA-sensitive enzyme solutions. Therefore, this method of extraction was established as the methodology of choice for all other subsequent experiments involving horizontal tube gel electroelution.

Table 9

A Comparison of the Average Ratios of Retention of Radiolabeled Materials in Gel Inserts After Various Forms of SDS Treatment and Gel Electroelutions.

The table below presents data on the average ratios of retention of gel inserts following several modifications in SDS extraction, gelation of inserts, and electroelution procedures. Methods of extraction in SDS included the following: 1) electroeluting SDS into the gel inserts for 30 minutes followed by a quiescent incubation prior to electroelution {Experiments 2, 3, and 6}, 2) overlaying an SDS agarose plug with liquid gel inserts at 37°C followed by a quiescent incubation prior to gel electroelution {Experiment 4}, and 3) extraction of gel inserts at room temperature in 1% SDS in TE for 3 hours or overnight with agitation prior to electroelution {Experiments 7 and 8}. Incubations in RNAse A during the course of electroelution was also performed in Experiments 6 and 8. Electroelutions were performed at either 37°C (Experiments 2, 3, and 4) or at room temperature (Experiments 6, 7, and 8). The "Exp" column indicates the number of the experiment. [a]8 indicates no incubation with RNAse A. [b]8 indicates an incubation with RNAse A after 3 hours of electroelution followed by three more hours of electroelution. The "Table" column indicates the table in the Dissertation (if any) from which the data was derived. The "Insert" column refers to the method in which the inserts were gelated. "N" is non-melted inserts, "M" is melted inserts, "R" is melted and regelled inserts (Table 7), "L" is liquid inserts which have not been gelled prior to SDS extraction, and "G" is liquid inserts which have been gelled prior to electroelution (Table 8). The "Buffer" column indicates the composition of the electroelution buffer, Tris-EDTA (TE) or Tris-magnesium chloride (TM). The "Hours" column refers to the hours of electroelution. The "Volts" column indicates the voltage applied during the electroelution. Gel inserts contained cells or nuclei radiolabeled with [^{14}C]thymidine, [^{14}C]uridine, or [^{14}C]leucine. The retention ratios for these three radiolabels are found under the columns labeled "Thymidine, "Uridine, and "Leucine" respectively. In Experiments 3 and 4, data from cells and isolated nuclei containing DNA radiolabel were averaged together. Experiments 6 and 7 are not illustrated in other tables or figures. Data used from Experiments 7 and 8 were pooled and averaged. Standard errors are indicated where appropriate.

Exp	Table	Insert	Buffer	Hours	Volts	Thymidine	Uridine	Leucine
2	5	N	TE	3	40	0.34 ± 0.02	0.24 ± 0.02	0.06 ± 0.05
3	7	N, M, R	TE	15	35	0.33 ± 0.03	----	0.05 ± 0.01
4	8	N, L, G	TM	8	20	0.91 ± 0.01	----	----
6	-	N	TM	9	50	0.41	0.16	0.13
7	-	R	TM	3	40	0.70	0.09	0.03
8[a]	20	R	TM	6	40	0.47	0.05	----
8[b]	20	R	TM	6	40	0.36	0.03	0.05

Discussion

Any conclusions drawn from the results discussed in Table 2 must be tempered by the lack of radiolabeled data confirming that the ethidium bromide generated bands were either DNA or RNA. Nonetheless, the large bands generated during electroelution from lysed CHO cells extracted in 2M NaCl and their absence from CHO cell nuclei extracted in 2M NaCl suggest the presence of high levels of cytoplasmic DNA in these cells. The large bands generated from the fibroblasts tested may also be cytoplasmic in origin although this was not confirmed. The results further indicate the presence of a form of nuclear DNA superstructure in salt extracted nuclei which is sensitive to treatments with proteinase K, SDS, urea, and alkali denaturation. Some of this sensitivity is probably due to the presence of Okazaki DNA fragments which have not yet been incorporated into large molecular weight DNA. However, the results of experiments presented later involving radiolabeled mouse L-1210 cells indicated that as much as 67% of the DNA could be removed by electroelution in cells which are being inhibited from replicating by serum arrest (Table 5). Such a sizeable loss of DNA from cells undergoing serum arrest can hardly be accounted for by Okazaki fragment loss. Therefore, the majority of this loss must be accounted for by the nature of the DNA superstructure associated with these cells.

The results in Table 3 indicate that the ability of gel inserts to retain uridine radiolabeled material after extraction in SDS and electroelution was not significantly affected by incubations in RNAse A or proteinase K but was affected by incubations in phospholipase C and DNAse I. In an earlier non-labeled experiment, incubation of inserts in the presence of proteinase K and RNAse A released a small of amount of material during electroelution (Plate XXIIf). However, it could not be determined which enzyme (if either) may have been responsible for the loss. A later experiment has revealed that retention of uridine radiolabeled material by inserts is slightly reduced when they are incubated in RNAse A followed by electroelution (Table 20).

Submersible horizontal tube gel electroelution may be used as an assay procedure for determining the relative retention of DNA, RNA, and protein radiolabel by gel inserts. Analysis of the parameter changes indicated that the greatest retention of DNA and the smallest retention of RNA and protein occurred when gel inserts were first extracted overnight in SDS-TE buffer followed by electroelution in TM buffer. The

importance of the way in which SDS is used in the extraction of RNA and protein label was clearly indicated by these experiments. Protein was easily removed either by overnight extraction in SDS or by electrophoresing SDS into the insert. RNA was more thoroughly removed by an overnight extraction of the insert in SDS-TE buffer followed by electroelution.

The inability of the exonuclease activities of DNA polymerase I to affect DNA label retention (Table 4) may be due to several reasons. The exonuclease activities may have been inhibited in the LE agarose in which they were impregnated even though DNAse I activity was still present (Maniatis, 1982). The time of incubation and/or the enzyme concentration may have been insufficient to detect any activity on large DNA molecules. And the exonuclease activities may have simply had no effect on DNA retention. Such possibilities require further investigation.

The ability to distinguish between relative retention of DNA label by gel inserts which were either non melted, melted or (liquid), or melted and regelled or (gelled) indicated that such differences in retention were very dependent upon the buffer system used (Tables 7 and 8). It was hypothesized that melted and regelled inserts would have the greatest amount of DNA label retention whereas non-melted gel inserts contained aqueous pockets after cell lysis in which the DNA existed in a non-trapped state similar to that which exists in melted gel inserts. It was interesting to learn that non-melted gel inserts retained more label than the other types when TE buffer was used but they contained less DNA label when TM buffer was used. TM buffer also retained much more of the DNA in all groups than did TE buffer. The retention differences seen in cell gel inserts electroeluted in TM buffer were not seen in nuclei. Reasons for these different responses remain unclear.

CHAPTER V

MATRIX AND NON-MATRIX DNA, RNA, AND PROTEIN RETENTION STUDIES

Introduction

During the course of early studies with the alkaline elution of X-ray damaged DNA through polycarbonate filters it was discovered by Dr. Nirendra Singh that a portion of the DNA was resistant to alkaline elution and that once bound to the filter it was resistant to even large doses of X-ray irradiation (data not shown). It occurred to me that such retention may be due to remnants of the DNA superstructure which was discussed in Chapter IV. I hypothesized that this DNA was located at the DNA domain attachment sites within the nuclear matrix. If so, then, matrix DNA should be preferentially retained more than non-matrix DNA on polycarbonate filters following alkaline elution. A similar situation should also occur during the course of electroelution, i.e., matrix DNA should be preferentially retained more than non-matrix DNA within gel inserts following electroelution. Therefore, the focus of these studies was on the ability of matrix DNA to be selectively retained more than non-matrix DNA under conditions of alkaline elution and gel electroelution.

Results

DNA, RNA, and Protein Retention Studies During Alkaline Elution on Polycarbonate Filters

The results shown in Tables 10 and 11 are from alkaline elution experiment 1 in which DNA was incubated in polycarbonate filter units for 1 hour at 37^{o}C in NaOH, pH 13.5 followed by rapid elution of the DNA with injections of NaOH through the filters. As shown in Table 10, data row 6 the majority of the DNA (60-65%), RNA, (64%)

Table 10

The Separation of Nuclear DNA into Matrix and Non-matrix Fractions for Alkaline Elution Through Polycarbonate Filters (Filter Experiment 1)

Mouse L-1210 cells were double radiolabeled with either [^{3}H]thymidine and [^{14}C]leucine or [^{14}C]thymidine and [^{3}H]uridine. The nuclei were purified and digested for 5 minutes in DNAse I and the reaction was terminated on ice with the addition of EDTA. The digested nuclei were extracted in 2M NaCl and centrifuged to generate the non-matrix supernatant fraction and the pelleted matrix fraction. The CPM shown in the table should be multiplied by 1000.

Nuclear Fraction	[^{3}H]Thy	[^{14}C]Thy	[^{3}H]Uri	[^{14}C]Leu
Matrix Label	131	521	213	1880
Non-Matrix Label	70	345	118	526
Total Label	201	866	331	2406
% of Total Label in Matrix	65	60	64	78

and protein labels (78%) resided within the matrix fractions. Matrix DNA generally contains 5% or less of the total DNA (Basler et al, 1981; Smith et al, 1984). Therefore, the label described in these two tables will be referred to as matrix-enriched label. Table 11 indicates the total amount of matrix-enriched and non-matrix labels applied and retained by the filters in data rows 1,2, 4, and 5 and it indicates the percent retention of the label in data rows 3 and 6. This percent retention is graphically illustrated in Figure 6. The ratios of matrix-enriched to non-matrix labels applied to filters is shown in data row 7 and the ratios of matrix-enriched to non-matrix labels retained by filters is shown in data row 8. The ratios of the percent matrix-enriched to non-matrix labels retained by filters is shown in data row 9. This ratio is called the retention index. When it is greater than 1 this indicates that the matrix sample is being retained more than the non-matrix samples by filters during alkaline elution. In this experiment all of the matrix-enriched label was incubated in the filters whereas only a portion of the non-matrix label was applied to its respective filters because the large volume of the non-matrix extract precluded the incubation of the entire volume unless most of it was injected through the filter.

Table 11

The Alkaline Elution of Matrix and Non-matrix DNA, RNA, and Protein Radiolabeled Materials Through Polycarbonate Filters (Filter Experiment 1)

Matrix and non-matrix samples (Table 10) were injected through filter units containing polycarbonate filters. The units were sealed on the bottom, filled with NaOH at pH 13.5, and incubated for 1 hour at 37°C. They were then rinsed with fresh NaOH solution to elute as much of the radiolabel as possible. The CPM shown in the table should be multiplied by 1000. The percent matrix or non-matrix retained is calculated by dividing the matrix or non-matrix counts retained by their respective applied counts and multiplying by 100%. The ratio of applied matrix to non-matrix is calculated by dividing the matrix counts applied by the non-matrix counts applied. The ratio of retained matrix to non-matrix is calculated by dividing the matrix counts retained by the non-matrix counts retained. The retention index is calculated by dividing the percent matrix retained by the percent non-matrix retained.

Nuclear Fraction	[^{3}H]Thy	[^{14}C]Thy	[^{3}H]Uri	[^{14}C]Leu
Matrix Applied	131	521	213	1880
Matrix Retained	11	41	13	180
% Matrix Retained	8	8	6	10
Non-Matrix Applied	20.1	63.6	25.9	107.8
Non-Matrix Retained	1.0	1.0	0.5	1.8
% Non-Matrix Retained	5	2	2	2
Ratio of Applied Matrix to Non-matrix	6.5	8.2	8.2	17. 4
Ratio of Retained Matrix to Non-Matrix	11	41	26	100
Retention Index	1.6	4	3	5

It was decided that preinjection of the non-matrix fraction through the filters prior to incubation would bias the results more than the addition of less material. These differences in the addition of matrix-enriched and non-matrix labels to the filters are reflected in data column 7. Six to 8 times more DNA and RNA and labels were applied to matrix-enriched filters than to non-matrix filters. Seventeen times more protein label was applied to the former than to the latter filters. The ratios of retained matrix-enriched to non-matrix labels were quite high, ranging from 11 to 41 for DNA label, 26 for RNA and 100 for protein labels. However, when the data is

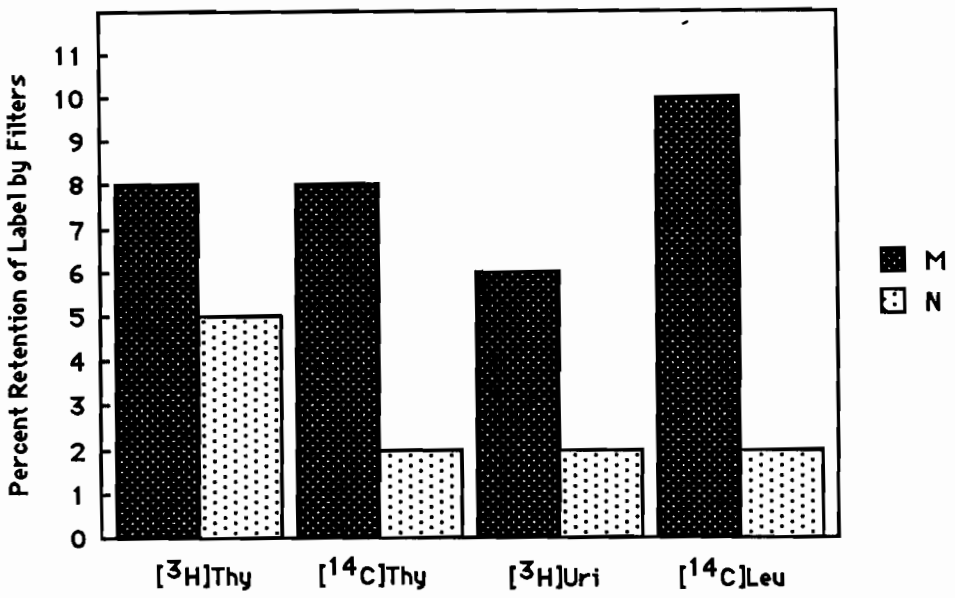


Figure 6

The Relative Retention of Matrix and Non-matrix DNA, RNA, and Protein Radiolabels by Polycarbonate Filters Following Incubation and Elution in Alkali (Filter Experiment 1).

Data is from Table 11, data rows 3 and 6.

normalized against the amount of labels applied these ratios drop considerably. The percentage of matrix-enriched DNA retained (data row 3) is 8% for DNA, 6% for RNA, and 10% for protein labels. The same figures for non-matrix labels (data row 6) are 2 to 5% for DNA, 2% for RNA, and 2% for protein labels. These results are shown in graphical form in Figure 6. When these data are used to obtain the retention indices shown in data row 9 the results indicated that matrix-enriched labels were retained more than non-matrix labels by as much as 1.6 to 4 times for DNA labels, 3 times for RNA label, and 5 times for protein label.

The results obtained from this experiment were interesting but not conclusive. The experiment was repeated again using more stringent conditions. The samples were incubated in 0.3 M KOH for 16 hours at 37^{o}C prior to rapid alkaline elution. Furthermore, the matrix DNA was more thoroughly digested by DNAse I this time such

Table 12

The Separation of Nuclear DNA into Matrix and Non-matrix Fractions for Alkaline Elution Through Polycarbonate Filters (Filter Experiment 2)

Mouse L-1210 cells were double radiolabeled with either [^{3}H]thymidine and [^{14}C]leucine or [^{14}C]thymidine and [^{3}H]uridine. The nuclei were purified and digested for 5 minutes in DNAse I and the reaction was terminated on ice with the addition of EDTA. The digested nuclei were extracted in 2M NaCl and centrifuged to generated the non-matrix supernatant fraction and the pelleted matrix fraction. The CPM shown in the table should be multiplied by 1000.

Nuclear Fraction	[^{3}H]Thy	[^{14}C]Thy	[^{3}H]Uri	[^{14}C]Leu
Matrix Label	389	146	318	54
Non-matrix Label	5023	2038	1293	460
Total Label	5412	2184	1611	514
% of Total Label in Matrix	7.2	6.7	19.7	10.5

that the percent of the DNA, RNA, and protein labels contained in this fraction were reduced to 6 to 7% , 20%, and 11% respectively (see Table 12, data row 4). Because of this reduction in label content the term matrix-enriched was no longer substituted for "matrix". This resulted in the addition of less matrix than non-matrix labels to the filters (Table 13, data rows 1 and 4) which shifted the ratios of matrix to non-matrix labels applied to filters down to as low as 0.07 to 0.08 for DNA, 0.25 for RNA, and 0.12 for protein labels respectively (Table 13, data row 10). The ratios of retention of matrix to non-matrix label was also shifted in favor of non-matrix retention. DNA, RNA, and protein labels ratios were reduced to 0.46 to 0.75, 0.67 and 0.50 respectively. However, when the label retained was normalized against the label applied and converted to percentages the matrix label was found to be more retained than the non-matrix label as seen in Table 13, data rows 3 and 6. This data is graphically illlustrated in Figure 7. The retention indices were 7 to 10 for DNA, 3 for RNA, and 3.7 for protein labels, respectively. The numbers obtained for RNA and protein retention are in close agreement to what was found in the first experiment but the DNA retention was much greater in the second than in the first experiment. Since

Table 13

The Alkaline Elution of Matrix and Non-matrix DNA, RNA, and Protein Radiolabels Through Polycarbonate Filters (Filter Experiment 2a)

Matrix and non-matrix samples (Table 12) were injected through filter units containing polycarbonate filters. The units were sealed on the bottom, filled with 0.3 N KOH and incubated for 16 hours at 37°C. They were then rinsed with fresh KOH solution to elute as much of the radiolabel as possible. The CPM shown in the table should be multiplied by 1000. The percent matrix, non-matrix, or B-10 label retained is calculated by dividing the matrix, non-matrix, or B-10 counts retained by their respective applied counts and multiplying by 100%. The ratio of applied matrix to non-matrix is calculated by dividing the matrix counts applied by the non-matrix counts applied. The ratio of retained matrix to non-matrix is calculated by dividing the matrix counts retained by the non-matrix counts retained. The retention index is calculated by dividing the percent matrix retained by the percent non-matrix retained.

Nuclear Fraction	[^{3}H]Thy	[^{14}C]Thy	[^{3}H]Uri	[^{14}C]Leu
Matrix Applied	195	73	159	27
Matrix Retained	0.13	0.03	0.10	0.03
% Matrix Retained	0.07	0.04	0.06	0.11
Non-matrix Applied	2510	1020	647	230
Non-matrix Retained	0.28	0.04	0.15	0.06
% Non-matrix Retained	0.01	0.004	0.02	0.03
B-10 Label Applied	67	123	198	213
B-10 Label Retained	0.01	0.02	0.06	0.00
% B-10 Label Retained	0.01	0.01	0.03	0.00
Ratio of Applied Matrix to Non-matrix	0.08	0.07	0.25	0.12
Ratio of Retained Matrix to Non-matrix	0.46	0.75	0.67	0.50
Retention Index	7	10	3	3.7

more non-matrix than matrix label was applied to the filters in the second experiment it seems safe to conclude that the differences in retention seen between matrix and non-matrix fractions are not due to non-specific binding because of concentration effects. This conclusion is supported further by a control filter to which was applied cell medium (B-10) in which the cells had been grown in the presence of the

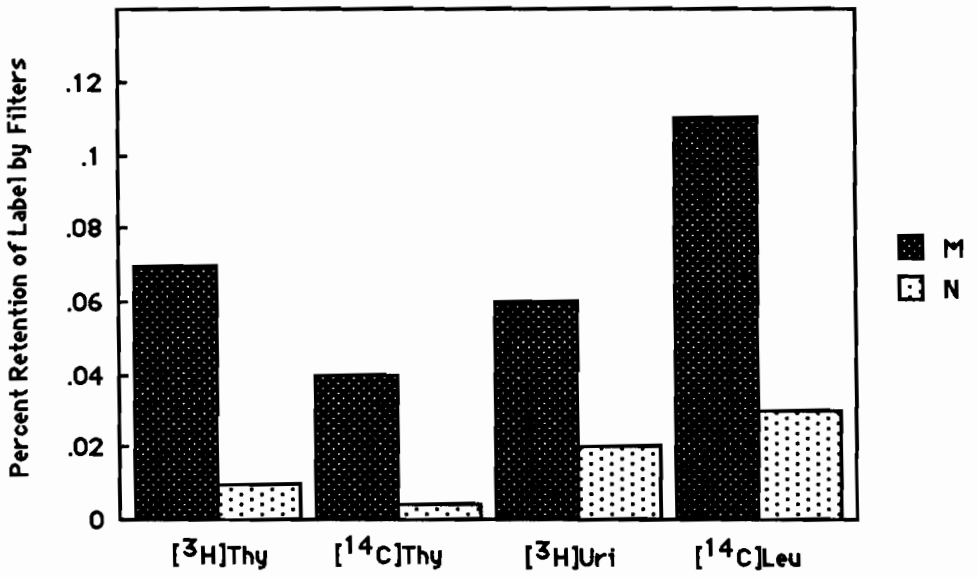


Figure 7

The Relative Retention of Matrix and Non-matrix DNA, RNA, and Protein Radiolabeled Materials by Polycarbonate Filters Following Incubation and Elution in Alkali

Data is from Table 13, data rows 3 and 6.

radiolabeled isotopes. As can be seen in Table 13, data row 9 the percent retention of the B-10 label was very similar to the percent retention seen for non-matrix label (Table 13, data row 6). This supports the conclusion that retention of non-matrix label by filters during alkaline elution is largely non-specific in nature whereas retention of matrix label by filters appears to be due to an inherent difference between matrix and non-matrix DNA, and their RNA, and protein constituents. This is graphically illustrated in Figure 8 where the non-specific binding of B-10 label has been subtracted from the matrix and non-matrix bound labels. All of the bound matrix label remains greater than zero whereas all of the bound non-matrix label is reduced to zero or less except for bound protein label.

The results described in Table 14 are from samples in the second experiment that were incubated in the presence of RNAse A after alkali incubation and elution. The

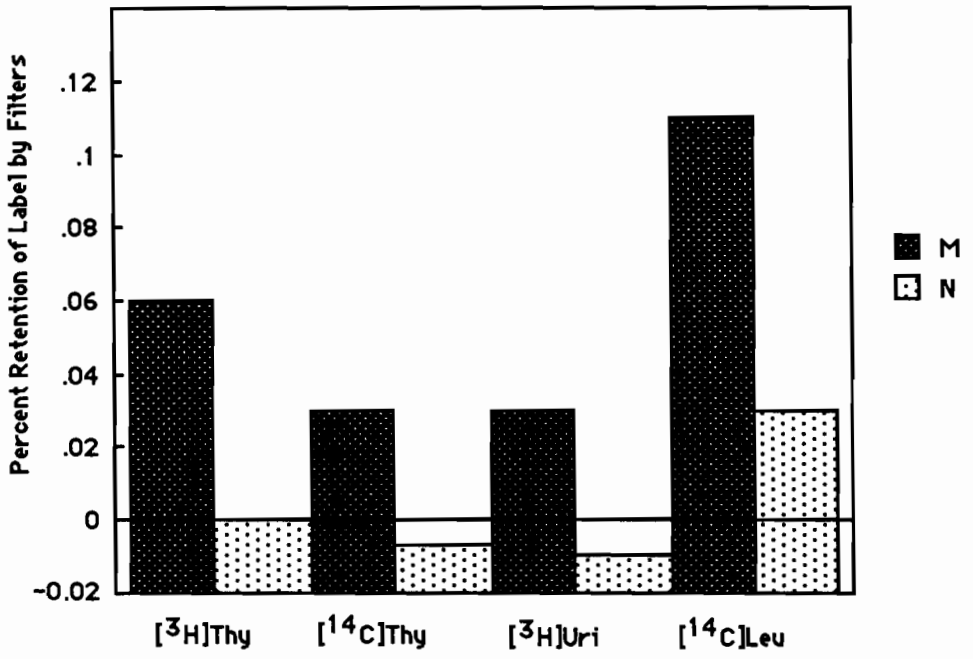


Figure 8

Figure 7 Results Minus Non-specific Retention of Radiolabeled Materials in B-10 Medium

Data is from Table 13. Data row 9 was subtracted from data rows 3 and 6.

filters were rinsed in PBS and incubated in RNAse A followed by a second elution in PBS. This incubation appeared to have no discernable negative effect on matrix RNA label retention (data rows 1 and 2) but did appear to affect the retention of non-matrix RNA label (data rows 3 and 4). The ability of RNAse A incubation to reduce the level of retention of DNA or protein in either matrix or non-matrix fractions was not detected. However, the apparent "increase" in retention seen in the [^{3}H]thymidine labeled DNA matrix fraction after RNAse A incubation indicates problems with experimental error. Therefore, while it is tempting to say that the RNAse A incubation destroyed only the non-matrix RNA such a conclusion must be tempered with the knowledge that these differences fall within the range of experimental error as seen in this particular system.

Table 14

The Alkaline Elution of DNA, RNA, and Protein Radiolabeled Materials Through Polycarbonate Filters Followed by Exposure to RNAse A and Additional Elution (Filter Experiment 2b)

Matrix and non-matrix samples were injected through filter units containing polycarbonate filters. The units were sealed on the bottom, filled with 0.3N KOH and incubated for 16 hours at 37°C. They were then rinsed with fresh KOH solution to elute as much of the radiolabel as possible. The units were rinsed in PBS and filled with a buffer solution. One set of units received buffer solution alone while a second set received buffer containing RNAse A. All units were incubated for 1 hour at 37°C and then eluted a second time using PBS. The CPM shown in the table should be multiplied by 1000.

Nuclear Fraction	[^{3}H]Thy	[^{14}C]Thy	[^{3}H]Uri	[^{14}C]Leu
Matrix Retained (No RNAse A)	0.13	0.03	0.10	0.03
Matrix Retained (RNAse A)	0.20	0.03	0.10	0.02
Non-matrix Retained (No RNAse A)	0.28	0.04	0.15	0.06
Non-matrix Retained (RNAse A)	0.29	0.03	0.10	0.05

Matrix and Non-matrix DNA and RNA Retention Studies During Agarose Gel Electroelution

A second assay system was designed to verify the results obtained from the alkaline elution filter experiments. This system involved testing the ability of gel inserts to retain matrix and non-matrix label following electroelution. During the early stages of development many kinds of parameters were being simultaneously analyzed in an attempt to gain a broader spectrum of understanding relative to matrix and non-matrix retention phenomena by gel inserts following electroelution. In the experiment described in Tables 15 and 16 three parameters were adjusted to determine their effects on the relative retention of matrix and non-matrix DNA label by gel inserts following electroelution: cell cycle kinetics (log phase versus serum arrested cells), pre-photography of gel inserts in U.V. light versus no pre-photography, and treatment of gel inserts with or without DNAse I. In this study whole nuclei samples were

Table 15

Total [^{14}C]Thymidine CPM in Gel Inserts Incubated in 40 Units/ml of DNAse I

The samples below were derived from either whole nuclei (M + N) or from non-matrix fractions (N). The "real" matrix fractions (M) were discarded because of clumping. Samples were derived from cells grown during conditions of serum arrest (SA) or during log phase (LP). The 50 minute counts were obtained from samples in aquasol which were 9 months old.

SA/LP	Nuclear Fraction	CPM
SA	(M + N)	533
"	N	382
LP	(M + N)	1063
"	N	1018

substituted for matrix because of problems with clumping of the matrix fractions. Hence the study conducted involved a comparison of DNA retention in matrix plus non-matrix fractions (M + N) versus the non-matrix fraction (N) alone. Only the data obtained from gel inserts treated with DNAse I were used in this analysis since the (M + N) DNA samples were actually salt extracted intact nuclear DNA which had never been exposed to DNAse I prior to gel insert formation. When all gel inserts were exposed to DNAse I this insured that most of the topological constraints imposed by intact DNA loops in intact nuclei would be destroyed. Therefore, any preferential retention of matrix DNA over non-matrix DNA would be due to other factors, e.g., a reticulated network of broken loops which migrate more slowly than linear DNA.

In later assays involving submersible horizontal tube gel electroelution, total counts were determined by adding up the counts electroeluted into a tube gel(s) plus the counts retained by the respective insert. However, in the assay discussed here total counts were determined using non-electroeluted inserts (Table 15) which were used in the compilation of the data described in Table 16. The latter table contains data for the relative retention of (M + N) and (N) samples together with retention indices (data column 3). The retention index is a ratio of the relative retention ratios of (M + N) to (N). A retention index

Table 16

Relative Retention of [^{14}C]Thymidine Radiolabeled Materials in Gel Inserts Incubated in 40 Units/ml of DNAse I Prior to Electroelution

The samples below were derived from either whole nuclei (M + N) or from non-matrix fractions (N). The "real" matrix fractions (M) were discarded because of clumping. Samples were derived from cells grown during conditions of serum arrest (SA) or during log phase (LP). Samples were either pre-stained in ethidium bromide and photographed in wells prior to and after electroelution or stained and photographed after electroelution (Plates XXIIIc and d). The purpose of this procedure was to determine if pre-photography had any effect on the relative retention of DNA by gel inserts. The non-electroeluted counts shown in Table 15 were divided into the electroeluted counts shown below in data column 1 to yield the ratios shown in data column 2. The respective (M + N) ratios were divided by the N ratios to obtain the retention indices shown in data column 3. Finally, all (M + N) and (N) retention ratios respectively were pooled, averaged, and displayed in data rows 9 and 10 along with their respective standard errors. The average retention index (R.I.) is shown in data column 3, row 9. The 50 minute counts were obtained from samples in aquasol which were 9 months old.

SA/LP	Nuclear Fraction	Pre-photo (Y/N)	CPM	Relative Retention	R. I.
SA	(M + N)	Y	10	0.019	2.4
"	N	"	3	0.008	---
"	(M + N)	N	7	0.013	1.3
"	N	"	4	0.010	---
LP	(M + N)	Y	10	0.009	1.5
"	N	"	6	0.006	---
"	(M + N)	N	12	0.011	1.8
"	N	"	6	0.006	---
Average	(M + N)	-	--	0.013 ± 0.002	1.6
"	N	-	--	0.008 ± 0.001	---

greater than 1 indicates that (M + N) samples are being retained by inserts to a greater extent than (N) samples. The data displayed is related to two of the three parameters previously discussed: cell cycle kinetics and photographic conditions used. The results did not suggest any significant differences in radiolabel retention by inserts because of changes in these two parameters and so the data was pooled, averaged, and displayed along with the standard error in data column 2, rows 9 and 10 reducing the data to a total of four gel

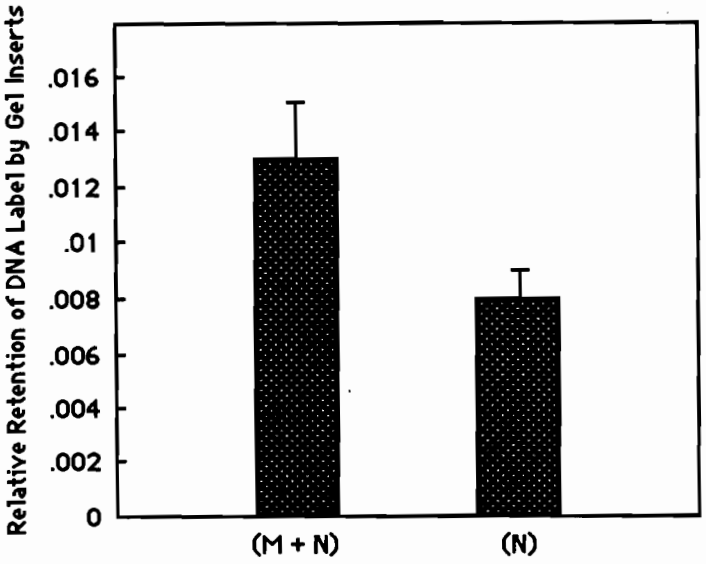


Figure 9

Graphical Illustration of Data Rows 9 an 10 From Table 16

inserts per (M + N) and (N) sample. The average relative retention ratios were 0.013 and 0.008 for (M + N) and (N) samples respectively. This is shown graphically in Figure 9. The retention index (1.6) is shown in data column 3, row 9. Matrix DNA was being retained 1.6 times more by gel inserts than non-matrix DNA.

The observation that matrix DNA was being retained more by inserts than non-matrix DNA was interesting but required further study which was more focused toward this phenomenon. Table 17 is displaying (M + N) and (N) data from mouse L-1210 cells which had been double radiolabeled with [^{3}H]thymidine and [^{14}C]uridine. The (M + N) fraction had been subjected to DNAse I treatment and extracted in 2M NaCl but had not been centrifuged to separate out the matrix pellet from the non-matrix supernatant because of clumping problems. The (N) samples were isolated in the usual manner. The major problem with this technique is that the non-matrix component of the (M + N) fraction may be as high as 98% or more of the total DNA and may quench the retention signal from the matrix component. One-half of the inserts were exposed to proteinase K following extraction in SDS and the other half were sham incubated. All inserts were then subjected to

electroelution. The results shown in data rows 1 and 2 indicated that incubation in proteinase K did not increase the loss of label in any of the inserts tested. Because of this, data rows 1 and 2 were pooled to generate data row 3 which is graphically depicted in Figure 10. The pooled retention ratios for [^{3}H]thymidine and [^{14}C]uridine were found to be 0.20 and 0.25 for (M + N) samples an 0.15 and 0.13 for (N) samples respectively (Table 17). These data demonstrate that the relative retention by gel inserts of (M + N) DNA and RNA was higher than that of (N) DNA and RNA, in agreement with the findings discussed in Table 16. The retention indices for DNA and RNA were 1.3 and 1.9 respectively (data row 4).

A final experiment was performed to test the affects of both alkali and proteinase K treatment on the ability of gel inserts to retain matrix and non-matrix DNA and RNA. In this study both the matrix and non-matrix fractions were used even though the possibility existed that pellet clumping might interfere with sample aliquoting in the matrix fraction. The direct comparison of matrix to non-matrix components was felt to be so important at this stage that problems with clumping would have to be tolerated. In order to minimize concentration effects, the non-matrix fraction (constituting 98% of the total DNA) was diluted down to the same concentration as the matrix fraction prior to gel insert formation. The inserts were exposed to proteinase K or sham incubated as before, equilibrated in either "neutral" (pH 8.0) or alkali buffer, and electroeluted in neutral and alkali gels respectively. The results are shown in Table 18. These data are depicted graphically in Figure 11 (thymidine results) and Figure 12 (uridine results). As can be seen in Figure 11 all of the matrix samples were retained to a much higher degree than their non-matrix counterparts regardless of treatment with proteinase K or alkali. In Figure 12 the RNA label is more highly retained in the non-matrix fraction under neutral conditions but this retention reverses under alkaline conditions; probably because of RNA hydrolysis. Treatment with proteinase K affected the retention of matrix DNA under neutral conditions and to a lesser extent under alkaline conditions but had little affect on the retention of non-matrix label under either neutral or alkaline conditions. A similar response occurred for the RNA label under neutral conditions. Under alkaline conditions the matrix RNA label retention was not perturbed by proteinase K treatment but a small loss in non-matrix RNA retention was seen. The retention indices are presented in Table 19. The affects of exposure to proteinase K and alkali are reflected in these indices. The retention index for thymidine after exposure to proteinase K drops from 5.5 to 4.0 under conditions of neutrality and from 8.3 to 7.8 under conditions of alkalinity. This indicates that the relative retention

Table 17

Relative Retention of (M + N) and (N) [^{3}H]Thymidine and [^{14}C]Uridine Radiolabeled Materials by Gel Inserts After Proteinase K Treatment

Mouse L-1210 cells were double radiolabeled with [3H]thymidine and [14C]uridine in high serum medium for 24 hours followed by incubation in low serum medium for 24 hours without radiolabel. Purified nuclei were isolated, subjected to DNAse I treatment and the reaction terminated by the addition of EDTA. The nuclei were extracted in 2M NaCl and divided into two sets. One set was separated into matrix and non-matrix fractions and the matrix pellet was discarded. The other set was not separated into fractions but used as an (M + N) fraction. This allowed the material to be aliquoted in a homogenous form during the formation of gel inserts. The inserts were extracted overnight in SDS, rinsed, and incubated in the presence or absence of proteinase K for 18 hours at 37°C. The inserts were subjected to horizontal tube gel electroelution and the counts retained by the inserts and collected by the tube gels were determined by liquid scintillation. The counts for each insert and its respective tube gel were added together and referred to as total counts per set. This number was divided into the counts retained by the insert of that set to determine the relative retention of label by the gel insert following electroelution. These numbers were averaged and indicated in the table along with standard errors. Because very little difference was noted between the relative retention data for sham and proteinase K treated gel inserts these numbers were averaged and standard error determined (data row 3). The retention indices (R. I.) in data row 4 were calculated from data row 3 by dividing (M + N) samples by their respective (N) samples.

	(M + N)		(N)	
Treatment	[^{3}H]Thymidine	[^{14}C]Uridine	[^{3}H]Thymidine	[^{14}C]Uridine
Sham	0.20 ± 0.02	0.22 ± 0.02	0.15 ± 0.01	0.13 ± 0.01
Proteinase K	0.19 ± 0.03	0.27 ± 0.08	0.15 ± 0.01	0.13 ± 0.01
Average	0.20 ± 0.02	0.25 ± 0.05	0.15 ± 0.01	0.13 ± 0.01
R. I.	1.3	1.9	----	----

of matrix DNA has been reduced by exposure to proteinase K under both neutral and alkaline conditions. The reduction of the RNA retention index from 0.9 to 0.4 under conditions of neutrality indicates that the relative retention of matrix RNA has been reduced by exposure to proteinase K. However, the exposure of RNA to proteinase K followed by electroelution in alkali reverses the data in two ways: the retention index is greater than one instead of less than one and it is increased still further after exposure to proteinase K. RNA hydrolysis in alkali probably explains the reverse

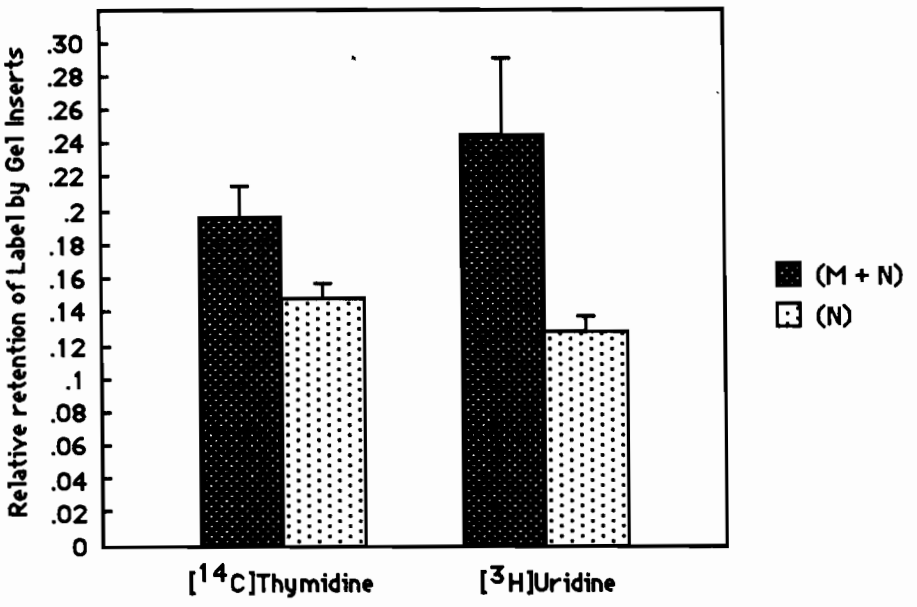


Figure 10

Graphical Representation of Data Row 3 From Table 17

in the retention index. The increase in the index following exposure to proteinase K reflects the reduction in non-matrix RNA retention resulting from enzyme treatment. This was not observed for the non-matrix DNA label (Figure 11).

Table 18

Relative Retention of Matrix and Non-matrix [^{3}H]Thymidine and [^{14}C]Uridine Radiolabeled Materials by Gel Inserts After Proteinase K and/or Alkali Treatments

Human Reh cells were double radiolabeled with [^{3}H]thymidine and [^{14}C]uridine in high serum medium for 24 hours followed by a 24 hour incubation in low serum medium without radiolabel. The nuclei were purified and used in the production of matrix and non-matrix fractions. The non-matrix fraction which comprised 98% of the total DNA counts was diluted down to the level of the matrix fraction prior to gel insert production. Gel inserts were made from both fractions, extracted overnight in SDS, rinsed, and equilibrated in TE buffer. They were then incubated for 25 hours at 37^{o}C in buffer with or without proteinase K. They were removed and equilibrated for 2 hours in either TE buffer, pH 8.0 or 50 mM NaOH, 1 mM EDTA prior to electroelution in neutral and alkaline slab gels respectively. The slab gel lanes were cut out, melted, and aliquots used to determine the counts electroeluted from each gel insert. Each slab lane count was added to its respective electroeluted insert count to obtain total counts for that set. This was divided into the counts obtained for the electroeluted gel insert to establish the relative retention ratio of that gel insert for DNA and RNA labels. The retention ratios were averaged and are presented below together with standard errors.

	Matrix		Non-matrix	
Treatment	[^{3}H]Thymidine	[^{14}C]Uridine	[^{3}H]Thymidine	[^{14}C]Uridine
Neutral, no Pk	0.71 ± 0.06	0.44 ± 0.06	0.13 ± 0.01	0.51 ± 0.02
Neutral, Pk	0.52 ± 0.02	0.22 ± 0.02	0.13 ± 0.01	0.52 ± 0.07
Alkali, No Pk	0.50 ± 0.03	0.17 ± 0.02	0.06 ± 0.01	0.08 ± 0.01
Alkali, Pk	0.47 ± 0.01	0.17 ± 0.04	0.06 ± 0.01	0.06 ± 0.02

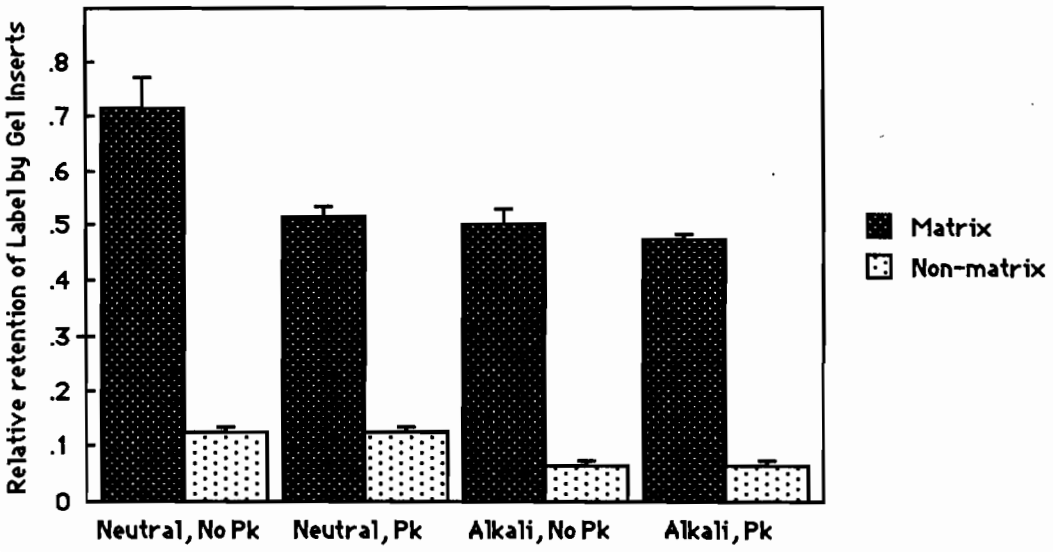


Figure 11

The Relative Retention of Matrix and Non-matrix DNA Radiolabeled Materials by Gel Inserts After Electroelution in the Presence or Absence of Alkali and Proteinase K

From data columns 1 and 3, Table 18.

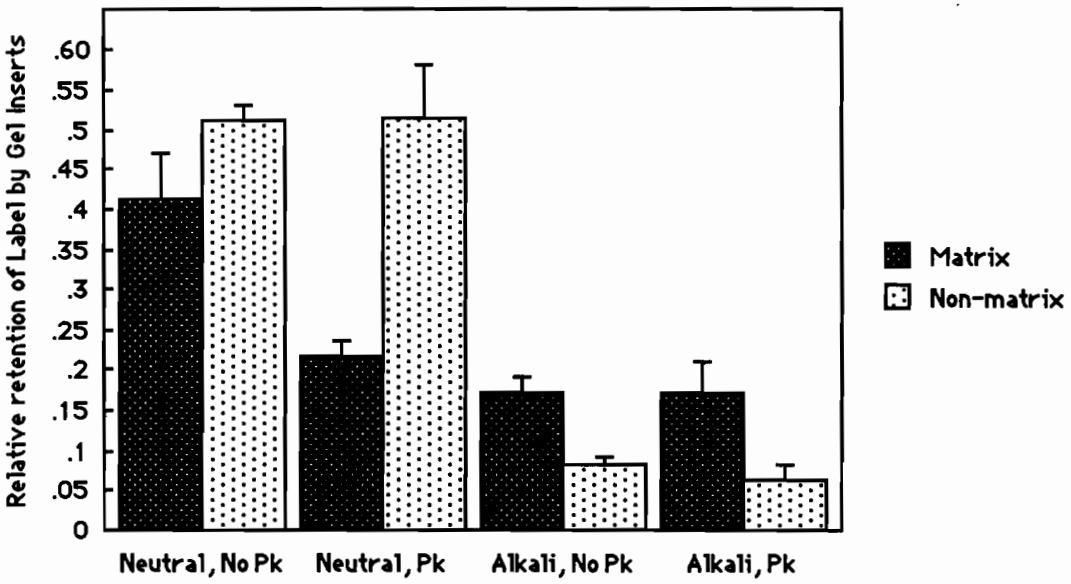


Figure 12

The Relative Retention of Matrix and Non-matrix RNA Radiolabeled Materials by Gel Inserts After Electroelution In the Presence or Absence of Alkali and Proteinase K

From data columns 2 and 4, Table 18.

Table 19

Retention Indices Derived From Data in Table 18

The data presented below was derived from Table18 by dividing data from columns 1 and 2 by data from columns 3 and 4 respectively.

Treatment	[^{3}H]Thymidine	[^{14}C]Uridine
Neutral, no Pk	5.5	0.9
Neutral, Pk	4.0	0.4
Alkali, No Pk	8.3	2.1
Alkali, Pk	7.8	2.8

Discussion

It could be argued that the "preferential retention" of matrix over non-matrix label in these experiments is due to a greater average DNA fragment size in the matrix fraction because it is more protected from DNAse I digestion. Such a possibility seems unlikely, however, in the case of the filter retention experiments where such retention should be more size-independent in the case of DNA fragmented by DNAse I and exhaustively denatured in alkali. Therefore, the combined results obtained from alkaline elution and gel electroelution experiments suggest that differences in retention between matrix and non-matrix fractions may be the result of differences between their DNA superstructures and their associations with RNA and protein constituents.

Classical RNA molecules should have been completely destroyed by the conditions of incubation imposed in Experiment 2. Nonetheless, RNA labeled material was still capable of being retained by filters containing either matrix or non-matrix fractions. Freely diffusable proteins, even in a denatured form should pass rapidly through the large pores of the polycarbonate filters used in the alkaline elution experiments. Even so, protein label was still being retained in both fractions. The results from the B-10 label retention experiment suggest that retention of non-matrix DNA and RNA label is not above background retention. If so, then, the protein retention observed in the non-matrix fraction must be due to factors other than association with DNA. However, the results from the B-10 experiment should be considered carefully because the B-10 medium which was used was removed from the cell pellet supernatant and could, therefore, contain DNA from lysed cells.

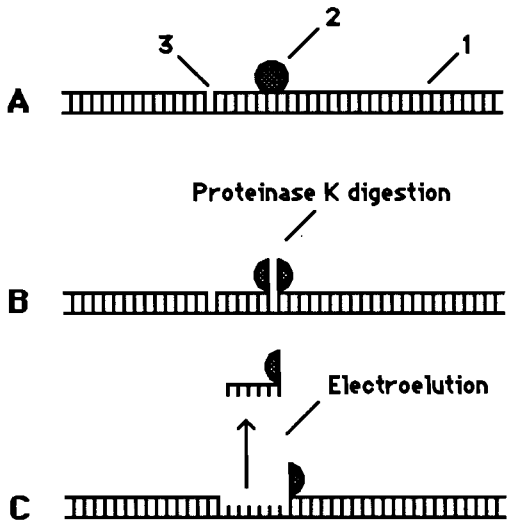
The retention of alkali resistant matrix RNA label could be a direct result of its intimate association with DNA molecules via covalent interactions (Birkenmeyer et al, 1987). A similar case could be stated for protein label(Liu et al, 1983; Sander and Hsieh, 1983; Cook, 1984; Osheroff and Zechiedrich, 1987). Some of the "covalently bound" non-matrix RNA and protein label could have originated from the matrix as supported by evidence discussed in Chapter VII. In order for RNA to be retained with DNA under alkaline conditions it must be protected from hydrolysis by covalently bonding the 2' position of the ribose sugar to another molecule. However, the possibility for explaining RNA resistance to RNAse A and alkali is that the uridine label is converted into thymidine via ribonucleotide reductase and incorporated into the DNA.

When whole cells are lysed in 2M NaCl and non-ionic detergent the resulting nucleoids appear as giant networks of loops of DNA emerging from the nuclei (McCready et al, 1980). A similar situation occurs when cells containing metaphase chromosomes are lysed under similar conditions (Mullinger and Johnson 1980; Laemmli et al, 1977; Paulson and Laemmli,1977). It appears that the nuclear matrix and chromosomal scaffolding are very similar in structure and composition (Bekers et al, 1986). Transmission electron microscopy indicates that the DNA loops which are released from the scaffolding of histone-depleted metaphase chromosomes are non-branched and return back to the same location within the scaffolding. In contrast, the structure of scaffold DNA which is located at points where the beginning and ending of the DNA loops converge together is obscured from view (Laemmli et al, 1977; Paulson and Laemmli, 1977). Assuming that matrix and scaffold DNA's and non-matrix and loop DNA's are the same, then, the preferential retention of matrix over non-matrix DNA on filters during alkaline elution and during gel electroelution may occur because the matrix DNA exists in a non-linear structure imposed upon it by covalently bound RNA or protein which is responsible for establishing the DNA loops structures within the scaffold or nuclear matrix. This non-linear structure may be responsible for the retention of high molecular weight DNA observed in the DNA retention studies described in Chapter IV. If this is the case, then, this structure may be resistant to the effects of both proteinase K and RNAse A enzymes in intact whole nuclear DNA. However, evidence for this suggestion is predicated on the assumption that losses in RNA label observed in the data in Table 3 reflect similar losses in DNA label during electroelution. This assumption is valid only if the RNA label being retained is covalently bound to the DNA. Evidence which suggests that such is the case is presented in Chapter VI in general and more conclusively in the data presented in Table 24. On the other hand, retention of matrix DNA during gel electroelution is partially reduced by proteinase K. Since this reduction does not completely abolish the preferential retention of matrix DNA over non-matrix DNA, some other molecule besides protein must be involved in establishing this preferential retention. It is not difficult to envision an alkali resistant RNA molecule which is resistant to RNAse A because of a protecting group at the 2' hydroxyl position. Alkali resistant RNA tetranucleotides have been isolated that are methylated in the 2' position of alternating ribose sugars (Maden and Salim, 1974; Rottman, 1978; Nishimura, 1979). RNA

nucleotides phosphorylated in the 2', 3', and 5' positions have also been isolated (Konarska et al 1982; Wallace and Edmonds, 1983; Ruskin et al, 1984, Kiberstis et al, 1985; Arnberg et al, 1986; Cech, 1986; Peebles et al, 1986; Van der Veen et al, 1986). The alkali resistance of these nucleotides has been demonstrated (Wallace and Edmonds, 1983; Ruskin et al, 1984; Kiberstis et al, 1985). In addition, alkali resistant nucleotides which appear to be RNA have been found covalently attached to DNA at origins of replication (Birkenmeyer et al, 1987). It is more difficult to envision a protein which is completely resistant to proteinase K; especially after SDS extraction.

The partial reduction in matrix DNA retention by proteinase K during gel electroelution may be due to the "nicking" of the DNA at protein bridges which are near the DNA domain attachment sites. It has been established that proteinase K relaxes supercoiling (Cook, 1984). This was supported by evidence in Chapter III. Therefore, a covalent protein linker must be involved. The likely candidate for such a linker is Topoisomerase II (Liu et al, 1983; Sander and Hsieh, 1983; Berrios et al, 1985; Earnshaw et al, 1985; Earnshaw and Heck, 1985; Cockerill and Garrad, 1986; Osheroff and Zechiedrich, 1987). However, treatment of gel encapsulated intact DNA with proteinase K does not result in a visible increase in DNA loss during electroelution as compared to untreated controls (Table 3, Plates XXIIf, XXIIIb, and XXIV). Therefore, protein cannot be responsible for the majority of the DNA retention. Although double strand breaks cannot be ruled out, the detection of loss of DNA retention in matrix samples after proteinase K digestion could be the result of DNA nicking by proteinase K at a point very close to a random single stranded cut by DNAse I. The small fragment generated may be incapable of resisting electroelution (Figure 13). In intact DNA studies such a loss would be visually undetectable. Although supercoiling would be disrupted, the DNA superstructure responsible for retention in gel inserts would remain intact. Evidence that single stranded nicks may cause a release of DNA fragments under neutral conditions during gel electroelution is suggested by occasional increases in the band length of ethidium bromide stained material during the electrophoresis of X-ray irradiated CHO cells (data not shown).

My initial hypothesis is that a protein such as Topoisomerase II resides near the DNA domain attachment sites (probably on both sides of a loop) and these sites are composed of covalent bridges between alkali resistant RNA and DNA (see Appendix D).



'Figure 13

Explanation for the Partial Loss of Matrix DNA Retention During Electroelution as a Result of Pre-Incubation in Proteinase K

Figure 13a illustrates double stranded DNA (1), topoisomerase II (2), and a single strand DNA nick on the upper strand (3). The topoisomerase II is covalently bound to the DNA, forming a single strand bridge on the upper strand. In Figure 13b the topiosomerase has been cleaved in half by proteinase K, generating a second single strand nick which is very close to the first nick. In Figure 13c the electroelution of the DNA results in the removal of this small fragment. If the fragment is located in an A+T rich region close to the DNA attachment site, then, it will be even easier to remove during electroelution.

CHAPTER VI

PARTIAL CHARACTERIZATION OF RNA ASSOCIATED WITH HIGH MOLECULAR WEIGHT DNA

Introduction

In this chapter the focus of the studies is on the nature of the bonds which are responsible for the establishment of DNA superstructure. The general method of approach was based on results obtained in Chapter IV. The cells or their nuclei were incorporated into gel inserts, extracted in SDS, and electroeluted. In one study the cells were radiolabeled with either [^{14}C]thymidine, [^{14}C]uridine, or [^{14}C]leucine and treated under various conditions to determine the extent of retention of RNA and protein label along with the DNA label following electroelution. The gel inserts were then exhaustively digested in DNAse I and the incubation solution was subjected to ethanol precipitation. The resulting pellets were found to contain about 1% of the total RNA label or 0.2% of the total protein label. These results suggested that the protein and/or RNA label being retained along with the DNA could be responsible for the establishment of the DNA superstructure responsible for DNA retention by gel inserts as shown in previous chapters. Exposure to either proteinase K or RNAse A prior to electroelution of intact cellular DNA did not release any detectable uridine labeled material label (Chapter IV). However, the more refined procedure of horizontal tube gel electroelution was able to detect a slight loss of DNA and RNA radiolabeled material from gel inserts during electroelution following incubation in RNAse A (Table 20). The results in Chapter V indicated that most of the DNA matrix superstructure responsible for the preferential retention of matrix DNA over non-matrix DNA was still intact even after a combination of proteinase K and alkali treatments. These results suggested that the bonds responsible for the retention were either not RNA or protein or were in forms resistant to these enzymes. As previously discussed it is

difficult to envision a protein with a configuration so novel that it can resist proteinase K treatment. It is not difficult to envision an RNA species with a protecting group at the 2' hydroxyl position that is resistant to RNAse A and alkali (Chapter V).

For the reasons cited above, the studies were further focused on the characterization of the RNA label associated with this high molecular weight DNA. In order to inhibit the conversion of uridine label into thymidine via the ribonucleotide reductase pathway all cells were grown in the presence of deoxythymidine, deoxycytosine, deoxyguanosine, and deoxyadenosine. The cells were subjected to conditions favorable for serum arrest to remove as much as possible the covalently bound Okasaki RNA primer label. Under these conditions of cell growth partial characterization of the DNA-associated RNA (DAR) by alkali hydrolysis revealed that at least 20% of it was resistant to alkali and could not be rendered alkali sensitive by alkaline phosphatase treatment, indicating that free phosphate groups were not responsible for the majority of the resistance. The relative affinity of this alkali resistant RNA (ARR) for the DNA was tested further by double labeling the cells with DNA and the RNA labels. It was learned that the rate of loss of DNA was closely coupled with the rate of loss of the ARR. Mass spectrometry data indicated that the uracil content of ARR was 100 times greater than in whole cell DNA. Together, these data provided evidence for the presence of a novel species of RNA tightly associated with the DNA.

Results

Minimizing Erroneous Labeling by Uridine

Cells were grown in high serum medium containing radiolabeled uridine for 24 hours then shifted to low serum medium containing no radiolabel for an additional 24 hours. The purpose of the shift to low serum was to inhibit DNA synthesis and chase label out of any transient RNA. It was thought that by doing this, conventional radiolabeled Okazaki RNA primers would be eliminated. In all of these studies, exogenous deoxynucleosides were added to both high and low serum medium in order to suppress the conversion of uridine label into thymidine label via the ribonucleotide reductase pathway (Thelander and Reichard, 1979). The effects of such exposure are

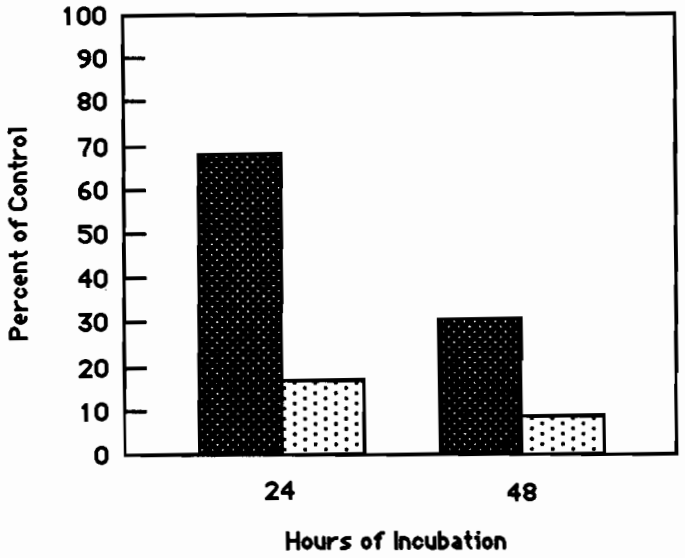


Figure 14

Growth kinetics in cells incubated in low serum

The dark histogram indicates cell population density in low serum as a percentage of the cell population density observed in high serum after 24 and 48 hours of incubation. The light histogram indicates incorporation of thymidine radiolabel at 24 and 48 hours of incubation as percentages of thymidine incorporation at "0" hours of incubation in low serum containing 50 µg/ml each of deoxyguanosine, deoxyadenosine, deoxycytidine, and deoxythymidine.

illustrated in Figure 14. Cell densities after 24 hours of exposure to low serum medium dropped to 68% of their high serum counterparts. Cell densities were increased in low serum medium with deoxynucleosides (data not shown) but thymidine uptake was only 17% that observed at zero time. At 48 hours, values for both cell density and thymidine uptake had decreased still further but unfortunately cell viability had dropped to 80%.

DAR Oligomers

Gel inserts containing cells radiolabeled with [^{14}C]thymidine, [^{14}C]uridine, or [^{14}C]leucine were prepared, extracted overnight in SDS, electroeluted for 3 hours, removed and subjected to incubations at 37 ^{o}C for 16 hours in RNAse A buffer with or

without 20 units/ml of the enzyme, electroeluted for 3 more hours, and incubated at 37°C overnight in DNAse I buffer with or without 20 units/ ml of the enzyme (see Figure 15 for protocol). The final incubation buffers were subjected to ethanol and/or trichloroacetic acid precipitation. The percent of total counts recoverable by the precipitation procedures are shown in Table 20. Since both procedures yielded similar results only the ethanol precipitated data will be discussed. Sham incubation resulted in the retention of 47.0% of the DNA label with no recovery in the supernatants and 1.4% (25 CPM) being recovered in the pellet. After incubation in DNAse I the percent retention in the insert dropped dramatically to only 7.7% with 34.1% of the label being recovered in the supernatants and 5.7% (119 CPM) recovery in the pellet. Pre-treatment with RNAse A followed by electroelution resulted in a slight decrease in insert retention to 5.8% with less material being recovered in the supernatants (31.5%) and the pellet (2.8% {40 CPM}). Therefore, total losses of DNA label from inserts attributable to RNAse A digestion was 7.9%. These losses probably occurred during the second electroelution step. The uridine labeled insert which was subjected to DNAse I digestion alone had retained only 0.4% of the total label with 3.8% and 1.0% (268 CPM) recovery of the label in the supernatants and pellets respectively. Pre-treatment of a uridine labeled insert with RNAse A followed by electroelution and DNAse I digestion resulted in the same amount of insert retention (0.4%) as obtained without RNAse A pre-treatment with about one-half as much recovery in the supernatant (1.9%) and essentially the same amount of recovery in the pellet (1.1% {310 CPM}). Therefore, total losses of RNA label from inserts attributable to RNAse A digestion was 1.8%. The loss of recovery in the supernatant suggested that this portion of the label was susceptible to RNAse A treatment and was removed during the second electroelution step. Inserts labeled with leucine retained only 0.2% of their label after RNAse A treatment followed by electroelution and DNAse I digestion. Recovery in the supernatants and pellet were only 0.1% and 0.2% (19 CPM) respectively.

Whether any of the material retained by the inserts had properties differing from the extracted material or remained associated with the inserts because of diffusion was not determined. The results of this study partially characterized the RNA species as associated with the DNA as existing in an oligomeric form (trichloroacetic acid or ethanol precipitable) which is resistant to RNAse A treatment.

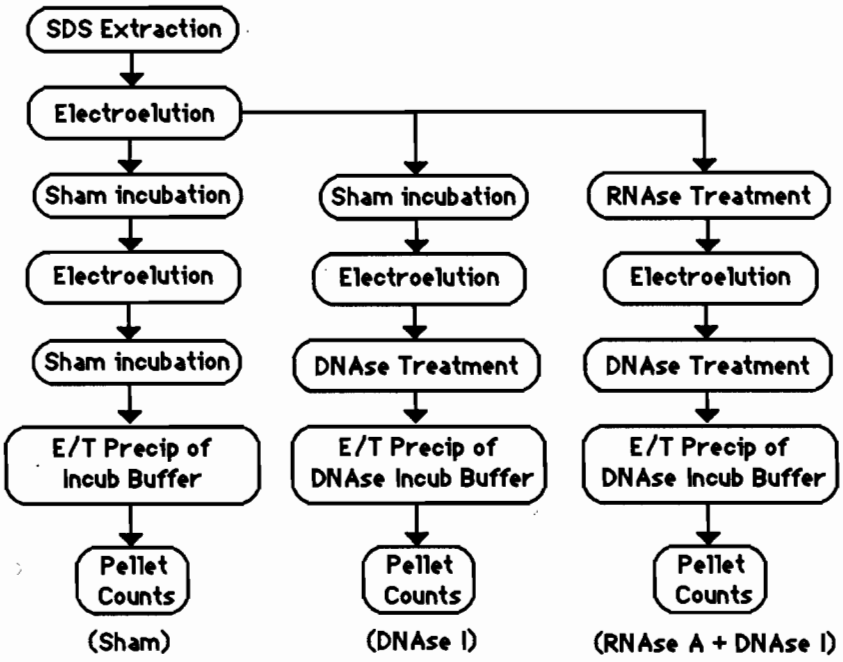


Figure 15

Flow Chart for DAR Isolation

Cells were prepared in gel inserts, SDS extracted, and run in parallel through the above series of electroelutions, incubations, and precipitations in ethanol and trichloroacetic acid. All samples were counted by liquid scintillation (Table 20).

Table 20

Percent of Total [^{14}C]Thymidine, [^{14}C]Uridine, and [^{14}C]Leucine Radiolabeled Materials Isolated From Single Labeled Gel Inserts and Their DNAse I Incubation Buffers

Mouse L-1210 cells were incubated in high serum medium for 24 hours in the presence of 50 ug/ml of thymidine, deoxyguanosine, deoxyadenosine, and deoxycytidine. At the end of this time the cells were radiolabeled with either [^{14}C]thymidine, [^{14}C]uridine, or [^{14}C]leucine and incubated an additional 24 hours. The cells were then incubated for 24 hours in low serum medium containing deoxynucleosides but no radiolabel. The cells were incorporated into gel inserts which were extracted overnight in 1% SDS buffer. The inserts were electroeluted and then incubated in the presence or absence of RNAse A. Data columns 3, 5, and 6 are from gel inserts pre-incubated in RNAse A. After the incubation period the gel inserts were electroeluted a second time and then incubated in the presence or absence of DNAse I. Data column 1 was derived from an insert which was not incubated in DNAse I. (See flow chart, Figure 15). The oligomers in the DNAse I incubation buffer were precipitated in either ethanol or trichloroacetic acid, centrifuged, and the pellets rinsed twice prior to solubilization and counting. The data is presented as percentages of total counts recovered. [a]D = DNAse I treatment; R, D = RNAse A treatment followed by DNAse I treatment, S = sham incubation. [b]The majority of these counts were in SDS extraction buffers and "pre-RNAse A" tube gels. [c]Percent of total CPM remaining per gel insert. [d]Percent of total CPM within supernatants and the ethanol precipitated pellets from DNAse I incubation buffers.

Radiolabel	Thymidine	Thymidine	Thymidine	Uridine	Uridine	Leucine
(D,R,S,)[a]	S	D	R, D	D	R, D	R, D
			Ethanol Precipitated Counts			
Other[b]	51.6	52.5	59.9	94.8	96.6	99.5
Insert[c]	47.0	7.7	5.8	0.4	0.4	0.2
Sup[d]	0.0	34.1	31.5	3.8	1.9	0.1
Pellet[d]	1.4	5.7	2.8	1.0	1.1	0.2
			Trichloroacetic Acid Precipitated Counts			
Other[b]	51.8	56.4	68.8	96.0	97.2	99.7
Insert[c]	47.1	8.3	6.6	0.4	0.4	0.2
Sup[d]	0.0	29.0	21.0	2.7	1.6	0.0
Pellet[d]	1.1	6.3	3.6	0.9	0.8	0.1

ARR Oligomers

The DAR was further characterized by determining its sensitivity to conditions of alkali hydrolysis that are routinely used to completely destroy all forms of alkali-sensitive RNA (Bock, 1968). After RNAse A treatment and electroelution the samples were thoroughly digested with 20 units/ml of DNAse I at 37°C for 23.5 hours to solubilize as much of the DNA as possible. After ethanol precipitation and a rinse the unbuffered pellets were exposed to either pre-neutralized KOH in buffer or to 0.1 N KOH without buffer and boiled for 20 minutes prior to neutralization and ethanol precipitation. The results of this procedure are shown in Table 21. As much as 40% of the DAR label recovered in Tris, NaCl, magnesium (245 CPM) and 70% of the label recovered in Tris, NaCl, EDTA (208 CPM) were insensitive to alkali hydrolysis under the conditions used. The alkali-treated DNA label recovered in Tris, NaCl, magnesium versus Tris, NaCl, EDTA contained 0.7% and 1.0% of the total cellular DNA label respectively as compared to 0.9% and 1.8% in their untreated counterparts. Therefore, the ability to precipitate the DNA label in Tris, NaCl, magnesium versus Tris, NaCl, EDTA buffers after alkali hydrolysis was found to be reduced to 78% and 56% of their respective non-alkali treated controls .

Partial Characterization of ARR by Alkaline Phosphatase

The fact that at least 40% of the DAR was alkali resistant suggested that the ARR was chemically modified at the 2' hydroxyl position of the ribose sugar. The most likely chemical modifications would be phosphate groups (Konarska, et al, 1982; Wallace and Edmonds, 1983; Ruskin, et al, 1984; Kiberstis et al, 1985; Arnberg et al, 1986; Cech, 1986; Peebles et al, 1986; Van der Veen et al, 1986) and/or methyl groups (Maden and Salim, 1974; Rottman, 1978; Nishimura, 1979). The ARR was initially characterized with alkaline phosphatase to determine if a free phosphate group at the 2' position of the ribose moiety was responsible for the alkali resistance (Wallace and Edmonds, 1983). Removable of this phosphate group by alkaline phosphatase would convert the ARR into an alkali-sensitive form which would then be susceptible to alkaline hydrolysis (Figure 41). The data for these experiments are presented in Table 22 and analyzed in Table 23. Gel inserts were exposed to DNAse I for 22.5 hours at 37°C and ethanol precipitated in Tris, NaCl, magnesium in the presence of 250 µg/ml carrier DNA. In three out of four data points

Table 21

Recovery of Ethanol Precipitated Materials Radiolabeled with Either [^{14}C]Thymidine or [^{14}C]Uridine Following Alkali Hydrolysis or Sham Incubation of DNAse I Extracts of Gel Inserts

Mouse L-1210 cells were radiolabeled with either [^{14}C]thymidine or [^{14}C]uridine as described in Table 20. The cells were incorporated into gel inserts which were extracted overnight in 1% SDS buffer. The inserts were electroeluted and incubated in DNAse I. The DNAse I buffer was ethanol precipitated in either Tris, NaCl, magnesium or Tris, NaCl, EDTA buffers, centrifuged, rinsed once to remove buffer, and subjected to 100^{o}C for 20 minutes in the presence of either Tris buffered solution, pH 8.0 or in 0.1 N KOH. After hydrolysis the tubes containing KOH were neutralized and buffered. All solutions were ethanol precipitated, centrifuged, and the pellets rinsed twice, resolublized, and counted. The data is presented as percentages of total counts recovered. [a]Percent of total cellular DNA or RNA label isolated in Tris, NaCl, magnesium without carrier DNA [b]Percent of total cellular DNA or RNA label isolated in Tris, NaCl, EDTA with 250 µg/ml carrier DNA

Radiolabel	Thymidine	Thymidine	Uridine	Uridine
Treatment	Buffer	KOH	Buffer	KOH
TNM[a]	0.9	0.7	1.0	0.4
TNE[b]	1.8	1.0	0.5	0.3

established for [^{14}C]thymidine the counts were essentially background (Table 22, data column 1). The one errant thymidine count (20 CPM) must logically be attributed to experimental error. As can be seen from Table 23 the ARR radiolabel comprised about 80% of the total [^{14}C]uridine label (63 out of 79 CPM and 188 out of 243 CPM assuming no alkaline phosphatase effect). The apparent partial sensitivity to alkaline phosphatase was seen only when the samples were incubated in 50 units/ml of alkaline phosphatase at 37 ^{o}C for 3 hours followed by only 3 hours of electroelution (see Table 22, column 3 and Table 23). However, this 7% loss in label (243 CPM to 200 CPM) occurred regardless of any additional exposure to alkali. Assuming the loss is the result of alkaline phosphatase treatment, then, one must also assume that only a small portion of the ARR was susceptible to the enzyme. Therefore, most of the ARR is protected from alkali hydrolysis by either non-phosphate bonds or by phosphate bonds interconnected with other molecules.

Table 22

Effect of Alkaline Phosphatase and Secondary Alkali Hydrolysis on the Amount of DNA and ARR Recovered From Ethanol Precipitated DNAse I Extracts of Gel Inserts

Mouse L-1210 cells were radiolabeled with either [^{14}C]thymidine or [^{14}C]uridine as described in Table 20. The cells were incorporated into gel inserts and extracted overnight in 1% SDS buffer. They were electroeluted and incubated in DNAse I solution. The DNAse I solution was ethanol precipitated in Tris, NaCl, magnesium with carrier DNA. The pellets were centrifuged down and rinsed once to remove buffer. They were incubated at 100°C for 20 minutes in either 0.1N KCl or 0.1 N KOH. The KOH samples were neutralized and all samples were buffered in a solution compatible with alkaline phosphatase (AP). Samples were incubated at 37°C in the presence or absence of alkaline phosphatase, ethanol precipitated, centrifuged, and rinsed once to remove buffer. They were incubated at 100°C for 20 minutes in either 0.1N KCl or 0.1N KOH. The samples were ethanol precipitated, centrifuged, rinsed twice, and the pellets resolublized and counted for 50 minutes. The actual counts recovered (minus background) are shown in the table in data rows 4 through 7. Samples in data columns 1 and 2 were part of the same experiment. Samples in data columns 3 and 4 were part of a similar but second experiment. Samples in row 1 were not exposed to alkali or alkaline phosphatase. Samples in row 2 were exposed to alkali twice but not to alkaline phosphatase. Samples in row 3 were exposed to alkali once and then to alkaline phosphatase. Samples in row 4 were exposed to alkali, alkaline phosphatase, and then to alkali again. Pellets contain less than 0.6% of total cellular CPM.

Hours of Electroelution	8	8	8	3
Units/ml of AP	25	25	50	50
Hours of Incubation	1	1	3	3
Radiolabel (CPM)	**Thymidine**	**Uridine**	**Uridine**	**Uridine**
Controls	1	81	77	243
KOH Twice	20	60	59	200
KOH + AP	1	66	61	179
KOH + AP + KOH	1	65	66	185

Table 23

Pooled [^{14}C]Uridine Radiolabel Data From Table 22

Data from [^{14}C]uridine radiolabeled samples in Table 22 was derivatized in two ways: assuming alkaline phosphatase (AP) exerted no effect on the system or assuming that it did had a partial effect on the system. Under the section of Table 23 in which no alkaline phosphatase effect was assumed all data from columns 1 and 2 representing samples which were not treated with alkali or alkaline phosphatase were averaged. Similar datum from column 3 was not altered. All data representing samples which were treated with alkali and/or alkaline phosphatase were pooled in the following manner: The data from columns 1 and 2 from Table 22 were averaged together. The data under column 3 was averaged together. Under the section of Table 23 in which an alkaline phosphatase effect is assumed the data was processed as follows: Data from columns 1 and 2 were excluded from this compilation. All of the original data from column 3 of Table 22 are shown in this section except that all alkaline phosphatase treated data were averaged together. *This is the standard error and n = the number of data points.

No AP effect assumed	Columns 1 + 2	n	Column 3	n
Control CPM, no alkali nor AP	79 ± 2*	2	243	--
Average non-control CPM	63 ± 2*	6	188 ± 6*	4
Percent loss	20	--	23	--

AP effect assumed	Column 3	n
Control CPM, no alkali nor AP	243	--
Alkali treated only CPM	200	--
Average AP-treated CPM	182 ± 2*	2
Total percent loss	25	--
Possible percent loss due to AP	7	--

The Relative Resistance of DNA and ARR to SDS Extraction and Electroelution

The 33% loss of total RNA label observed in the alkaline phosphatase experiments when the electrophoretic time was increased from 3 hours to 8 hours (Table 22) was a cause for concern. This loss in counts suggested that it might be possible to remove all of the "DAR" from the remaining high molecular weight DNA by simply extending the time of electroelution. Therefore, an experiment was performed which involved double labeling the cells with [^{3}H]thymidine and [^{14}C]uridine. Gel inserts containing 9 million nuclei were made from nuclei which had been stored in liquid nitrogen. The gel inserts were electroeluted for 9 hours and 18 hours respectively. Interestingly, the

storage of nuclei under these conditions altered the sample such that there was an extensive loss of both thymidine and uridine label during electroelution. The percent retention of label after 9 hours of electroelution was 2.64% for DNA and 1.28% for the ARR form of DAR label (Table 24). After 18 hours of electroelution these numbers dropped to 0.51% and 0.25% respectively. The total DNA and RNA label is probably underestimated because some of it is lost following KOH hydrolysis. However, the ratio of ARR/DNA label was almost identical for both 9 and 18 hour samples (0.138 versus 0.142) as was the ratio of label loss for DNA and ARR label respectively (0.164 versus 0.169). This reinforces the idea that high molecular weight DNA and ARR are intimately associated and cannot be separated by SDS extraction and electro-elution alone, and that the DNA and the ARR are covalently bonded together as are the Okazaki DNA fragments and RNA primers during DNA replication.

The Relative Base Composition of ARR

A sample of electroeluted ARR from whole mouse L-1210 cells was analysed by mass spectrometry by Dr. Dave Bergtold at the National Bureau of Standards in Gaithersburg, Maryland. He digested the ARR by acid hydrolysis to release all the bases and compared base ratios of the ARR sample to those of total cell DNA isolated from herring sperm DNA. He compared spectrometric peaks of thymine to all other base peaks and the results he obtained are shown in Table 25. These results indicated that the pyrimidine content of ARR was much higher than the purine content and that thymine was the principal pyrimidine with uracil comprising about 3% of the total. However, when the uracil content of ARR was compared to that observed in whole cell herring sperm DNA it was found to be 100 times higher, suggesting that the uridine radiolabel of earlier experiments had not been extensively converted into thymidine prior to its incorporation into ARR. Therefore, at least 3% of the ARR appeared to be composed of RNA with the remainder being DNA.

Table 24

Comparative Loss of [^{3}H]Thymidine and [^{14}C]Uridine Double Radiolabeled Materials After SDS Extraction and Gel Electroelution

Mouse L-1210 cells were double radiolabeled with [^{3}H]thymidine and [^{14}C]uridine as described in Table 20. Gel inserts contained nuclei which had been frozen in liquid nitrogen prior to use. The inserts were extracted overnight in 1% SDS buffer and electroeluted for 9 and 18 hours respectively. Gel tubes were replaced after 3 and 9 hours of electrophoresis. Data row 1 contains the total alkali resistant counts collected from one set of gel inserts together with an average gel tube total count. Data row 2 contains all the alkali resistant counts recovered from the gel inserts alone. Data row 3 is the percentage of total counts recovered from the gel inserts compared to the average total gel tube counts and alkali resistant gel insert counts. Data row 4 is the ratio of ARR counts divided by the alkali-treated DNA counts for 9 and 18 hours of electroelution respectively. Data row 5 is the ratio of the 18 hour ARR or alkali-treated DNA counts divided by their 9 hour counterparts.

Nine Hours Electroelution			Eighteen Hours Electroelution		
Thymidine		Uridine	Thymidine		Uridine
95399		26898	101054		29255
2964		408	486		69
3.1		1.5	0.5		0.2
---	0.138	---	---	0.142	---
---		---	0.164		0.169

Table 25

Relative Base Ratios of DNA and RNA Purines and Pyrimidines to Thymine in Mouse L-1210 Cell ARR and in Herring Sperm DNA as Determined by Mass Spectrometry

Samples include commercially prepared whole herring sperm DNA and unlabeled whole mouse L-1210 cell ARR isolated from electroeluted inserts by exhaustive DNAse I digestion, alkali hydrolysis, and ethanol precipitation as described in Table 20. Samples were acid hydrolysed, free bases were analyzed by mass spectrometry, and relative peak sizes obtained were compared to thymine peaks. Column 2 compares uracil to thymine, column 3, cytosine to thymine; column 4, adenine to thymine; and column, 5 guanine to thymine. The ratios obtained for ARR were divided by the ratios obtained for herring sperm DNA to obtain the relative ratios of uracil, cytosine, guanine, and adenine in ARR to whole cell herring DNA. These results are shown in row 4.

Sample	U + T	C + T	A + T	G + T
Herring DNA	0.00028	0.15	0.620	0.280
Mouse ARR	0.02700	0.19	0.087	0.029
ARR + DNA	96.43	1.27	0.14	0.10

Discussion

The results indicate that the ability of DAR to remain associated with high molecular weight DNA is highly resistant to SDS extraction and electroelution but not to DNAse I followed by electroelution. The resistance of DAR to the inhibition of DNA synthesis together with its tight association with DNA suggests that it could be a modified Okazaki RNA primer that remains covalently bound to the DNA at the end of replication in a form that renders much of it insensitive to RNAse A and alkali. Evidence for alkali resistant RNA primers within origins of replication has been cited (Birkenmeyer et al, 1987). DAR can be ethanol or TCA precipitated after DNAse I treatment from the buffer in which the gel inserts are incubated. The ability to recover the DAR from the incubation buffer suggests that it has been converted into a freely diffusable form. And its capacity for ethanol precipitation suggests that this form must be oligomeric although it is uncertain how much of this capacity is due

strictly to the labeled RNA alone as opposed to its association with DNA fragments or exogenously added protein in the form of DNAse I. The fact that the recovery of ethanol and trichloroacetic acid precipitated DNA and RNA pellets was reduced following exposure to RNAse A could be interpreted in two ways: 1) The DNAse I activity of the RNAse A preparation was not completely destroyed by the procedures used (Maniatis, 1982) or 2) RNA responsible for the retention of the DNA was damaged by the RNAse A digestion resulting in the loss of the DNA during electroelution. If the latter explanation is correct, then, only a very small amount of the total RNA is responsible for the retention of a much larger amount of DNA as evidenced by the small RNA loss resulting from RNAse A digestion and electroelution (Table 20). This hypothetical RNA may be analagous to alkali sensitive linkers which punctuate the DNA at domain-sized intervals (Filippidis and Meneghini, 1977). Why the recovery of RNA label is more sensitive to alkaline hydrolysis than to RNAse A digestion in not clear unless most of the loss is simply due to inefficiency in recovery after alkali treatment as suggested by the losses observed in the accompanying DNA samples (Table 21). Of course the loss of DNA label following alkaline hydrolysis could also be explained by the loss of RNA which is sensitive to both RNAse A and alkali, generating DNA fragments which are recovered less efficiently by ethanol precipitation (see Figures 44 and 45).

These observations support the idea that some portion of the RNAse A/alkali sensitive DAR is connected to a small portion of the DNA and is responsible for its retention during electroelution and recovery by ethanol precipitation. However, this portion of the DAR is not critical in the ability of gel inserts to retain the majority of the DNA during electroelution. The possible significance of RNAse A/alkali sensitive sites within the DNA in terms of DNA superstructure has been discussed in Chapter I. A more thorough discussion of the implications of these observations will be presented in Chapter IX and in Appendix D.

The presence of a portion of the DAR which is recalcitrant to alkali hydrolysis under the conditions used here is an important discovery which must be carefully scrutinized. These results can be explained in at least four ways: 1) the RNA was not "completely" hydrolyzed, 2) the presence of 8 μg of DNAse I in each sample during boiling resulted in a denatured protein-nucleic acid precipitate which trapped otherwise soluble counts, 3) the recovered uridine label was converted into thymidine via pathways such as ribonucleotide reductase and incorporated into DNA, and 4) the

recovered uridine label has been incorporated into an alkali resistant form of RNA. The first explanation is unlikely because it has been reported that unbuffered RNA samples less than 3 mg/ml in concentration can be completely degraded to mononucleotides by boiling for 20 minutes in 0.1 N KOH (Bock, 1968). The total amount of DNA and RNA label present within each unbuffered reaction tube after DNAse I digestion indicates that probably only 100-200 ng of each nucleic acid was present. Furthermore, prior to alkali hydrolysis the pellets were rinsed in buffer-free ethanol with salts to remove traces of buffer which might "dampen" the effects of the KOH during heating. The second explanation is not as easily dismissed even though 18% to 60% of the RNA pellet hydrolyzed under these conditions and the resulting pellets were rinsed twice to remove all traces of soluble label prior to counting. The possibility of uridine to thymidine conversion through such pathways as ribonucleotide reductase was remote for three reasons: 1) Such incorporation would be minor compared to the uptake of thymidine label by these same cells, but the percent uridine label which was ARR was as much as one-third to one-half of the percent of thymidine label incorporated into DNA isolated under the same conditions (Table 21), and the total counts isolated were 100-200 times higher in the ARR fraction than in the DNA fraction even though the specific activity of the radiolabeled thymidine and uridine were equivalent (data not shown), 2) The cells had been grown in the presence of deoxynucleosides to inhibit the conversion of uridine into thymidine via the ribonucleotide reductase pathway (Thelander and Reichard, 1979), and 3) direct analysis of the relative base composition of mouse L-1210 cell ARR by mass spectrometry indicated a uracil content which was 3% of the total thymine content and almost 100 times higher than the uracil content of whole cell herring sperm DNA (Table 25).

I concluded that the majority of these alkali resistant uridine counts represented a form of low molecular weight RNA which were tightly associated with much larger pieces of DNAse I-resistant DNA; the latter of which were responsible for the ability of the ARR to precipitate in ethanol and trichloroacetic acid.

RNA must be chemically modified at 2' ribose positions to render it inert to the effects of alkali (Michelson, 1963). The largest alkali-resistant RNA's isolated to date are ribosomal RNA's which are methylated at the 2' position of the ribose sugar and are four nucleotides in length (Maden and Salim, 1974). It is unlikely that such oligonucleotides would be capable of ethanol precipitation under the conditions used

here. It is even less probable that they would be present to undergo such a precipitation procedure following such extensive treatments as SDS extraction and electroelution. The results obtained from the alkaline phosphatase experiments suggest that ARR is at best only slightly sensitive to the enzyme. Therefore, it appears that alkali resistance is not due to the presence of free phosphate groups at the 2' positions of the ribose RNA moiety. This does not rule out the possibility that diphosphate bridges might exist in these positions. If they did they could be responsible for the covalent attachment of the DNA domain origins of replication to each other or to other components within the nuclear matrix (see Appendix D).

CHAPTER VII

THE ASSOCIATION OF ARR WITH MATRIX AND NON-MATRIX DNA

Introduction

In the last chapter it was hypothesized that DAR could be responsible for establishing the DNA superstructure discussed in earlier chapters. Since the bonds responsible for establishing this superstructure were hypothesized to exist at the DNA domain attachment sites with the confines of the nuclear matrix the DAR should be present within matrix DNA and absent from non-matrix DNA. The existence of ARR within the DAR provided a unique probe for testing this hypothesis. Cells were double labeled with thymidine and uridine radioisotopes and serum-arrested in medium without radiolabel in order to compare the ratio of ARR to DNA label in both matrix and non-matrix fractions. The nuclear DNA was separated into matrix and non-matrix fractions, extracted in SDS, and purified by hydroxylapatite batch elution. This material was dialyzed, ethanol precipitated, and subjected to alkali hydrolysis as before. The ratio of DNA to ARR for the matrix fraction was divided by the DNA to ARR ratio of the non-matrix fraction and the resulting number was called the predilection index. An index greater than 1 would indicate that the ARR had a greater predilection for the matrix than the non-matrix region of the nucleus. All predilection indices generated were greater than 2 and more extensive matrix DNA digestions coupled with improved matrix and non-matrix DNA separations generated indices as high as 18.6. This indicated that the matrix ARR was very tightly bound to the nuclear matrix and may very well lie within the DNA domain attachment sites. However, only about 20% of the total ARR was found associated with the matrix DNA. What was the function of the remaining ARR which was found in the non-matrix fraction? It was hypothesized that this ARR may be a form which has been chemically altered resulting in its detachment from the nuclear matrix. It was hypothesized that some of the nuclear matrix ARR

may become methylated during the process of differentiation resulting in the loss of DNA attachment sites and DNA loop fusions. Models explaining these processes are discussed in this chapter and in Appendix D. Cells double labeled with uridine and methyl-methionine radioisotopes were processed as before and ARR and alkali-resistant methylated oligomers ($ARCH_3$) were isolated. The ratio of $ARCH_3$ to ARR was determined for both matrix and non-matrix fractions and the non-matrix ratio was divided by the matrix ratio. This number was called the methylation index. An index greater than 1 would indicate that the non-matrix ARR was more methylated than the matrix ARR. The methylation index was found to be 2.17 which supported the hypothesis that non-matrix ARR was chemically different from matrix ARR due to a methylation process.

Results

Association of the ARR with the Nuclear Matrix

Table 26 contains [^{3}H]thymidine and [^{14}C]uridine counts for mouse nuclear matrix and non-matrix samples. It includes data on pre-DNAse samples (those not yet exhaustively incubated in DNAse I) and samples exhaustively incubated in DNAse I once or twice. Those samples exposed to DNAse I include those heated to 100^{o}C for 20 minutes in 0.1 M KCl (pre-ARR samples), and those heated to 100^{o}C for 20 minutes in 0.1M KOH (ARR samples). The samples exposed to DNAse I twice include only ARR samples. The pre-DNAse I matrix and non-matrix samples taken together are considered here as total nuclear DNA. The pre-ARR samples included thermostabile alkali sensitive RNA and ARR. When the samples were digested two times in DNAse I prior to alkali hydrolysis the levels of matrix DNA and ARR were not affected but the non-matrix DNA and ARR counts dropped from 217 and 192 to 60 and 109 respectively (Table 26). This suggested that two DNAse I digestions were more efficient in removing susceptible DNA from the non-matrix fraction than one digestion alone.

Experiments with DNAse I digestion and hydrodynamic shearing of nuclear DNA into matrix and non-matrix fractions demonstrated that not all of the matrix DNA fraction

(the pellet resulting from centrifugation) was removed during matrix and non-matrix fractionation procedures. Even during a simple decantation of the supernatant the "stringiness" of the DNA was sufficient to pull a portion of the pellet out with the decanted supernatant (non-matrix) DNA. When the supernatant DNA was underlayered with Sea Prep low melting temperature agarose, centrifuged as before, and chilled to gel the Sea Prep it was discovered that and 11% and 6% of the total DNA and RNA radiolabels isolated by conventional DNAse I digestion and 23% and 21% of the total DNA and RNA radiolabels isolated by hydrodynamic shear (vortexing) were being trapped in the Sea Prep underlayer (Table 27). Such an observation was critical in the proper analyses of the relative ARR content of matrix and non-matrix fractions.

Based upon the results shown in Tables 26 and 27 all subsequent matrix and non-matrix samples isolations included an additional purification step involving Sea Prep underlayering followed by two DNAse I digestions of all pre-ARR and ARR samples. Table 28 contains results from mouse cells and Table 29 contains similar data on human lymphocytes except for the absence of pre-ARR data. Low cell yields necessitated the exclusion of pre-ARR data as well as a reduction in the total number of aliquots counted. The higher labeling efficiency observed from the data in Table 28 as compared to Table 26 resulted from the removal of deoxynucleosides from the media.

Data from Tables 26, 28, and 29 were used in the computations shown in Table 30. These computations include the percentage of total nuclear DNA which is either matrix or non-matrix DNA, (data column 1), the percentage of total nuclear ARR which is either matrix or non-matrix ARR, (data column 2), and the percentage of pre-ARR for a given sample which is ARR, (data column 3). In data column 4 the ARR counts for a given sample have been divided by the pre-DNAse I thymidine counts for that same sample. When the matrix ARR/DNA ratio is divided by the non-matrix ARR/DNA ratio a "predilection index" is generated as shown in data column 5. A predilection index greater than 1 indicates that the ARR has a predilection for the nuclear matrix, i.e., it is more concentrated per unit of DNA in the matrix than in the non-matrix DNA. The computations for Table 26 indicate that 4.1% of the total nuclear DNA was isolated in the matrix fraction in the absence of Sea Prep underlayering. The percentage of ARR which was associated with the matrix fraction increased from 8.1% after one DNAse I digestion to 13.5% when a second set of samples were digested in DNAse I twice. The percentage of pre-ARR that was ARR after one DNAse I digestion was 17.5% and

Table 26

Double Radiolabel Counts Isolated From the Nuclear Matrix and Non-matrix of Mouse L-1210 Cells after One or Two DNAse I Treatments Followed by Heating in the Presence or Absence of Alkali

Mouse L-1210 cells were double labeled with [^{3}H]thymidine and [^{14}C]uridine as described in Table 20. Additional separation of the matrix and non-matrix fractions by Sea Prep underlayering was not used here. Purified matrix and non-matrix aliquots were counted or exhaustively digested in DNAse I once or twice. The DNAse I digests were heated to 100 ^{o}C for 20 minutes in either 0.1 M KCl or 0.1 M KOH, neutralized where appropriate, ethanol precipitated, rinsed twice, resolubilized, and counted. [a]Aliquots which were digested twice in DNAse I, [b]average and standard error, [c]number of aliquots counted.

Treatment	Nuclear Fraction	[^{3}H]Thymidine[b]	[^{14}C]Uridine[b]	N[c]
Pre-DNAse I	Matrix	259	591	1
0.1 M KCl, 100^{o}C	Matrix	37 ± 4	97 ± 11	2
0.1 M KOH, 100^{o}C	Matrix	7 ± 3	17 ± 1	2
0.1 M KOH, 100^{o}C	Matrix[a]	6	17	1
Pre-DNAse I	Non-matrix	6130	7785	1
0.1 M KCl, 100^{o}C	Non-matrix	774 ± 48	1371 ± 47	2
0.1 M KOH, 100^{o}C	Non-matrix	217 ± 16	192 ± 7	2
0.1 M KOH, 100^{o}C	Non-matrix[a]	60	109	1

14.0% for matrix and non-matrix fractions respectively. Similar information for samples incubated in DNAse I twice was not obtained. The predilection index increased from 2.1 for samples incubated once in DNAse I to 3.7 for samples incubated twice in DNAse I. In both cases the indices indicate that there is a predilection of the ARR for the nuclear matrix DNA, but the index is increased after a subsequent DNAse I incubation. This shift in the index is the result of a loss in non-matrix ARR during the second DNAse I incubation. The ARR concentration in the matrix fraction was unaffected by this additional incubation. One explanation for this loss is that the non-matrix DNA was incompletely digested after only one DNAse I digestion and served

Table 27

Trapping of DNA and RNA Radiolabels by the Sea Prep Underlayering Technique

Mouse L-1210 cells were radiolabeled as described in Table 20. Matrix and non-matrix fractions were separated using either conventional DNAse I digestion or hydrodynamic shearing. In the latter technique the nuclear pellets were subjected to extraction in 2M NaCl TE buffer in the absence of DNAse I digestion and vortexed intermittently for one minute prior to conventional fractionation. It was felt that mechanical shearing of the nuclear DNA may generate a "cleaner" separation of matrix and non-matrix fractions than DNAse I digestion. Because of time constraints, the technique was not utilized in any further studies. The results in data columns 1 and 2 are presented as percentages of total label isolated.

Fractionation Procedure	Nuclear Fraction	[^{3}H]Thymidine	[^{14}C]Uridine
DNAse I Digestion	Matrix	40	27
" "	Non-matrix	48	66
" "	Sea Prep	11	6
Hydrodynamic Shear	Matrix	7	5
" "	Non-matrix	70	74
" "	Sea Prep	23	21

as a carrier for non-matrix ARR label. It is for this reason that a second DNAse I digestion was carried out in this particular experiment and why all subsequent experiments utilized two DNAse I digestions.

The computations for Table 28 indicate that only 1.5% of the total DNA was associated with the nuclear matrix in the presence of Sea Prep underlayering. Since Sea Prep underlayering should increase rather than decrease this number, the reduction from 4.1% in computations for Table 26 to 1.5% in computations for Table 28 merely indicates that the DNAse I digestion was more efficient in the latter experiment, resulting in a decrease in matrix DNA content. In computations for Table 28, the percentage of ARR associated with the nuclear matrix after two DNAse I digestions was 21.3%. In computations for Table 26 this percentage was only 13.5%

Table 28

Double Radiolabel Counts Isolated From the Nuclear Matrix and Non-matrix of Mouse L-1210 Cells After Two Successive DNAse I Treatments Followed by Heating In the Presence or Absence of Alkali

Mouse L-1210 cells were double labeled in log phase with [^{3}H]thymidine and [^{14}C]uridine in the absence of exogenous deoxynucleosides. They were incubated for 24 hours in low serum medium to remove extraneous label and to inhibit further DNA replication. Additional separation of the matrix and non-matrix fractions was achieved by Sea Prep underlayering. Purified matrix and non-matrix aliquots were counted or exhaustively digested in DNAse I for two successive treatments. The DNAse I digests were heated to 100°C for 20 minutes in either 0.1 M KCl or 0.1 M KOH, neutralized where appropriate, ethanol precipitated, rinsed twice, resolubilized, and counted. [a]Average and standard error, [b]number of aliquots counted.

Treatment	Nuclear Fraction	[^{3}H]Thymidine[a]	[^{14}C]Uridine[a]	N[b]
Pre-DNAse I	Matrix	1291 ± 25	823 ± 8	3
0.1 M KCl, 100°C	Matrix	617 ± 8	255 ± 6	3
0.1 M KOH, 100°C	Matrix	278 ± 3	104 ± 4	3
Pre-DNAse I	Non-matrix	84513 ± 1188	6316 ± 102	3
0.1 M KCl, 100°C	Non-matrix	3211 ± 69	457 ± 7	2
0.1 M KOH, 100°C	Non-matrix	1393 ± 18	385 ± 10	3

in samples exposed to DNAse I twice in the absence of Sea Prep underlayering. How much of this increase in ARR content within the matrix fraction is the result of Sea Prep underlayering and/or experimental error is uncertain. In computations for Table 28 the percentage of pre-ARR which was ARR was 40.8% and 84.2% for matrix and non-matrix fractions respectively. This is a dramatic upward shift from the numbers tabulated in computations for Table 26 for samples exposed to DNAse I one time. One explanation for this shift could be that more of the alkali-sensitive RNA was destroyed during the longer incubation period in DNAse I thus increasing the relative amount of ARR within the pre-ARR sample. In samples exposed to DNAse I twice there was a dramatic upward shift in the predilection index from 3.7 in computations for Table 26 to 17.5 in computations for Table 28. This shift could be attributed to the

Table 29

Double Radiolabel Counts Isolated From the Nuclear Matrix and Non-matrix of Human Lymphocytes

Human lymphocytes were stimulated to replicate using phytohemagglutinin and double labeled with [^{3}H]thymidine and [^{14}C]uridine in the absence of exogenous deoxynucleosides. They were incubated for 24 hours in low serum medium to remove extraneous label and to inhibit further DNA replication. Additional separation of the matrix and non-matrix fractions was achieved by Sea Prep underlayering. Purified matrix and non-matrix aliquots were counted or exhaustively incubated in DNAse I for two successive treatments. The DNAse I digests were heated to 100^{o}C for 20 minutes in 0.1 M KOH, neutralized, ethanol precipitated, rinsed twice, resolubilized, and counted. [a]Average and standard error, [b]number of aliquots counted.

Treatment	Nuclear Fraction	[^{3}H]Thymidine[a]	[^{14}C]Uridine[a]	N[b]
Pre-DNAse	Matrix	539	250	1
0.1 M KOH, 100^{o}C	Matrix	36 ± 2	22 ± 0	2
Pre-DNAse	Non-matrix	45166	3093	1
0.1 M KOH, 100^{o}C	Non-matrix	636 ± 0	98 ± 4	2

more extensive digestion of the matrix DNA by DNAse I (from 4.1% in computations for Table 26 down to 1.5% in computations for Table 28) and/or to the use of the Sea Prep underlayering technique in the latter experiment which would remove more of the matrix DNA from the non-matrix DNA which would then be added back to the matrix fraction. In any case, the inverse relationship between matrix DNA content and the predilection index strongly indicates that the matrix ARR is located very near or at the nuclear DNA attachment sites.

The computations for Table 29 indicate that only 1.2% of the total DNA was associated with human lymphocyte nuclear matrix. Again, this number only reflects the relative efficiency of the DNAse I digestion during that particular experiment. The percentage of total ARR found within the matrix was 18.3% which is similar to the figure derived from computations for Table 28 for mouse nuclear matrix ARR (21.3%). The percentage of pre-ARR which is ARR was not determined due to a paucity of sample material. The predilection index (18.6) was found to be remarkably

Table 30

Data Derivatized From Tables 26, 28, and 29

Data column 1 represents percentages of total matrix and non-matrix (M + N) DNA prior to exhaustive DNAse I digestion. Data column 2 represents percentages of total matrix and non-matrix ARR isolated. Data column 3 represents the percentage of pre-ARR (within a given sample) which is ARR. Pre-ARR is a combination of alkali-sensitive RNA and ARR and its value is determined by heating RNA label at 100°C for 20 minutes in 0.1 M KCL. Data column 4 is the ratio of matrix or non-matrix ARR counts to their respective total DNA counts. Data column 5 is called the "predilection index" (P. I.) and it is determined by dividing the matrix ARR/DNA ratio by the non-matrix ARR/DNA ratio. An index greater than 1 indicates a predilection of the ARR for the nuclear matrix DNA. [a]These aliquots were digested only once in DNAse I. [b]These samples were isolated from cells which has been grown in the presence of 50 ug/ml deoxynucleosides. [c]Indicates which samples were or were not further purified with Sea Prep underlayering.

Table	Nuclear Fraction	Sea Prep[c] Underlayer	% DNA (M + N)	% ARR (M + N)	% ARR in pre-ARR	ARR + DNA	P. I.
26	Matrix[a,b]	No	4.1	8.1	17.5	0.0656	2.1
26	Non-matrix[a,b]	No	95.9	91.9	14.0	0.0313	---
26	Matrix[b]	No	4.1	13.5	---	0.0656	3.7
26	Non-matrix[b]	No	95.9	86.5	---	0.0178	---
28	Matrix	Yes	1.5	21.3	40.8	0.0806	17.5
28	Non-matrix	Yes	98.5	79.7	84.2	0.0046	---
29	Matrix	Yes	1.2	18.3	---	0.0408	18.6
29	Non-matrix	Yes	98.8	82.7	---	0.0022	---

similar to the one obtained for mouse cells (17.5) isolated under similar conditions, i.e., Sea Prep underlayering coupled with two DNAse I digestions. Such a finding not only reinforces the idea that matrix ARR may be near or at the nuclear matrix DNA attachment sites but also indicates that nuclear matrix ARR exists at these locations in different mammalian species. These results suggest that ARR sites within the nuclear

matrix are strongly conserved. Therefore, ARR must be performing some very basic and important function(s) associated with the nuclear matrix.

Chemical Differences Between Matrix and Non-matrix ARR's

The purpose of this experiment was to test the hypothesis that methylation of the 2' hydroxyl position of ribose moieties may confer alkali resistance to the non-matrix ARR whereas both both methyl and phosphate groups may be conferring a similar resistance to the matrix ARR. It was suggested that the methylation of ARR may serve as a means for inactivating the 2' hydroxyl ribose sites so they would be incapable of forming diphosphate bridges in response to an internal Okazaki RNA primer signal sequence during the initiaition of DNA synthesis (See Discussion). All of the non-matrix ARR would be methylated whereas only a portion of the matrix ARR would be methylated; the rest would be phosphorylated. Therefore, the ratio of methylated alkali resistant oligomers to ARR should be higher in the non-matrix than in the matrix fraction.

A presumptive test was performed to test this hypothesis by double labeling mouse cells with [^{3}H]methionine and [^{14}C]uridine in the presence of 50 ug/ml each of adenosine and guanosine to prevent the non-specific uptake of methyl label into purine biosynthetic pathways (Weinberg and Penman, 1968; Madem and Salim, 1974). Table 31 contains [^{3}H]methionine and [^{14}C]uridine counts for nuclear matrix and non-matrix samples purified with Sea Prep underlayering. It includes data on pre-DNAse samples (those not yet exhaustively incubated in DNAse I) and samples exhaustively incubated in DNAse I twice. Those samples exposed to DNAse I include those heated to 100^{o}C for 20 minutes in 0.1 M KCl (pre-ARR samples), and those heated to 100^{o}C for 20 minutes in 0.1M KOH (ARR samples). The pre-ARR samples include thermostabile alkali-sensitive RNA and ARR. Data from Table 31 were used in the computations shown in Table 32. These computations include the percentage of total nuclear ARR which is either matrix or non-matrix ARR, (data column 1), and the percentage of pre-ARR (for a given sample) which is ARR, (data column 2). In data column 3 the alkali resistant tritiated methylated counts ($ARCH_3$) for a given sample have been divided by the ARR counts for that same sample. When the non-matrix $ARCH_3$/ARR ratio is divided by the matrix $ARCH_3$/ARR ratio a "methylation index" is

Table 31

Radiolabel Counts Isolated From Mouse Cell Nuclear Matrix and Non-matrix Double Labeled With [^{3}H] Methionine and [^{14}C] Uridine

Samples are from mouse cells labeled in log phase with [^{3}H] methionine (Met) and [^{14}C] uridine. Cells were grown in the presence of 50 µg/ml of adenosine and guanosine to inhibit the uptake of labeled methyl groups into the purine biosynthetic pathway. They were incubated for 24 hours in low serum medium with adenosine and guanosine (but no exogenous deoxynucleosides) to remove extraneous label and to inhibit DNA replication. Additional separation of the matrix and non-matrix fractions was achieved by Sea Prep underlayering. Purified matrix and non-matrix aliquots were counted or exhaustively digested in DNAse I for two successive treatments. The DNAse I digests were heated to 100°C for 20 minutes in either 0.1 M KCl or 0.1 M KOH, neutralized where appropriate, ethanol precipitated, rinsed twice, resolubilized, and counted. [a]50 minute counts, [b]Average and standard error, [c]number of aliquots counted.

Treatment	Nuclear Fraction	[^{3}H] Met[b]	[^{14}C] Uridine[b]	N[c]
Pre-DNAse	Matrix	676 ± 62	624 ± 24	3
0.1 M KCl, 100°C	Matrix	116 ± 4	112 ± 3	2
0.1 M KOH, 100°C	Matrix[a]	7 ± 0	15 ± 0.6	3
Pre-DNAse	Non-matrix	4989 ± 57	3972 ± 49	3
0.1 M KCl, 100°C	Non-matrix	463 ± 3	338 ± 8	3
0.1 M KOH, 100°C	Non-matrix[a]	96 ± 1	94 ± 4	4

generated as shown in data column 4. A methylation index greater than 1 indicates that the non-matrix ARR is more "methylated" than the matrix ARR.

The percentage of ARR associated with the matrix was 13.8% and the percentage of pre-ARR that was ARR was 13.4% and 27.8% for the matrix and non-matrix samples respectively. These numbers are considerably lower than their thymidine/uridine labeled counterparts described for Table 28 in Table 30, data rows 5 and 6 and are instead more similar to the results shown for Table 26 in Table 30, data rows 3 and 4. Interestingly, the percentages of non-matrix pre- ARR's which are ARR's for both Table 28 in Table 30 and Table 32 is twice that for their matrix counterparts. Both

Table 32

Ratios of Alkali-Resistant [^{3}H]Methionine Labeled and [^{14}C] Uridine Labeled Oligomers

Data column 1 represents percentages of total matrix and non-matrix (M + N) ARR isolated. Data column 2 represents the percentage of pre-ARR (within a given sample) which is ARR. Pre-ARR is a combination of alkali-sensitive RNA and ARR and its value is determined by heating RNA label at 100°C for 20 minutes in 0.1 M KCL. Data column 3 is the ratio of matrix or non-matrix alkali resistant methylated ($ARCH_3$) counts to their respective ARR counts. Data column 4 is called the "methylation index" (M. I.) and it is determined by dividing the non-matrix $ARCH_3$/ARR ratio by the matrix $ARCH_3$/ARR ratio. An index greater than 1 indicates that the non-matrix ARR is more "methylated" than the matrix ARR.

Nuclear Fraction	% ARR (M + N)	% ARR in Pre-ARR	$ARCH_3$ + ARR	M. I.
Matrix	13.8	13.4	0.467	2.19
Non-matrix	86.2	27.8	1.021	---

sets of cells were incubated and processed at the same time and under similar conditions except for the addition of adenosine and guanosine to the methionine/uridine group. This suggests that the exogenous adenosine and guanosine may have influenced these percentages. The methylation index was found to be 2.19 which indicates that the non-matrix ARR may be more methylated than the matrix ARR. This supports the hypothesis that chemical differences exist between matrix and non-matrix ARR. The ability of adenosine and guanosine alone to completely inhibit non-specific methylation of RNA nucleotides has been met with variable results (Weinberg and Penman, 1968; Maden and Salim, 1974). It is possible that some non-specific methylation occurred in the ARR's thereby quenching the signal provided by the specific methylation which could be occurring in the 2' hydroxyl ribose position. If such is the case, then, the methylation index may be considerably larger than 2.19.

Discussion

The relationship between the pre-ARR isolated from matrix and non-matrix DNA and the DAR isolated from gel insert studies (Chapter VI) is not yet clear. In both cases there exists a combination of both alkali sensitive and alkali resistant RNA's. However, the combination of the two has varied widely from experiment to experiment. The DAR contains a percentage of ARR which ranges from 40% to 82% (Chapter VI). Matrix and non-matrix pre-ARR's contain percentages of ARR's ranging from 13% to 41% and from 14% to 84% respectively. The percentage of whole cell RNA which has been isolated as DAR by electroelution is less than 1% (Chapter VI) whereas matrix and non-matrix pre-ARR's can be isolated in quantities which are roughly 10 times this amount. This 10 fold difference in DAR and pre-ARR quantity may be attributable to radical differences in how DAR and pre-ARR are isolated. It seems reasonable to conclude that at least a portion of the matrix and non-matrix ARR's are equivalent to ARR isolated during gel electroelution studies and, therefore, are forms of DAR's. If these relationships can be established, then, the possibility that matrix ARR is somehow involved with permanent DNA attachment sites at origins of replication will be strengthened. Evidence that ARR is associated with origins of replication is already available (Birkenmeyer et al, 1987).

Differences in the (ARR/CH_3) ratios of matrix and non-matrix ARR's suggests that methylation may play an important role in ARR function. Methylation of DNA is associated with gene inactivation. A similar function could be involved with ARR methylation in the process of DNA loop fusions during cellular differentation (see Appendix D).

CHAPTER VIII

ELECTRON MICROSCOPY STUDIES ON MATRIX AND NON-MATRIX FRACTIONS

Introduction

The results obtained thus far suggested that an RNA species may be responsible for establishing DNA domain attachment sites within the nuclear matrix. It was felt that at this point in time a more direct approach should be undertaken for studying DNA superstructure. Therefore, purified matrix and non-matrix DNA samples were prepared for examination by transmission electron microscopy. The results of these examinations led to the discovery of DNA-like circles averaging 1 to 3 μ in circumference within both matrix and non-matrix samples. Although these circles could exist as single units, many of them were interconnected into a network. Also observed were partially fused or circular DNA-like networks, principally composed of dimers which were dimpled at the point of junction. It was hypothesized that the majority of the inner region between the two dimers had been lost and the dimpling effect was an indication of the presence of base pairing between the remaining portion of the junction. The circles were similar in size to DNA circles found in pig sperm, (Hotta and Bassell,1965), developing chicken lymphocytes (Delap and Rush, 1978), monkey cells, (Krolewski et al, 1982), human fibroblasts (Icard-Liepkalns et al, 1986) and in mouse cells (Sunnerhagen et al, 1986). The circular networks were similar to those found in chromosomes treated in alkali and urea (Comings and Okada, 1973; Sorsa, 1973; Sorsa and Castrodeza, 1976). The possibility existed that these networks were concatenants of viral-like DNA involved in "rolling circle" type replication (Bernstein and Bernstein, 1973). However, it was discovered that this network was not disrupted under conditions favorable for DNA denaturation or by enzymatic digestions with proteinase K. (Results with RNAse A digestion were too poor to evaluate). Therefore, the possibility that these networks consisted of replicating

DNA circles merely joined together by replication forks was not supported by this evidence. Digestion in DNAse I followed by heating generated many small fragments followed by a loss of circles. Together these results suggested that the circles were composed of DNA connected together by covalent interactions which did not involve proteins. I have hypothesized that these networks may be held together by covalent interactions between novel RNA molecules, some of which are RNAse A/alkali sensitive and are clipped out by endogenous RNAse activity resulting in the dimpled DNA-like dimers previously described.

Results

The results to be discussed in this chapter were obtained using a variety of methods and cell types. The samples were prepared from cells double labeled with [^{3}H]thymidine and [^{14}C]uridine. Radiolabel information on these samples can be obtained from Table 33 which includes cell types used, number of nuclei used per sample, sample counts, and ratios of sample counts to background. For purposes of clarity the methods and cell types used in obtaining these results are presented in tabular form in Table 34 which is also used as a legend reference for Plates I through XIV. The results are described in Table 35. All matrix and non-matrix samples were extracted in SDS, purified by hydroxylapatite batch elution, dialysed, and ethanol precipitated prior to preparation for analysis by transmission electron microscopy. For purposes of explanation, single circles and circular networks will be referred to as DNA.

With the exception of lymphocyte matrix samples all samples used for electron microscopy had double label counts which were at least 5 times greater than background. The former counts were only 1.5 to 1.8 times above background (Table 33). The poor labeling seen in the lymphocyte fraction appeared to be largely due to a problem in label uptake by the lymphocytes under the conditions used (see Materials and Methods).

As can be seen in Table 34 all samples were prepared for electron microscopy using a cytochrome C spreading technique. All of the samples shown here were stained in uranyl acetate. Most of these samples were either rotary shadowed or subjected to

Table 33

Double Radiolabel [^{3}H]Thymidine and [^{14}C]Uridine Counts Associated With Samples Used for Electron Microscopy

Column 1 indicates the cell type for each sample used: mouse = mouse L-1210 cells, human (L) = human peripheral lymphocytes, human (R) = human lymphoblastoma cells type Reh. Column 2 is the fraction type: M = matrix, N = non-matrix. Mouse samples were not separated into single and double stranded fractions. Only the double stranded fractions were used from the human samples. Column 3 indicates the number of nuclei used to obtain the counts denoted for each sample. Columns 4 and 5 are the average counts obtained for each sample for both thymidine (T) and uridine (U) radiolabels together with the standard errors. Columns 6 and 7 indicate the background counts for each sample type. Columns 8 and 9 are the ratios of average sample counts to their respective background counts.

Cell Type	Fraction	Nuclei per Sample ($X 10^6$)	Sample CPM		Background CPM		Ratio of Sample to Background	
			T	U	T	U	T	U
Mouse	M	3.00	175 ± 4	189 ± 1	35	29	5.0	6.5
Mouse	N	3.00	4376 ± 78	1246 ± 36	35	29	125.0	43.0
Human (L)	M	2.25	64 ± 1	40 ± 1	36	27	1.8	1.5
Human (R)	M	2.50	2699 ± 13	305 ± 3	36	27	75.0	11.3

low angle shadowing using pallidium-gold. Sample treatments prior to cytochrome C spreading varied. In the simplest cases the ethanol precipitated sample pellets were redissolved in TE buffer and used directly for cytochrome C spreading. These samples were referred to as controls. Samples were heated in one of two ways: A 10 µl volume of the redissolved pellets was heated for 1 minute at 100^{o}C followed by chilling on ice. In the second method the redissolved samples were mixed with cytochrome C spreading buffer with a final formamide concentration of 40% and heated in a 10 µl volume to 80^{o}C for 1 minute followed by chilling on ice. Both of these methods should denature any double stranded nucleic acids and disrupt any non-covalent branching of DNA resulting from replication and recombination. If samples were exposed to enzymes, then, these exposures were done prior to heating. In the case of DNAse I and phospholipase C, the ethanol precipitated pellets were redissolved in TN rather than TE buffer. Once dissolution was complete the buffer was adjusted to a final concentration

Table 34

Summary of Protocols Used for Obtaining the Results Displayed in the Electron Micrographs Illustrated in Plates I Through XIV

In column 3, UA indicates staining with uranyl acetate, RS signifies rotary shadowing with pallidium-gold,and LAS indicates low angle shadowing with pallidium-gold. In column 5 the mouse cells are an L-1210 suspension culture, the human cells (R) are a human lymphoblastoid cell line referred to as Reh cells and the human cells (L) are peripheral lymphocytes. Matrix and non-matrix fractions were extracted overnight in TE buffer containing 1% SDS. The mouse matrix and non-matrix fractions were purified from hydroxylapatite by batch elution in which single and double stranded fractions were not separated . The human matrix and non-matrix fractions were purified from the double stranded fraction of a hydroxylapatite batch elution. In column 6, samples heated to 100°C were heated in 10 µl volumes of buffer for one minute and chilled on ice prior to mixing with cytochrome C DNA spreading buffer containing 40% formamide. Samples which had been digested in enzymes were heated in this same solution. Samples heated to 80°C were removed from the incubation buffer and mixed with cytochrome C DNA spreading buffer containing 40% formamide prior to heating of 10 µl volumes for one minute at 80°C. This was followed by chilling on ice. In column 7, PK indicates digestion in 200 µg/ml of proteinase K at 37°C. PLC indicates digestion at 37°C in 40 units/ml of phospholipase C. Samples treated with DNAse I were kept at room temperature during the incubation and then subjected to 80°C where indicated. Samples treated for 15 seconds with DNAse I were incubated in 0.1 to 1.0 units/ml of the enzyme. Samples treated for 60 to 120 seconds with DNAse I were incubated in 10 units/ml of the enzyme.

Table 34

Plate		Visualization	Fraction	Cell Source	Heating	Enzyme Treatments
I	a, b	UA, RS	Matrix	Mouse	None	None
II	a, b	UA	Non-matrix	Mouse	None	None
III	a	UA	Matrix	Mouse	None	PK, 3 hours
III	b	UA, LAS	Matrix	Mouse	None	DNAse I, 60 sec
IV	a, b	UA	Matrix	Human, R	None	None
IV	c, d	UA	Matrix	Human, L	None	None
V	a	UA, RS	Matrix	Mouse	None	None
V	b	UA, RS	Matrix	Mouse	100°C	None
VI	all	UA, LAS	Matrix	Mouse	80°C	None
VII	---	UA, LAS	Matrix	Mouse	80°C	None
VIII	a	UA, RS	Matrix	Mouse	None	PK, 3 hours
VIII	b	UA, RS	Matrix	Mouse	100°C	PK, 3 hours
VIII	c	UA, RS	Matrix	Mouse	None	None
VIII	d	UA, RS	Matrix	Mouse	100°C	None
VIII	e	UA, RS	Matrix	Mouse	None	PK, 3 hours
VIII	f	UA, RS	Matrix	Mouse	100°C	PK, 3 hours
IX	---	UA	Matrix	Mouse	None	PK, 3 hours
X	a	UA, LAS	Matrix	Mouse	None	DNAse I, 60 sec
X	b	UA	Matrix	Mouse	None	DNAse I, 120 sec
XI	---	UA, LAS	Matrix	Mouse	80°C	DNAse I, 15 sec
XII	---	UA, LAS	Matrix	Mouse	80°C	DNAse I, 15 sec
XIII	a	UA, LAS	Matrix	Mouse	80°C	DNAse I, 60 sec
XIII	b	UA	Matrix	Mouse	80°C	DNAse I, 120 sec
XIII	c	UA	Matrix	Mouse	None	PLC, 3 hours
XIII	d	UA	Matrix	Mouse	None	PLC, 3 hours
XIV	---	UA	Matrix	Mouse	80°C	DNAse I, 120 sec

of proteinase K. DNAse I incubation parameters were much less stringent in that the incubation times did not exceed 2 minutes at room temperature using enzyme concentrations of 10 units/ml or less. Once the enzyme incubations were complete the samples were either used directly for cytochrome C preparations or heated by one of the two methods previously described.

Plates I and II are examples of mouse matrix and non-matrix DNA samples respectively which have not been subjected to either heating or enzyme incubations. In both plates the presence of interlocking circles which are heterogenous in size is obvious. In Plate Ib there appears to be connections between two linear strands and a circular structure. Since these circular networks have survived both an overnight extraction in 1% SDS buffer followed by purification on hydroxylapatite it is reasonable to conclude that they are probably circular DNA's or RNA's devoid of any protein which was not covalently attached to them. The circular networks observed in the non-matrix samples are truncated versions of the more elaborate networks observed in the matrix samples and may very well represent matrix contamination of the non-matrix. Evidence that matrix DNA may contaminate non-matrix samples has been presented in Chapter VII of this Dissertation.

Plate III contains examples of vesicular-like material isolated from mouse matrix DNA whose relationship to the DNA circles (if any) is unclear. The morphology is strikingly different from the circular networks observed in Plates I and II in that the vesicular networks are much less elaborate, more homogenous in size, and seem to exist primarily in partially "fused" pairs of "vesicles" which stain heavily within the boundaries of the "vesicles" (unlike the circles) suggesting the presence of a "membrane-like" covering over the entire surface of the structure. It is not clear if the enzyme incubations in either proteinase K or DNAse I generated these structures or if they were already present. Both Plates IIIa and b were stained but the latter was also subjected to low angle shadowing.

Plate I

Untreated Mouse Matrix DNA

Bars = 0.5 microns, see Table 34 for details

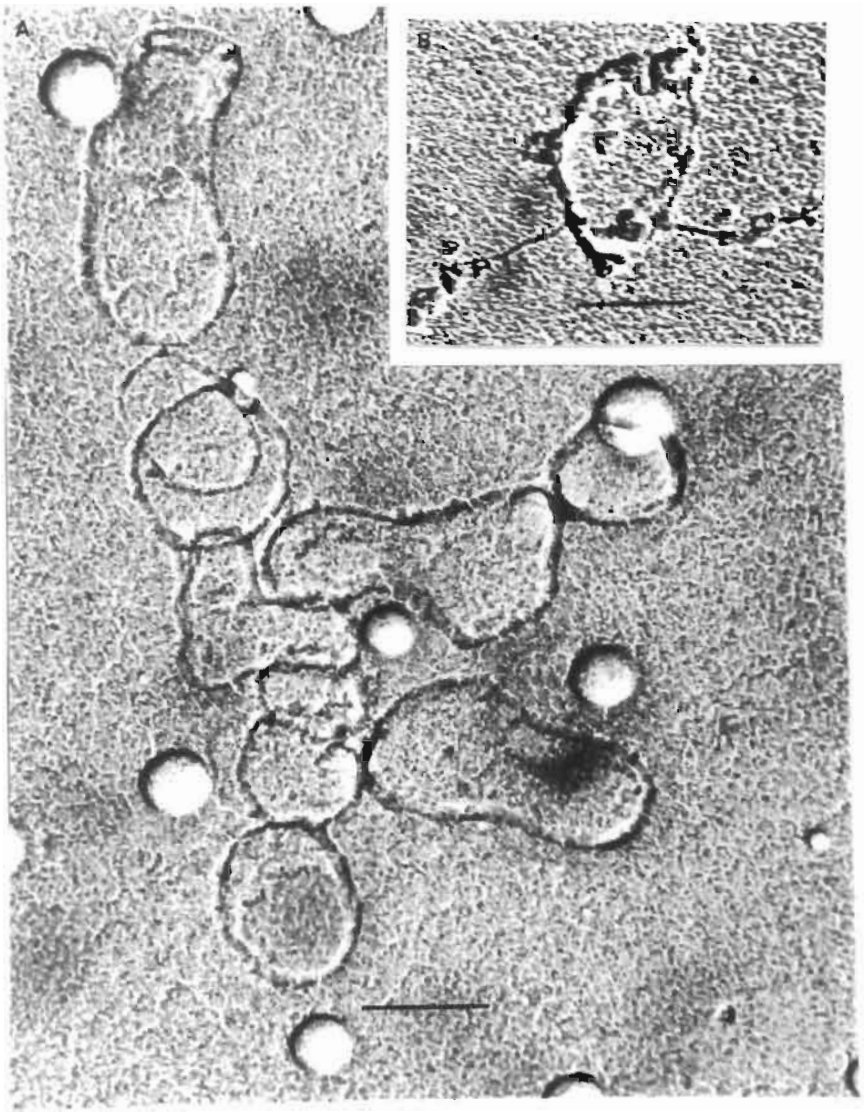


Plate II

Untreated Mouse Non-matrix DNA

Plate IIa, b bars = 2.0 and 0.1 microns respectively, see Table 34 for details

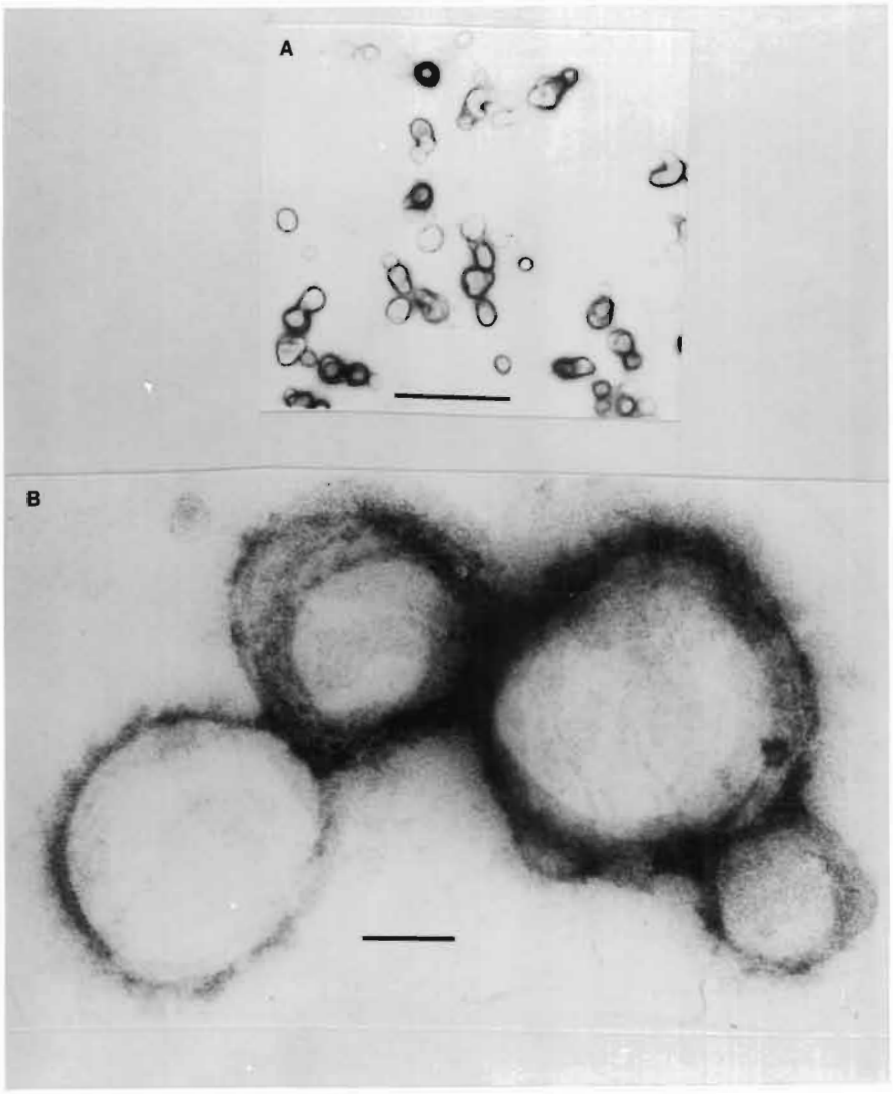


Plate III

Mouse Matrix "Vesicles" From Samples Treated With Either DNAse I or Proteinase K

Bars = 1.0 micron, see Table 34 for details

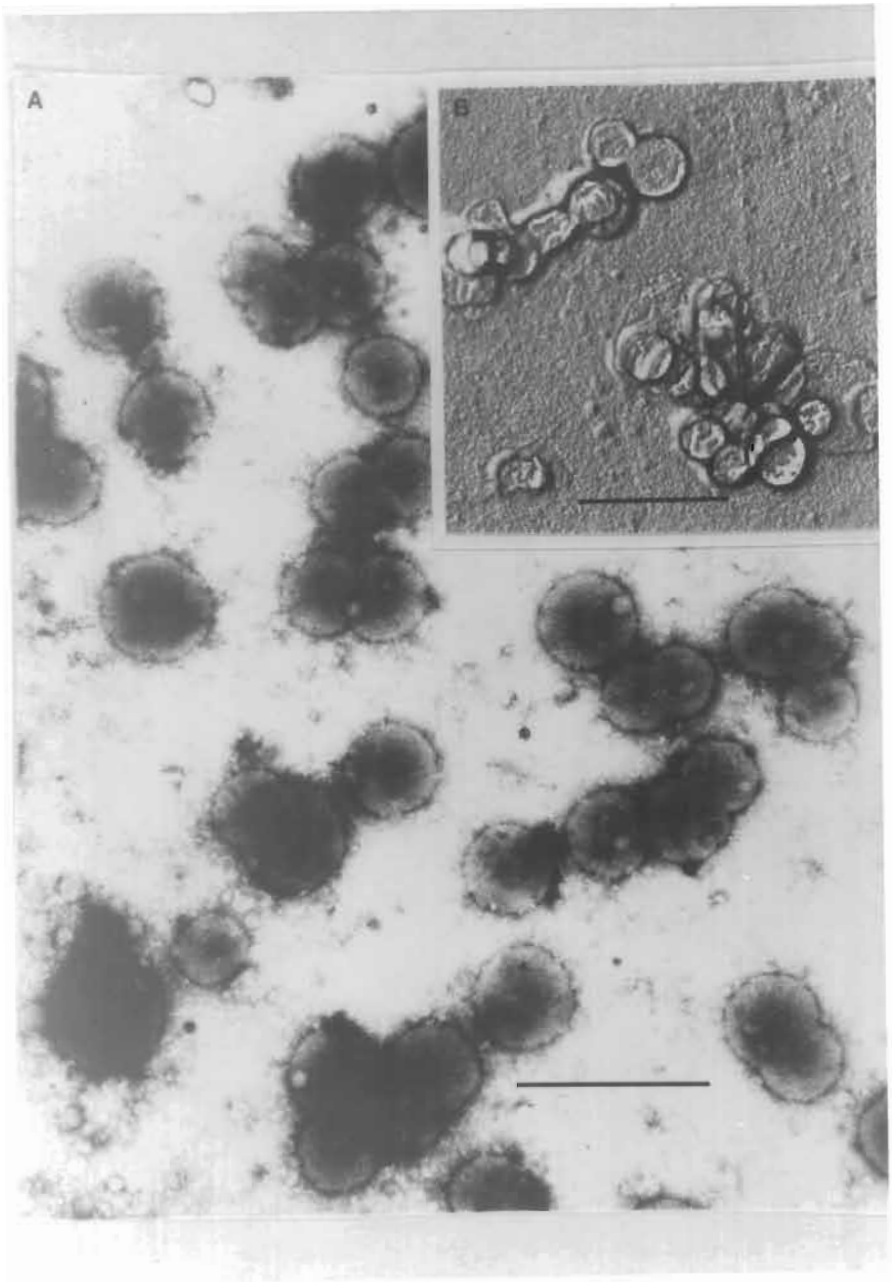


Plate IV includes matrix samples derived from both human lymphocyte (a and b) and Reh cells (c and d). These samples were isolated from the double stranded fractions of hydroxylapatite batch elutions. Otherwise, they were processed in a manner identical to the mouse matrix and non-matrix fractions previously described in Plates I and II. The photographs clearly demonstrate the presence of small circular networks which are morphologically very similar to the networks found in the mouse matrix and non-matrix fractions. Their presence suggests that these circular networks are not unique to mouse cells and that they exist in other mammalian species as well.

All of the remaining plates feature mouse matrix DNA samples which have undergone experimental manipulations. In Plate V the samples were divided into two groups: One group (a) served as a control and was treated as described before. The other group (b) was subjected to heating at 100^{o}C for 1 minute and chilled on ice. Both groups were then mixed with cytochrome C spreading buffer and used to make DNA spreads for electron microscopy. The control samples contained circular networks similar in morphology to those seen previously. Heating of the samples greatly distorted the morphology of the networks but evidence of surviving circular interconnections was still evident. This strongly suggests that the bonds responsible for the circular interconnections are covalent in nature and, therefore, resistant to denaturation by heat. In Plates VI and VII the samples were first mixed with the DNA spreading solution in a final formamide concentration of 40% and then heated to 80^{o}C for 1 minute followed by chilling on ice. The chilled samples were then used directly for making DNA spreads. These conditions should not only denature double stranded nucleic acids but should also inhibit renaturation upon lowering the temperature. The most striking result is the presence of "daisy-like" structures in which loops of DNA appear to form a rosette around a central core region (a and b). Also included are a plasmid-like circular molecule which appears to be partially denatured (c) and a very long strand of DNA which has been partially cropped (d). Plate VII is a higher magnification of Plate VIa. These unusual configurations further support the hypothesis that eukaryotic chromosomal DNA exists in a superstuctural form that is non-linear in nature which is established by covalent interactions between adjacent DNA molecules. The next series of experiments involved enzyme incubations with or without subsequent heating of the samples prior to DNA spreading.

Plate IV

Untreated Matrix DNA's From Human Reh Cells and Lymphocytes

Bars = 0.5 microns, see Table 34 for details

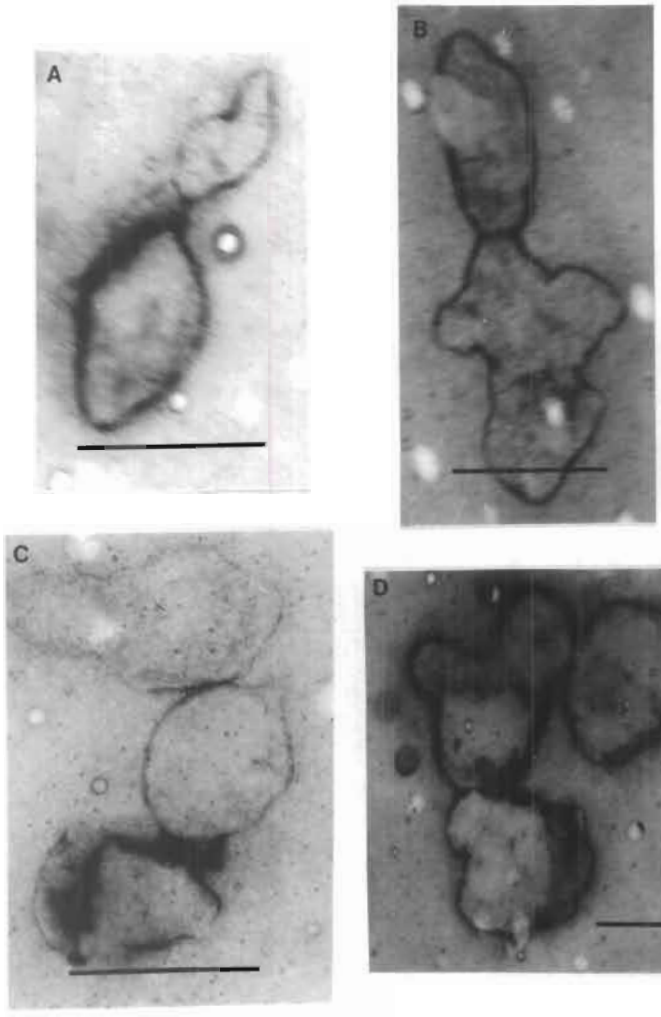


Plate V

Mouse Matrix DNA, Untreated or Heated to 100°C

Bars = 0.5 microns, see Table 34 for details

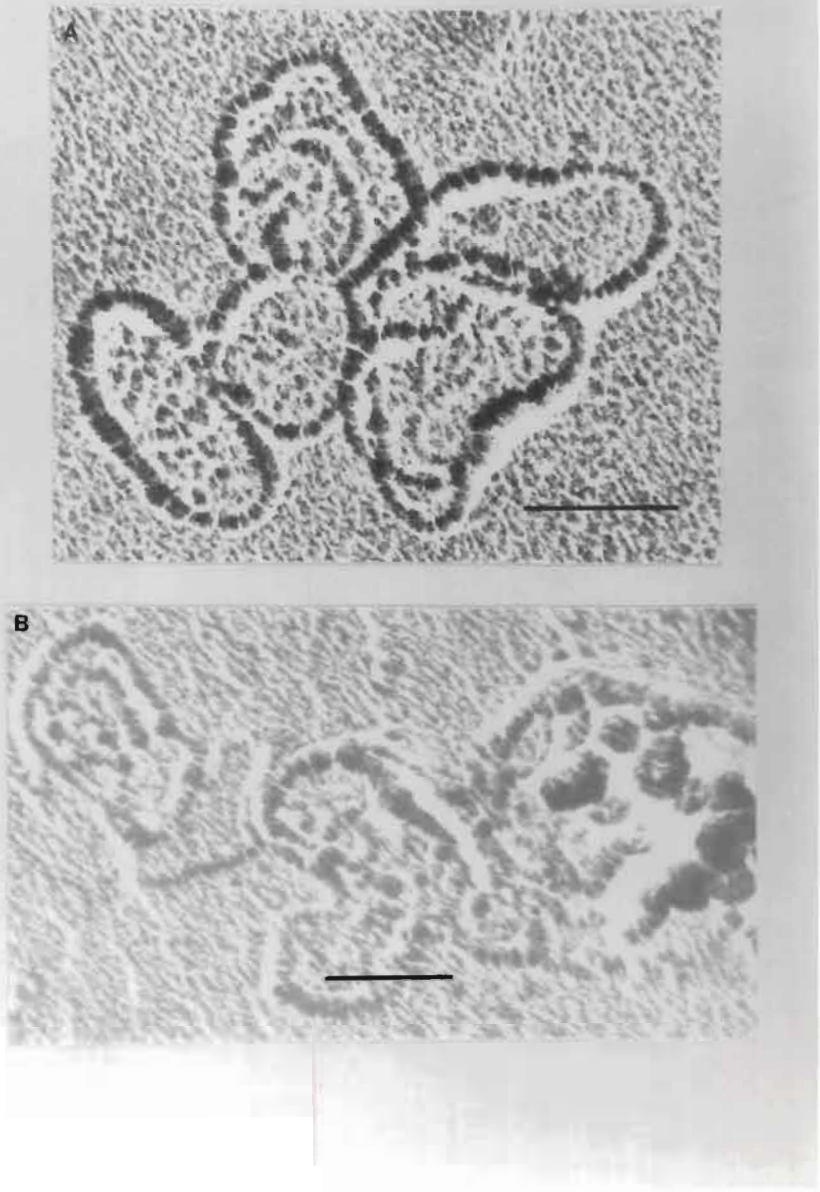


Plate VI

Mouse Matrix DNA Heated to 80°C in DNA Spreading Buffer Containing 40% Formamide

Plate VIa, c bars = 0.2 and 1.0 microns, Plate VIb, d Bars = 0.5 microns, see Table 34 for details

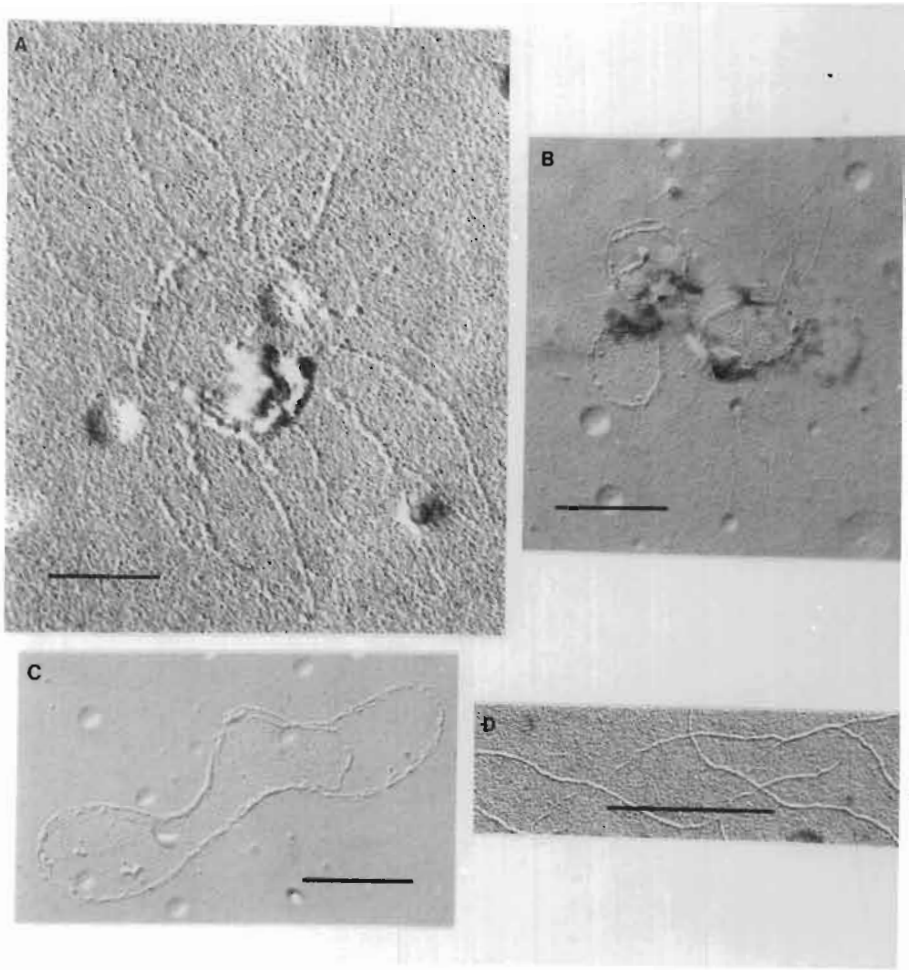


Plate VII

Enlarged View of Plate VIa

Bar = 0.1 microns, see Table 34 for details

In Plates VIII and IX the samples were subjected to digestion in 200 μg/ml of proteinase K for 3 hours at 37°C or sham incubated. The samples were then split into four groups: These groups are illustrated in Plate VII as follows: 1) undigested and unheated controls (c) similar to the samples described in Plates I and II, 2) undigested and heated to 100°C for 1 minute followed by chilling on ice (d), digested in proteinase K and unheated (a and e) and Plate IX, and digested in proteinase K and heated to 100°C (b and f). Samples in Plates VII and IX were stained but the former was also subjected to rotary shadowing. Although the morphology of the circles and networks was greatly altered there was no indication that treatment with proteinase K alone or in combination with heat denaturation disrupted the integrity of the circles or their interconnections. Therefore, it appeared certain that the interconnections were the result of non-proteinaceous covalent bonds. This conclusion supports evidence presented earlier in Chapter IV relating to the inability of proteinase K digestion to eliminate the ability of gel inserts to retain whole cell DNA during electroelution. It was stated in this chapter that retention of whole cell DNA by gel inserts during electroelution may be due to the presence of a non-linear DNA superstructure which is trapped by the agarose and remains trapped even after proteinase K treatment.

The remaining series of experiments discussed in Chapter VIII concerns the incubation of matrix DNA samples in either 0.1 to 10 units/ml of DNAse I for up to 2 minutes at room temperature or incubation in 40 units/ml of RNAse A or phospholipase C for 3 hours at 37°C. The only samples subjected to heating were those exposed to DNAse I. The conditions used for heating were identical to those described for samples in Plate VI in which the sample was mixed with cytochrome C buffer at a final formamide concentration of 40% and heated to 80°C for 1 minute followed by chilling on ice and DNA spreading. Plate X contains samples exposed to 10 units/ml of DNAse I for 60 and 120 minutes (a and b respectively). Both samples were stained but the former was also subjected to low angle shadowing. Although no visible damage appears to have occurred, a considerable amount of single stranded nicking must have taken place in order to explain the results seen in Plates XI through XIV.

Plate VIII

Mouse Matrix DNA Treated With Proteinase K or Sham Incubated With or Without Heating to 100°C

Plate VIIIb bar = 1.0 micron, all others = 0.5 microns, see Table 34 for details

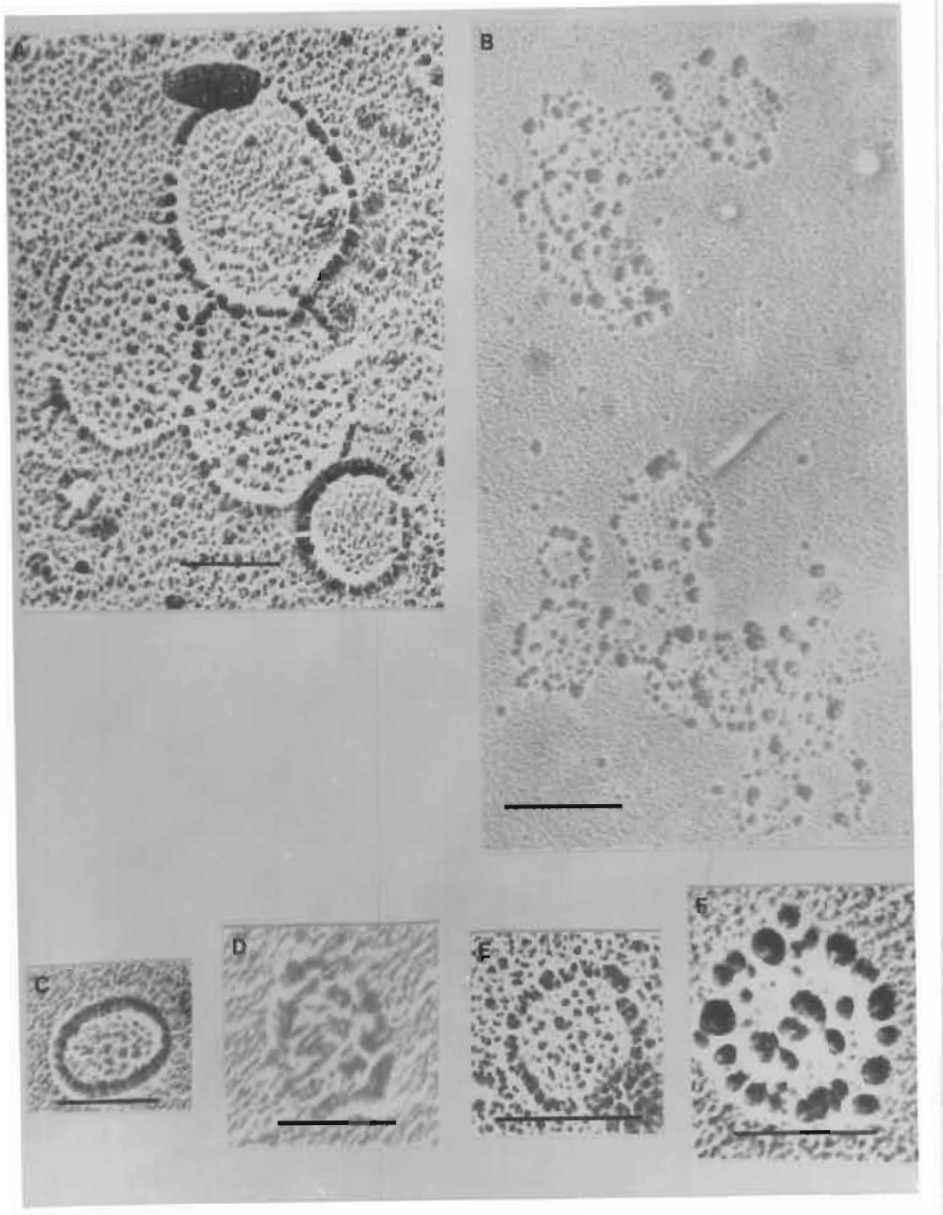


Plate IX

Mouse Matrix DNA Treated With Proteinase K

Bar = 0.2 microns, see Table 34 for details

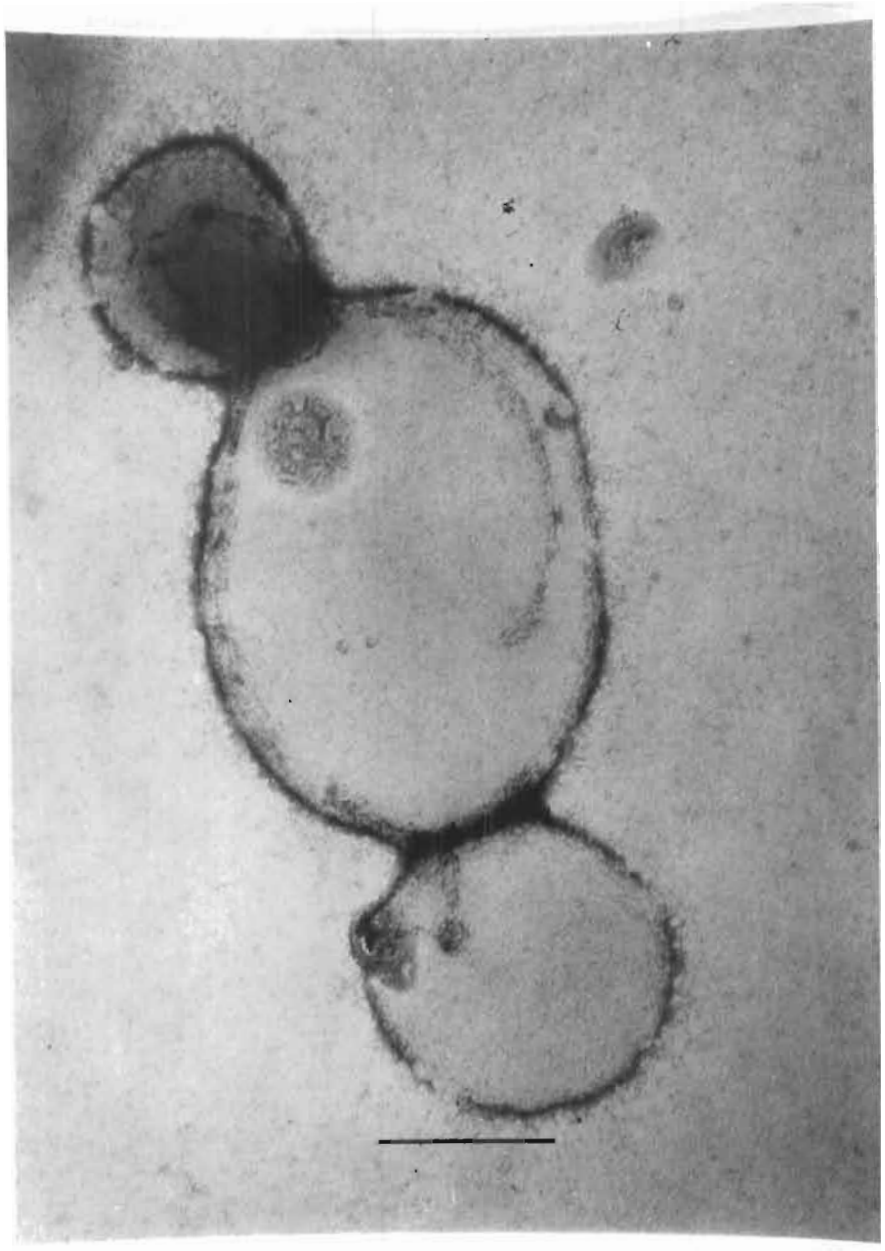
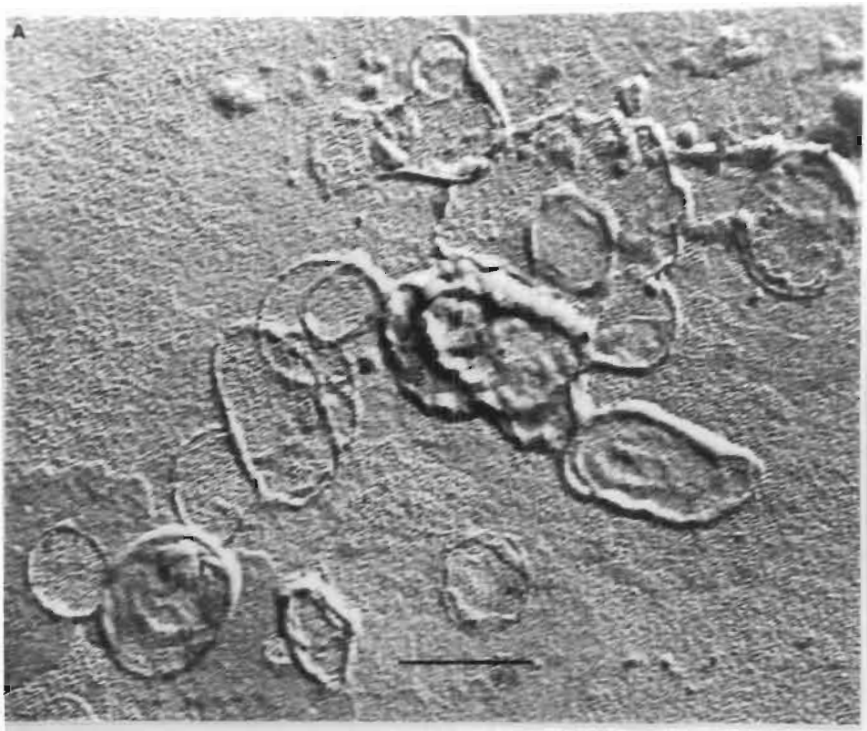
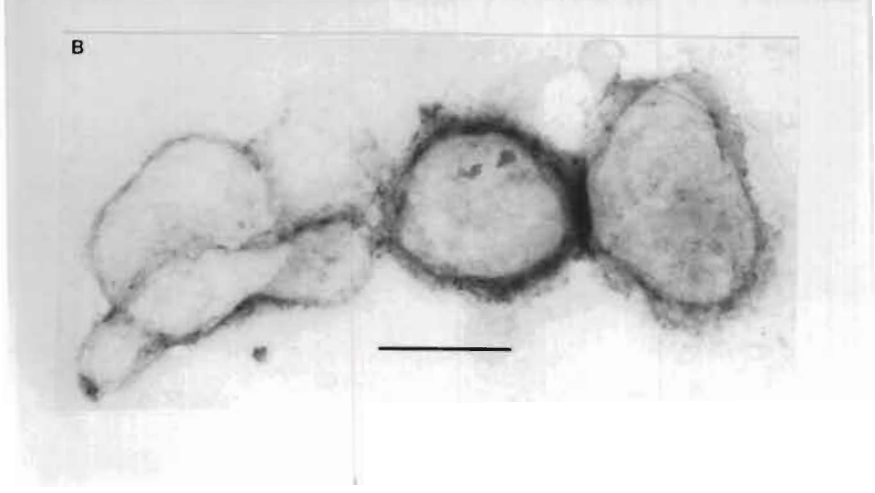


Plate X

Mouse Matrix DNA Treated With DNAse I

Bars = 0.5 microns, see Table 34 for details





In Plates XI and XII the samples were exposed to either 0.1 or 1.0 units/ml of DNAse I for only 15 seconds at room temperature prior to mixing the sample into cytochrome C buffer and heating it to 80^{o}C as described earlier. The results suggest that the stained DNA circles are breaking up into small linear fragments of DNA as a result of the combined actions of DNAse I digestion and heating in cytochrome C buffer. Furthermore, searches for intact circles or networks under these conditions were unsuccessful. The apparent ability of DNAse I to destroy these circles is the strongest direct evidence yet that they are composed largely of DNA. Similar fragments generated by DNAse I from samples incubated in 10 units/ml of DNAse I for 60 seconds and 120 seconds can be seen in Plate XIII (a and b respectively). Both samples were stained but the former was also subjected to low angle shadowing. Plate XIV is a magnfication of Plate XIIIb.

The ability of phospholipase C to disrupt the DNA circles was visible in Plate XIII (c and d). Both samples were negatively stained. This supports evidence described in Chapter IV relating to the ability of phospholipase C to reduce the ability of gel inserts to retain whole cell uridine label during electroelution. Evidence presented in that chapter and others suggest that this uridine label is intimately associated with the DNA and its subsequent loss reflects a loss in the integrity of the DNA with which it is associated. Additional evidence that phospholipase C can release matrix DNA fragments from the nuclear matrix has also been cited (Maraldi et al, 1984). The simplest explanation for these phenomena is that the phospholipase C is contaminated with an endonuclease whose activity becomes detectable when phospholipase C is used at high concentrations over extended periods of time. However, the possibility also exists that the phospholipase C is disrupting some sort of exotic phosphatidyl bond between nucleic acid and phospholipids.

The ability of RNAse A to disrupt circles or networks was not detectable (photograph not shown). However, the sample quality was so poor and quantities so low that any interpretations of the results would be equivocal.

Plate XI

Mouse Matrix DNA Treated With DNAse I Followed by Heating to 80°C in DNA Spreading Buffer Containing 40% Formamide

Bar = 0.2 microns, see Table 34 for details

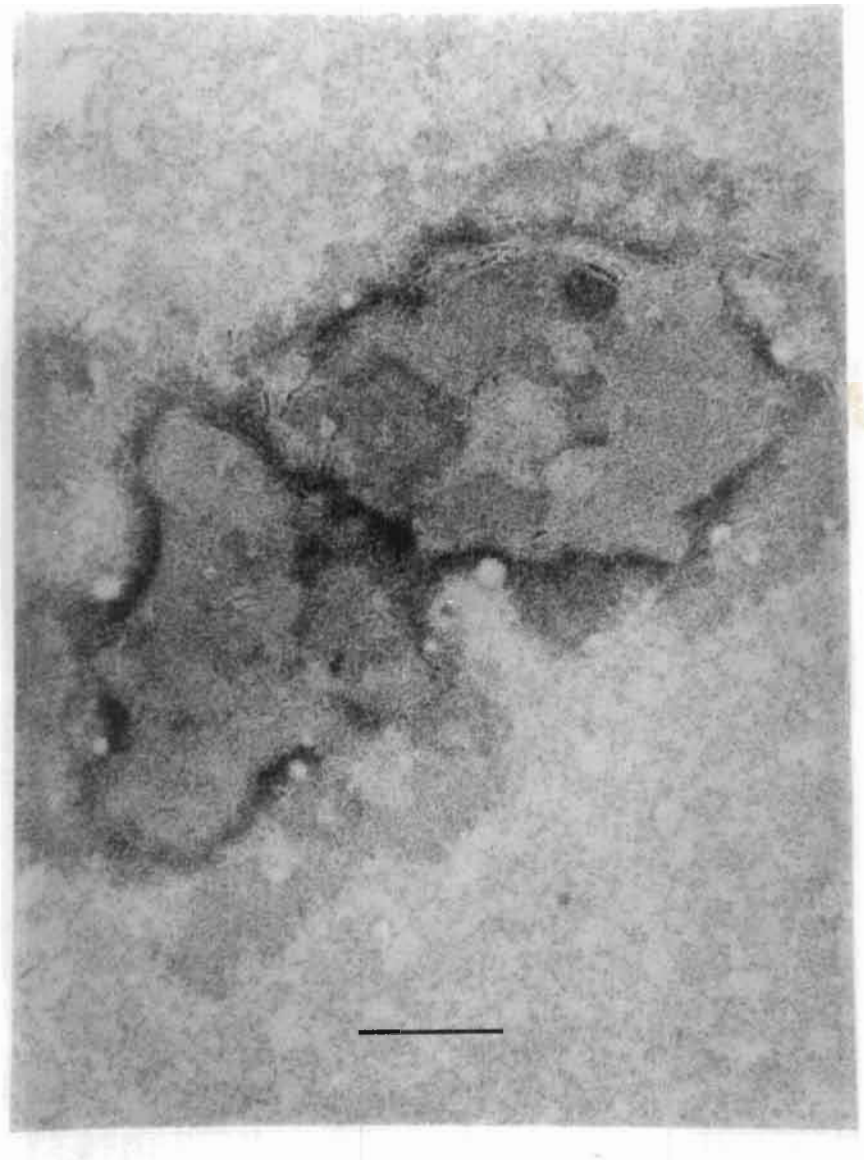


Plate XII

Mouse Matrix DNA Treated With DNAse I Followed by Heating to 80°C In DNA Spreading Buffer Containing 40% Formamide

Bar = 0.2 microns, see Table 34 for details

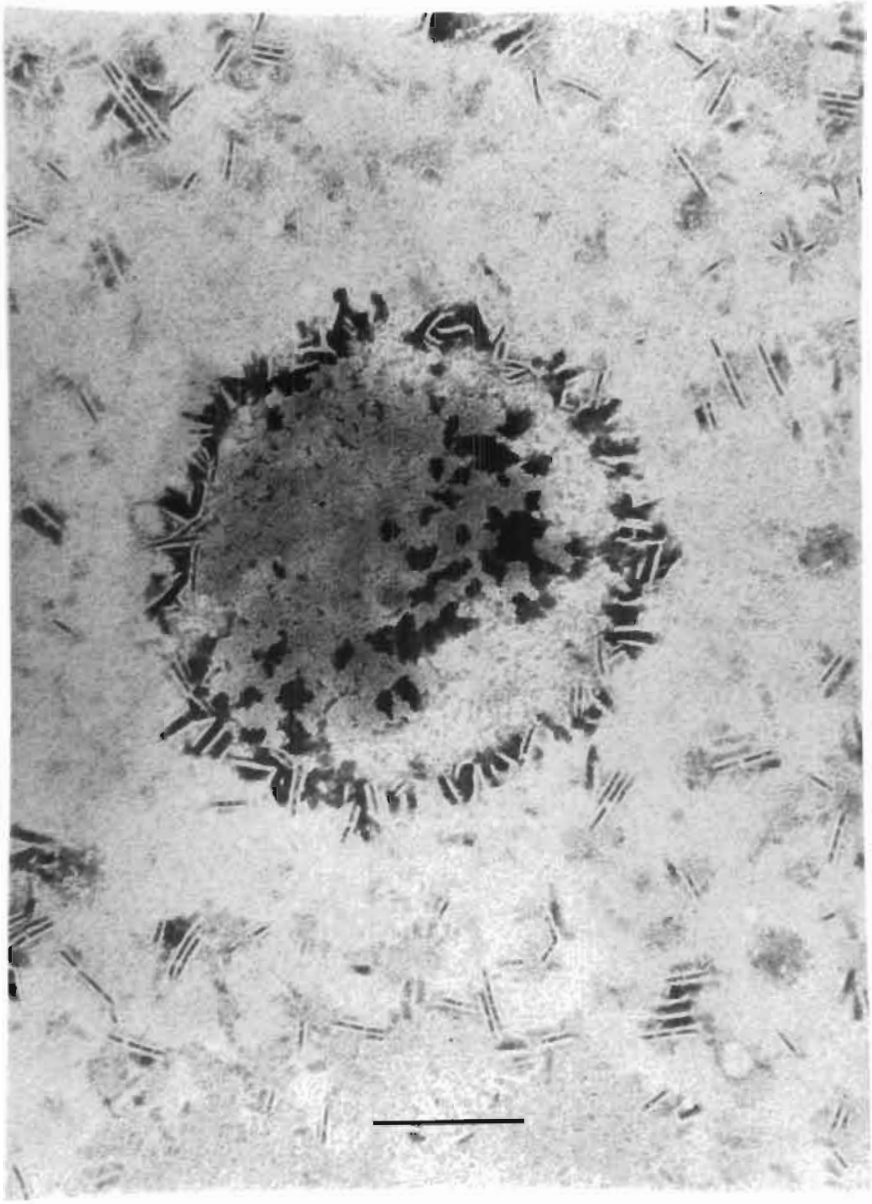


Plate XIII

Mouse Matrix DNA Treated With DNAse I or Phospholipase C, Samples Treated With DNAse I Followed by Heating to 80°C in DNA Spreading Buffer Containing 40% Formamide

Bars = 0.2 microns, see Table 34 for details

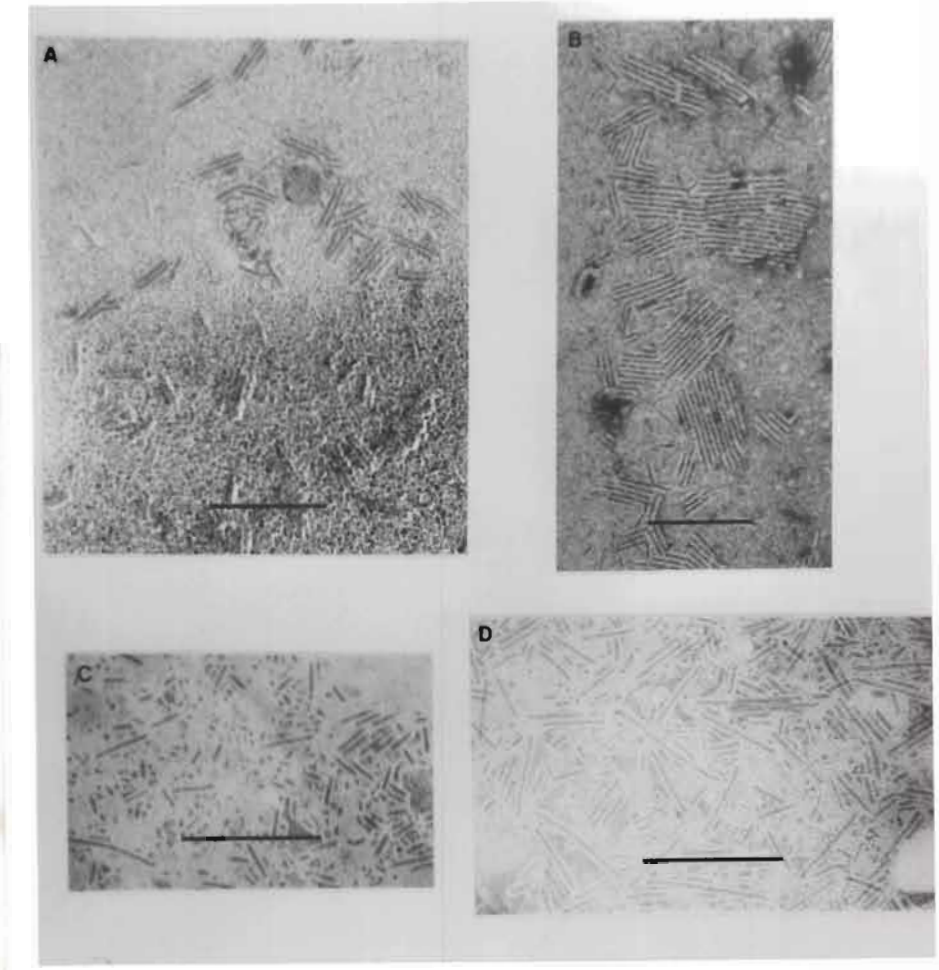


Plate XIV

Enlarged View of Sample Type Shown in Plate XIIIb

Bar = 0.05 microns, see Table 34 for details

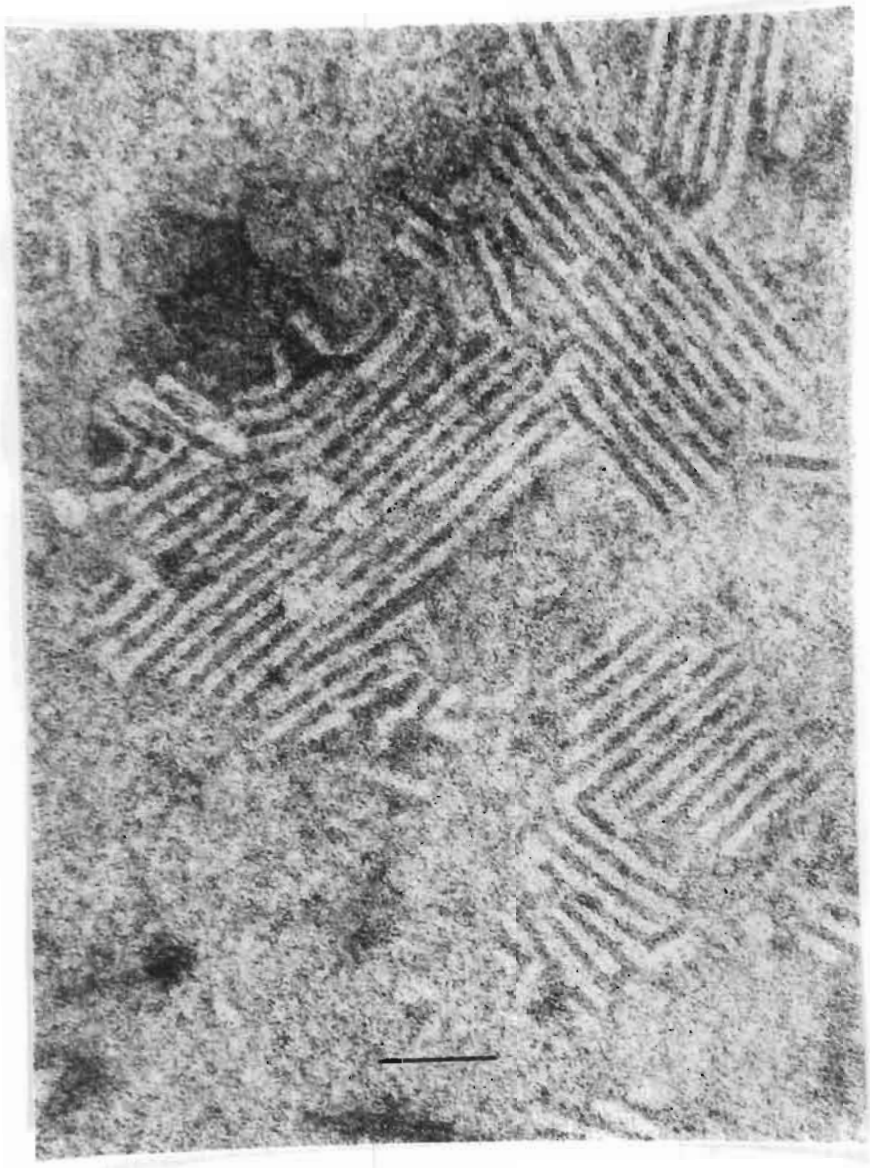


Table 35

Effect of Various Treatments on the Integrity of Matrix "DNA" Circles and Interconnections

Treatments are as follows: SDS =1% SDS extraction overnight at room temperature, alkali = alkali hydrolysis, 100°C = heating of a 10 µl volume of sample to 100°C for 1 minute followed by chilling on ice, 80°C = heating of a 10 µl volume of sample to 80°C in 40% formamide for one minute followed by chilling on ice, proteinase K = digestion in 200 µg/ml of proteinase K for 3 hours, at 37°C, phospholipase C = digestion in 40 units/ml of phospholipase C for 3 hours at 37°C, RNAse A = digestion in 40 units/ml of RNAse A for 3 hours at 37°C, DNAse I (low) = digestion in 0.1 to 1.0 units/ml of DNAse I between 15 to 120 seconds at room temperature, DNAse I (high) = digestion in DNAse I in concentrations, times, and temperatures greater than those used in these experiments. Cell types include mouse L-1210 cells (M), human lymphoblastoma cells, type Reh, (R), and human peripheral lymphocytes (L) *Samples were low in quantity and poor in quality.

Treatment	Cell Type	Visible Effect	
		Circles	Interconnections
SDS	M, R, L	None	None
SDS, alkali	-	----	----
SDS, 100°C	M	None	None
SDS, 80°C	M	None	None
SDS, proteinase K	M	None	None
SDS, proteinase K, 100°C	M	None	None
SDS, RNAse A*	M	None?	None?
SDS, phospholipase C	M	Destroyed	?
SDS, DNAse I (low)	M	None?	None?
SDS, DNAse I, (low) 80°C	M	Destroyed	?
SDS, DNAse I, (high)	-	---------	----

Plates XVa, b, c, d, and e are selected areas from Plates Ia, IIa, IIIa, IIIb, and Xa respectively. A typical circular interconnection is shown in (a). Configurations which appear to be fusion circles involving two smaller circles are seen in b through e. The forces responsible for the dimpling effect present in all of these fusion circles is interpreted in Plate XVI using a series of circular rubber bands which have been "superglued" together, forming an interconnecting joint. Plates XVIa, b, and c represent the intact interconnected joint, cutting of the joint, and the formation of the dimpled fusion molecule respectively. Figure 16 is a hypothetical illustration of the

molecular bonding which could be generating these effects. The nature and possible significance of this bonding is discussed in considerably more detail in Appendix D. The interconnecting bond between two circles is illustrated as a tetrameric base paired region which crosslinks the two circles together at both ends by RNAse A resistant ARR (a). The internal region is susceptible to both RNAse A digestion and alkali hydrolysis (b). Its subsequent destruction by either of these treatments generates the dimpling effect which is established by the ARR crosslinked hairpins (c).

Plate XV

A Composite Photograph of Samples Demonstrating the Presence of Fused, Dimpled "DNA" Circles

Plate XVa is an example of two networked "DNA" circles from Plate Ia. Plates XVb, c, d, and e are examples of fused, dimpled "DNA" circles from Plates IIa, IIIa. IIIb, and Xa respectively. Bars for Plates XVa, b, c, d, and e = 0.5, 2.0, 1.0, 1.0, and 0.5 microns respectively. See Table 34 for details

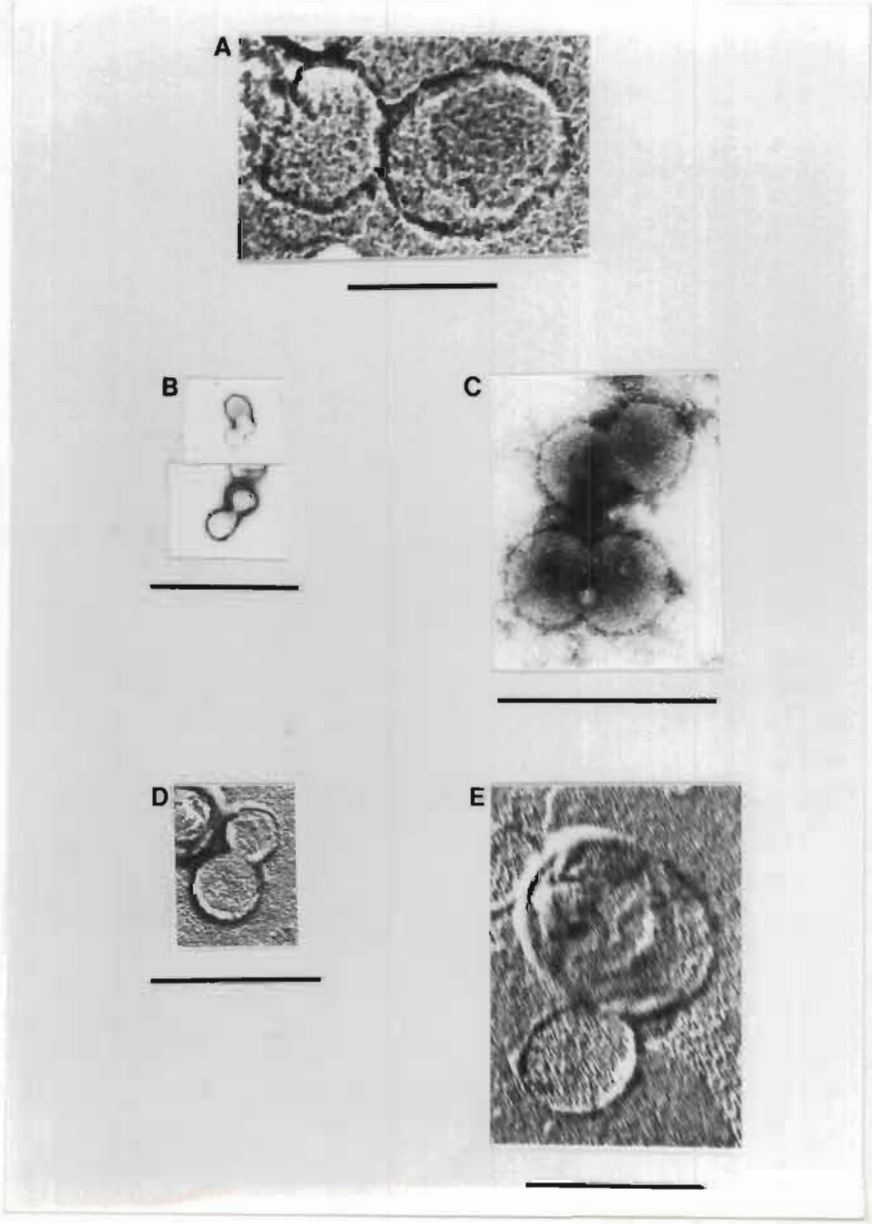
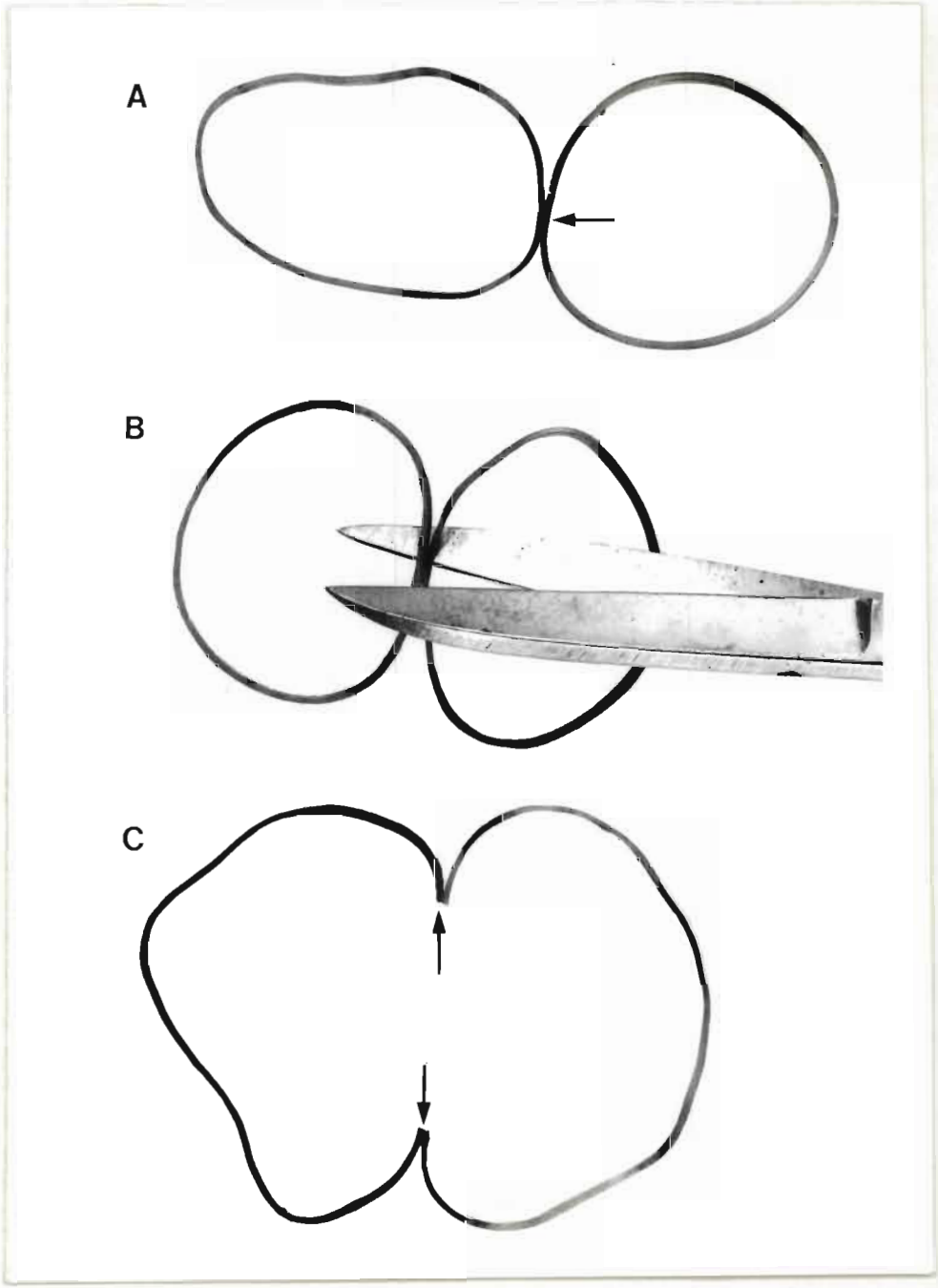


Plate XVI

Interpretation of How Fused, Dimpled "DNA' Circles May be Generated From Networked "DNA" Circles

Plate XVIa, b, and c represent networked DNA circles, the cutting of networked circles at the point of attachment, and the fusing and dimpling of the circles respectively. See Figure 16 for a possible explanation of this hypothetical event.



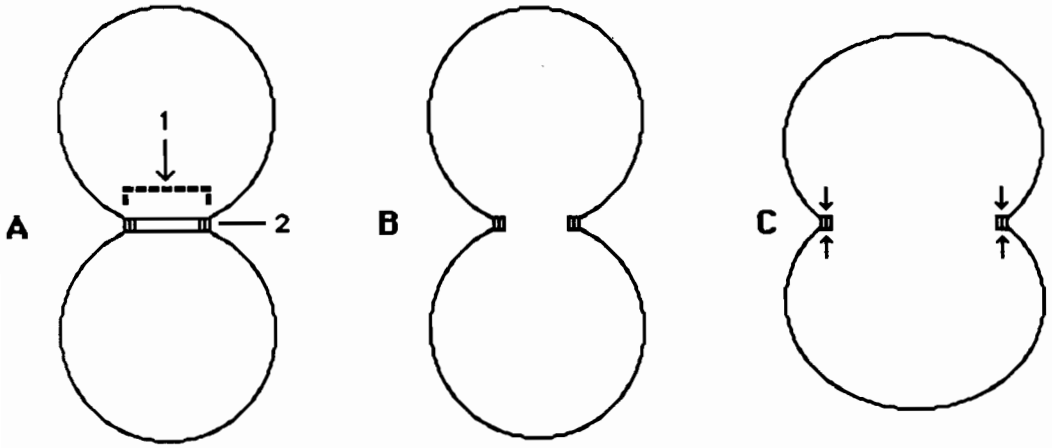


Figure 16

Hypothetical Model for Explaining the Dimpling Effect Observed Within the Fusion Circles Displayed in Plates XVb Through e

Two circles are interconnected by a tetrameric base paired region (1) which is flanked by covalently crosslinked ARR (2) as shown in (a). Hydrolysis and loss of the internal tetrameric region coupled with the retention of the ARR crosslinked hairpins (b) results in the dimpling effect observed in the resulting fusion circle (c). For additional information see Figures 40-45.

Discussion

The results described in Chapter VIII support the hypothesis that a non-linear DNA superstructure exists within eukaryotic matrix DNA that is non-proteinaceous in composition and involves covalent interactions between adjacent DNA circles. Whether these interconnections are composed of DNA, RNA, or some other molecule remains to be seen. The relationship of these interconnections to DNA domain attachment sites is unclear as is the signficance of the vesicular material shown in Plate III. The ability of two adjacent circles to "fuse" into a larger dimpled circle (Plate XVb-e) is intriguing and may provide clues as to the nature of the bonding between interconnected circles (Figure 16). Clearly the discovery of these novel DNA configurations within the nuclear matrix of mouse and human cells and their resistance to denaturation and proteinase K digestion warrents further investigations of their composition, function, mode of replication, presence in other cell types, and evolutionary origins.

CHAPTER IX

GENERAL DISCUSSION

Initial Hypotheses

In Chapter I of this Dissertation a set of four hypotheses were stated relevant to this study of eukaryotic DNA superstructure. These hypotheses were as follows:

1. In the absence of non-covalently bound material, intact eukaryotic DNA superstructure exists in a complex, non-linear form.

2. This non-linear DNA superstructure is established by permanent nuclear matrix DNA domain attachment sites.

3. The molecules and bonds responsible for establishing this superstructure are covalent crosslinks which are associated with RNA that is resistant to RNAse A and alkali.

4. The RNAse A/alkali resistant RNA associated with the non-matrix DNA is chemically different from its nuclear matrix counterpart, suggesting that functional differences exist between the two classes of RNA.

Evidence

Evidence for a Non-linear DNA Superstructure

Non-linear DNA structures such as circles are randomly trapped depending upon their size and degree of supercoiling when mixed with liquid agarose which is then gelled (Dean et al, 1973, Schindler et al, 1982). Linear DNA's larger than 20 kb can

also be trapped but much less efficiently than circles (Schindler et al, 1982). Branched DNA migrates more slowly during agarose gel electrophoresis than does a linear DNA fragment of comparable molecular weight (Bell and Byers, 1983). Large linear DNA's the size of chromosomes are capable of electroelution from an aqueous system into a gel (Schwartz and Cantor,1984 ; Van der Ploeg et al,1984). In this context an aqueous system also includes gel inserts in which cells are lysed after insert formation (Cook, 1984). Even so, much of the DNA (Table 9) is still resistant to electrophoresis (Cook,1984; Schwartz and Cantor,1984; Van der Ploeg et al,1984). These data suggest that when non-covalently bound materials are removed from intact eukaryotic DNA by agents such as SDS, a non-linear DNA superstructure remains which prevents all or part of the DNA from migrating in an electric field through an agarose gel.

Evidence for Non-linear DNA Superstructure in Matrix DNA

Early electron microscopy studies have revealed that chromatin and chromosomal loops originate and return back into the same area of the nuclear matrix or the chromosomal scaffolding (McCready et al, 1980; Mullinger and Johnson, 1980; Laemmli et al, 1977; Paulson and Laemmli, 1977). This suggests that the non-matrix DNA (the outer exposed loops) would be converted into linear DNA when nuclear DNA is separated into matrix and non-matrix fractions after DNAse I digestion and salt extraction. If matrix DNA also existed in a linear form in a similar size range as non-matrix DNA, then, following these treatments it should respond in a fashion similar to non-matrix DNA relative to DNA retention after exhaustive alkali treatment followed by alkaline elution on polycarbonate filters. It should also respond in a similar fashion to gel electroelution. Evidence suggests that matrix DNA is more resistant to alkaline elution after exhaustive treatment in alkali than is non-matrix DNA (Tables 11 and 13). Furthermore, when matrix and non-matrix DNA's are incorporated into gel inserts, extracted in SDS, and subjected to gel electroelution in the presence or absence of alkali, the matrix DNA is more resistant than non-matrix DNA to electroelution (Table 18). The combination of these two independent set of results suggests that matrix DNA is different from non-matrix DNA and exists in a non-linear form.

Evidence for the Presence of Covalently Linked Nuclear Matrix DNA

Theoretically, DNA can exist as a covalently bound non-linear superstructure in only one of two ways: 1) as a series of interlocking DNA circles 2) as linear or circular segments of DNA which are covalently bound to one another by non-DNA linkers. Evidence for the presence of the former DNA superstructure has been observed by autoradiography (Bernstein and Bernstein, 1973) and at least two kinds of catenated dimers have been observed (Lewin,1977). Complex networks of thousands of DNA circles can also be found within the kinetoplasts of Trypanosomes (Ray, 1987). Evidence for the presence of covalently linked chromatin circular networks is also available (Comings and Okada, 1973; Sorsa, 1973; Sorsa and Castrodeza, 1976). Evidence that matrix DNA may be comprised of complex networks of covalently linked circles resistant to denaturation and proteinase K digestion is described in Chapter VIII. The presence of similar but smaller structures in non-matrix fractions is probably the result of matrix contamination of the non-matrix fraction (Plate II). This evidence suggests that at least a portion of the non-linear matrix DNA superstructure presumed responsible for the preferential retention of matrix over non-matrix DNA during alkaline elution and gel electroelution may be in the form of the complex circular networks described in Chapter VIII.

Evidence for the Role of Non-DNA Linkers in
Establishing Non-linear DNA Superstructure

If it is assumed that non-linear DNA superstructure is the result of non-DNA covalent linkers, then, such linkers may be composed of protein, RNA, phospholipids, or some other class of molecules. Evidence that protein is involved in supercoiling has been presented (Cook, 1984). Evidence that protein is involved in at least some of the preferential retention of matrix DNA over non-matrix DNA has also been presented (Table 18). Furthermore, a small fraction of the total protein label remains tightly associated with the DNA during intact DNA retention studies even after a 24 hour radiolabel "cold chase" indicating that this protein is permanently associated with the DNA (Tables 5 and 20). It is also present during alkaline elution studies involving log phase cells (Tables 11 and 13). However, evidence provided in intact DNA studies (Table 3) and in matrix and non-matrix DNA gel electroelution studies (Tables 18 and 19) indicates that most of the intact DNA and matrix DNA retention attributable to

non-linear DNA superstructure is unaffected by treatments which should destroy protein linkers. Whether or not a small amount of DNA loss occurs following such treatments is uncertain. Although the loss (if any) of protein label resulting from these treatments was not directly monitored, the evidence indicates that the majority of non-linear DNA superstructure established by non-DNA linkers is not proteinaceous. Digestion of matrix fractions with phospholipase C releases DNA fragments (Maraldi et al, 1984). Evidence that phospholipids may be partially responsible for non-linear DNA superstructure is available from intact DNA studies involving digestion of DNA in phospholipase C (Table 3). However, other evidence suggests that such a digestion degrades the DNA into fragments similar to those observed during DNAse I digestions (Plate XIII). Therefore, the phospholipase C may contain an endonuclease activity. Because of this it cannot be stated with certainty whether digestion of intact DNA with phospholipase C results in a loss of retention because of digestion of phospholipids or because of the direct degradation of the DNA.

Evidence for the association of RNA with non-linear DNA superstructure in intact DNA studies even after a 24 hour radiolabel "cold chase" is shown in Tables 5, 20, 21, 22, 24, and 25. Retention of RNA under these conditions indicates that it is permanently associated with the DNA. Intact DNA retention is reduced slightly by treatment with heat-treated RNAse A suggesting that intact RNA may be necessary for at least some of the DNA retention (Table 20). Therefore, the effect of RNAse A digestion on intact DNA was analagous to the effect of proteinase K digestions on matrix DNA retention (Table 18). The association of RNA label with the remaining intact DNA was unaffected by treatment with RNAse A followed by electroelution. This could be interpreted by arguing that a low level of DNAse I activity was still present in the heat-treated RNAse A preparation which was responsible for the slight loss in intact DNA label following RNAse A digestion and electroelution. However, the RNA which is associated with the DNA contains an alkali resistant (ARR) subcomponent which may be resistant to RNAse A (Tables 21 and 22). Furthermore, during the course of electroelution, rates of DNA and ARR loss indicate that the two molecules are tightly linked (Table 24). This close asociation of ARR to DNA suggests that the uridine label may have been converted into thymidine prior to incorporation into the ARR. The evidence against this interpretation is as follows: 1) conditions favorable for conversion of uridine into thymidine were minimized by the incubation of cells in the

presence of exogenous deoxynucleosides prior to the addition of radiolabel (Thelander and Reichard, 1979), 2) the conversion of uridine label into thymidine followed by its incorporation into DNA should be much lower than the direct incorporation of thymidine label into DNA, and radiolabeled thymidine uptake was strongly suppressed by the presence of exogenous deoxynucleosides (Figure 14). However, the percent uridine label which was ARR was as much as one-third to one-half of the percent of thymidine label incorporated into DNA isolated under the same conditions (Table 21) and the total counts isolated were 100-200 times higher in the ARR fraction than in the DNA fraction even though the specific activity of the radiolabeled thymidine and uridine were equivalent (data not shown), and 3) mass spectrometry indicated that ARR contains about 100 times more uracil than does whole cell DNA (Table 25). Therefore, the current evidence supports the hypothesis that an RNAse A/alkali resistant RNA which is tightly associated with intact DNA could play a role in establishing a non-linear DNA superstructure which is responsible for intact DNA retention during gel electroelution.

Evidence That ARR is Concentrated in the Nuclear Matrix and May be Located at Permanent DNA Domain Attachment Sites

Some component of the ARR associated with DNA in intact DNA retention studies is concentrated in the nuclear matrix DNA even after a 24 hour radiolabel "cold chase" (Tables 26, 28, 29, and 31). Such an association indicates that matrix ARR is permanently associated with the DNA. Furthermore, the inverse relationship between the percentage of total nuclear DNA associated with the nuclear matrix and the ratio of matrix ARR to matrix DNA suggests that matrix ARR is closely associated with matrix DNA attachment sites (Table 30). Since the matrix ARR is present even after incubating radiolabeled cells for 24 hours in medium containing no radiolabel it must be associated with permanent attachment sites, and perhaps origins of replication. Evidence that ARR is associated with origins of replication has been cited (Birkenmeyer et al, 1987).

Evidence that Non-matrix ARR is Chemically Different From Matrix ARR

Only 20% of the nuclear ARR is associated with the nuclear matrix DNA even though it is highly concentrated there (Table 30). The possibility that some of the matrix

ARR is contaminating the non-matrix fraction is quite strong (Tables 27, 30, and Plate II). However, it is difficult to suggest that 80% of the matrix ARR is being retained by the non-matrix fraction when the non-matrix fraction was purified by Sea Prep underlayering (Table 30). It is also feasible to suggest that some of the non-matrix ARR is a legitimate component of the non-matrix fraction. If this is the case, then, how is it similar to or different from matrix ARR? Evidence suggests that non-matrix ARR is more methylated than matrix ARR (Table 32) and is, therefore, chemically different. The chemical differences in matrix and non-matrix ARR may indicate functional differences between the two classes of molecules. The "methylation" of non-matrix ARR may be correlated to its lack of association with DNA domain attachment sites (see Appendix D).

Summary and Conclusions

The observation that intact eukaryotic cellular DNA is capable of resisting electroelution following SDS extraction indicates that the DNA may be covalently linked together in a non-linear fashion. The molecular structure of DNA requires that any non-linear superstructure can only occur if the DNA exists as catenated circles or if it is covalently interconnected by non-DNA linkers. Electron microscopic evidence supports the presence of interconnecting non-DNA linkers as opposed to catenated circles of DNA. The tight association of RNA and protein to intact DNA even after conditions favorable for serum arrest coupled with a radiolabel "cold chase" suggests that one or both of these molecules could be non-DNA linkers which are associated with the DNA even during periods of non-replication. But the inability of proteinase K and RNAse A to disrupt the majority of the non-linear DNA superstructure suggests that neither protein nor RNA are involved as non-DNA linkers for the majority of the non-linear DNA superstructure. However, the presence of alkali resistant RNA which is tightly bound to intact DNA suggests that this RNA may also be resistant to the effects of RNAse A and, therefore, could be a non-DNA linker. The topology of DNA loops indicates that the most probable location of non-DNA linkers is within the nuclear matrix or chromosomal scaffolding at the level of the DNA domain attachment sites. Evidence indicates that the ARR is not only highly concentrated in the nuclear matrix but that it probably resides within the DNA domain attachment sites. Furthermore, it appears to be chemically and, therefore, functionally different from non-matrix ARR.

Taken together, this evidence supports the hypotheses that a non-linear eukaryotic DNA superstructure is being established at the DNA domain attachment sites within the nuclear matrix by ARR covalent crosslinks which remains intact even during periods of non-replication. And the most probable mode of interaction which would account for the alkali resistance of ARR and its ability to couple DNA molecules together would be phosphodiester covalent crosslinks between the 2' ribose positions of adjacent ARR linkers.

Current Hypotheses and Methods of Testing

Hindsight can be a remarkable attribute in the quest for scientific knowledge. It provides a data base on which to formulate experiments which should have been performed during the course of experiments already concluded. On a less satirical note, however, it also provides a framework for the development of entirely new kinds of experiments that go beyond the task of "mopping up" old data. Based upon this established data base the following hypotheses, subhypotheses and methods of testing are being proposed for post-doctoral investigations.

Hypothesis I

The eukaryotic chromosome does not contain a single linear piece of DNA but is instead composed of independently supercoiled interlocking circles of DNA connected together at their origins of replication within the permanent nuclear matrix DNA attachment sites. The bonding responsible for this non-linearity is RNAse A/alkali resistant RNA which is covalently crosslinked at 2' phosphodiester positions between adjacent replicons. The crosslinks flank a tetrameric base paired region within the origins of replication which is either RNAse A/alkali resistant (Type R) or sensitive (Type S) depending upon the presence or absence of Okazaki RNA primers within the region. Type R regions are associated with structural DNA such as centromeres and Type S regions are associated with DNA capable of gene rearrangements during developmental processes such as cellular differentiation, aging, and carcinogensis. For further details on Hypothesis I, see Appendix D.

Methods of Testing

This hypothesis can be tested using the phenomenon of DNA trapping in gel inserts. It has been established that when naked DNA is mixed with liquid agarose and trapped by gelling, the degree of trapping is a function of its size, circularity, and extent of supercoiling. Large, relaxed circles are trapped most effectively. Small linear molecules are trapped least effectively (Dean et al, 1973; Schindler et al, 1982). It is possible to extract cells or isolated nuclei embedded in low melting temperature gel inserts with SDS and then encapsulate the insert within a pipette section using high melting temperature agarose plugs. This capsule can be heated to 70^{o}C resulting in a melting of the insert without leakage from the capsule because of the high melting temperature agarose plugs (Chapter IV). The quiescent melting of the insert should release the SDS extracted DNA into an aqueous environment without extensive mechanical shear. The capsule can then be chilled on ice to regel the insert, thereby trapping the DNA within the agarose. The data presented in this Dissertation in Chapter IV relating to the phenomenon of DNA trapping in whole cells and isolated nuclei which have been extracted in SDS prior to melting and regelling is inconclusive. However, I feel that the major reason for this is the presence of supercoiled DNA circular domains which are not amenable to trapping. If the DNA is relaxed either by X-ray irradiation or proteinase K treatment, the DNA domains will open up and become more accessible for trapping. There should be a positive correlation between the degree of trapping and X-ray dose in the dose area which yields predominantly single stranded nicks if more than one independently supercoiled circle is being relaxed.

Subhypothesis 1

The radiolabeled uridine associated with total ARR or matrix and non-matrix ARR has not been altered by alternative pathways such as ribonucleotide reductase or by heating in 0.1N KOH.

Method of Testing

Perform mass spectrometry on acid-hyrdolyzed ARR and determine base ratios as before but use whole mouse L-1210 cell DNA as a control rather than herring sperm DNA. This will also determine if guanosine and adenosine levels are significantly different from whole mouse cell DNA.

Subhypothesis 2

Matrix ARR is located at the DNA domain attachment sites.

Method of Testing

If this is so, then, increases in DNAse I digestion of nuclei should decrease the total DNA associated with the matrix with little effect on matrix ARR levels. Therefore, a graph of matrix ARR/DNA ratios against the percent of DNA remaining associated with the matrix should reveal an inverse relationship between the two variables.

Subhypothesis 3

ARR is alkali resistant because of either methyl or phosphate groups attached to the 2' hydroxyl of the ribose moiety. The methyl groups reside within both the matrix and non-matrix DNA fractions but the phosphate groups reside only within the matrix DNA.

Methods of Testing

An RNA processing activity has been discovered that debranches RNA lariats (Ruskin and Green, 1986). This 2' phosphodiesterase could be used to probe for the hypothetical ARR attachment sites for 2' RNA crosslinks. Detachment of ARR sites by a 2' phosphodiesterase should generate loop deletions and not loop fusions, the latter of which may occur in prokaryotes (see Hypothesis II). Therefore, this method of testing may not only provide clues to the nature of ARR sites but may also provide additional information relative to differences in prokaryotic and eukaryotic DNA attachment sites based upon whether a predominance of loop fusions or deletions occur upon treatment with this enzyme.

Current evidence suggests that non-matrix ARR is more methylated than matrix ARR by at least a factor of 2. A similar experiment can be performed with [^{32}P] inorganic phosphate. If an extra phosphate is located at the 2' position of the ARR ribose in only matrix ARR, then, the ratio of [^{32}P] labeled matrix ARR to [^{32}P] labeled non-matrix ARR should be greater than 1 and theoretically as high as 2 assuming every matrix ARR ribose moiety contained an extra phosphate group. It is possible, however, to generate 2' phosphates without an additional phosphate by

triester bonding between phosphates and ribose sugars on opposing nucleic acid strands. Experiments with alkaline phosphatase and alkali hydrolysis suggest that this is not the case, however.

Matrix and non-matrix ARR M/U and P/U ratios may be evaluated by triple labeling cells with [^{3}H]methyl-methionine, [^{14}C]uridine, and [^{32}P] inorganic phosphate. Non-specific background labeling by methylation and conversion of uridine label into thymidine could be reduced by the addition of exogenous guanosine, adenosine, formate, and all four deoxynucleosides. Ethanol precipitated DNA product containing ARR would be subjected to alkali hydrolysis and ethanol precipitated to remove all alkali sensitive uridine label. The pellets would be resuspended and exhaustively digested in a DNAse I, 3' and 5' exonuclease cocktail to completely remove all the DNA from the ARR. The removal of the DNA is important for evaluating the ARR P/U ratios because of the phosphate background generated by the presence of DNA. The efficiency of this digestion in removing DNA could be monitored initially using cells double labeled with {^{3}H]thymidine and [^{14}C]uridine. The final product would be subjected to column chromatography and all fractions would be counted and plotted to determine the efficiency of thymidine and uridine separation by the location of the thymidine and uridine peaks within the fractions. Once an efficient DNA digestion and thymidine-uridine separation protocol has been established the cells would be triple labeled as described above, the digested ARR would be subjected to column chromatography, and fractions counted to determine if the triple label peak is migrating properly through the column. Counts from the triple label peak which are not in danger of overlapping the invisible thymidine peak will be used to determine M/U and P/U ARR ratios for matrix and non-matrix samples. If the hypothesis is correct, then, the P/U ratio should be greater in matrix than non-matrix samples and the M/U ratio should be smaller in matrix than non-matrix samples.

The ARR product could also be analyzed by mass spectrometry to determine its exact molecular composition and its relative concentration if appropriate standards are available. A 2'3'5' phosphorylated RNA nucleotide could be created as a standard and a protocol is available for producing 2'3'5' adenosine. It may be possible to use this protocol to produce other 2'3'5' standards as well. Alkali hydrolyzed ribosomal RNA could be used as alkali resistant 2'-0-methylated RNA nucleotide standards.

Subhypothesis 4

At least some of the independently supercoiled DNA domains are interlocked by RNA linkers sensitive to RNAse A/alkali and treatments with these reagents result in DNA domain fusions with the concommittent release of a small piece of interconnecting DNA. Treatment of intact DNA domains with proteinase K does not release any DNA which would suggest (but not confirm) that increases in trapping by proteinase K are due to relaxation of supercoiling only and not due to DNA domain fusions. The hypothetical construction of the tetrameric region and its response to various sequential treatments is described next in terms of Forms I through Forms IV ARR.

Form I ARR

The ARR 2' phosphate groups serve as bridges between two circular DNA domains which are base paired into a tetrameric region at their origins of replication. This region is flanked by ARR which covalently links the DNA domains together by phosphate bridges. The superstructural configuration comprising two or more DNA domains can be categorized based upon whether or not any RNAse A/alkali sensitive RNA exists between the ARR linkers. Type R represents structural DNA (centromeric?) which is devoid of any RNAse A/alkali sensitive RNA and is mainly composed of very small circles of interconnected DNA molecules analogous to those described in Chapter VIII. Another term for these structures is "static" loops indicating their intrangience to change. Type S represents functional DNA which is involved in gene rearrangements during cellular differentiation. Interconnecting linkages between DNA domains in this category contain a tetrameric region composed of DNA Okasaki segments which are 100 to 150 nucleotides in length alternating between Okasaki RNA primers which are 10 nucleotides in length. All of the RNA primers are alkali sensitive RNA (ASR). The flanking ARR segments are derived from RNA primers and are composed of either 100% ARR or a combination of ARR and ASR. However, no ASR exists outside the central tetrameric region. Destruction of the RNAse A/alkali sensitive RNA within the tetrameric region of these "fluid" loops results in the fusion of the two adjacent DNA domains sharing the terameric region.

Form II ARR

After exhaustive DNAse I digestion the resulting product is a central tetrameric region flanked on both ends by two double stranded linear DNA segments which are separated from the central region by ARR. The outer double stranded DNA segments consists of two daughter strands which are assymetric in length because of unequal DNAse I digestion.

Form III ARR

Heat denaturation splits the entire Form II ARR complex down the middle as well as removing small fragments of DNA from the flanking DNA regions nicked by DNAse I. This results in greater assymetry in the outer flanking regions with respect to fragment size and A/T and G/C ratios. The split daughter fragments contain two non-complementary single stranded DNA fragments at each end which are connected to a central region which is one-half of the original tetrameric region, and, therefore, double stranded.

Form IV ARR

Upon alkali hydrolysis the ASR segments with the central region are destroyed resulting in the loss of central region DNA and a splitting of the remaining Form III ARR complex into two components. How much of the central region is destroyed depends upon whether the ARR flanking regions are completely ARR or contain an ASR component. If the ARR regions are completely ARR, then, a segment of central region DNA will survive at one end of each of the Form IV ARR complexes resulting in a complex which is separated by ARR into an asymmetric non-complementary end composed of two non-daughter strands and a symmetric complementary end composed of two daughter strands. If the ARR contains an ASR component, then, the entire central region will be lost and the Form IV ARR complex will contain only an ARR hairpin connected to two non-daughter, non-complementary strands.

Methods of Testing

If some of the DNA domains are interlocked by RNAse A/alkali sensitive linkers, then, treatment of relaxed DNA domains with either RNAse A or alkali prior to melting and regelling should increase trapping even more because DNA domain

fusions generate bigger domains which are trapped more effectively than smaller domains. If DNA which has been relaxed by proteinase K can be trapped even more effectively after RNAse A treatment, this would indicate that at least some of the DNA domains are being separated by RNA and not protein linkers.

It has been demonstrated that treatment of inserts containing radiolabeled DNA with RNAse A followed by electroelution and exhaustive DNAse I digestion results in a loss of about 25% of the DNA radiolabel from the insert and about 50% of the ethanol or trichloroacetic acid DNA pellets recovered from the DNAse I digestion (Table 20). Therefore, treatment of intact DNA with RNAse A appears to facilitate the release of a small portion of the DNA during electroelution. This experiment could be further refined using samples derived from cells double labeled with either [^{3}H]thymidine and [^{14}C]leucine or [^{3}H]thymidine and [^{14}C]uridine to determine if treatment with alkali or RNAse A or proteinase K causes any additional releasing of DNA radiolabel together with RNA or protein radiolabel from gel inserts containing intact, supercoiled DNA during electroelution. In the first set of experiments, whole cells or isolated nuclei would be extracted in SDS prior to use. Gel inserts could be rinsed and sham incubated or exposed to RNAse A followed by exposure to proteinase K along with appropriate sham incubated controls. The inserts would then be divided into two additional groups: One group would be sham incubated and the other would be incubated in alkali for 30 minutes to one hour followed by neutralization. If the ability of thymidine and uridine labels to be retained by gel inserts during electroelution is unperturbed by treatment in proteinase K, but slightly perturbed by RNAse A and alkali after protein label has disappeared, then, this confirms the hypothesis that protein is not responsible for the retention of the intact nuclear DNA during electroelution but RNA which is RNAse A/alkali sensitive is responsible for a small amount of DNA retention. The continued presence of RNA label together with the DNA could be interpreted as either a novel form of RNA which is insensitive to RNAse A and alkali treatments or the conversion of RNA label into DNA. If RNAse A and alkali have already been shown to generate DNA domain fusion, then, this small loss of DNA supports the hypothesis that a small piece of interconnecting DNA is being lost during DNA domain fusions.

In the past, eukaryotic nuclei have been subjected to alkali hydrolysis, heating, and exposure to RNAse A and proteolytic enzymes in order to understand the nature of the constraining forces (DNA domain attachment sites) which establish the DNA as

independently supercoiled domains. The results of studies have been based on the premise that reduction in supercoiling as measured by ethidium bromide binding assays is an indication of the loss of the constraints (Cook and Brazell, 1976; Cook et al, 1976; Cook and Brazell, 1978). However, in studies with prokaryotes involving RNAse A incubations the evidence suggests that the DNA may be folded into independently supercoiled DNA domains by RNA linkers which may be destroyed without a concommittent loss in supercoiling (Worcel and Burgi, 1972; Pettijohn and Hecht, 1973). A similar situation could exist in eukaryotes. If so, then, the following set of experiments could be performed to test this hypothesis: Eukaryotic nuclei exposed to alkali followed by neutralization or to RNAse A should undergo a certain degree of loop fusion following exposure to 1% SDS, 4M urea , and perhaps even to 2M NaCl depending upon the proportion of DNA constraints which are sensitive to these treatments. They could be irradiated with X rays and subjected to nucleoid-type sedimentation analyses against controls not treated with RNAse A or alkali to determine if either of these treatments have caused an increase in the average loop size. If loop fusions have occurred as a result of treatments with RNAse A or alkali, then, this supports the hypothesis that RNAse A/alkali sensitive RNA exists at the junction of some DNA domains and its destruction results in the concommittent fusion of these DNA domains.

Purified matrix DNA samples can be exposed to RNAse A and alkali and compared to appropriate controls under the electron microscope. If the linkers contain RNAse A/alkali sensitive regions, then, the circles will fuse into larger circles which may contain visible linker fragments. The average circular size will be much greater in samples exposed to RNAse A or alkali than in the control samples.

Matrix samples could be electroeluted after exposure to RNAse A to see if such treatment causes any loss in DNA retention. Treatment with alkali has already demonstrated such losses but the reason for the losses could be due to denaturation of small pieces of DNAse I nicked DNA (Table 18). If similar losses occur under conditions of neutrality after RNAse A treatment, then, this supports the hypothesis that a portion of the matrix DNA superstructure is held together by RNAse A/alkali sensitive bonds.

Nuclei can be purified from cells triple labeled as described in Subhypothesis 3 and treated with RNAse A or alkali. They can then be subjected to a brief treatment with

DNAse I to generate matrix and non-matrix fractions. Some of the RNAse A/alkali sensitive attachment sites may detach in the presence of 2M NaCl and be released into the non-matrix fraction. These fractions would be analyzed as described in Subhypothesis 3 to determine if either the RNAse A or alkali treatments have decreased the matrix P/U ratio and slightly increased the non-matrix P/U ratio. The effect on the M/U ratios should be just the reverse. These results would support the conclusion that loop fusions have occurred as a result of RNAse A and alkali treatments resulting in the release of ARR phosphate bridges from the nuclear matrix into the non-matrix.

The matrix DNA associated with DAR after exhaustive DNAse I digestion could be used to generate DNA probes for visualizing DAR-rich DNA sequences via recombinant DNA technology. Southern blots of these clones could be screened against electrophoretic agarose gels containing matrix and non-matrix RNAse A or alkali hydrolysed DAR's. If DNA loop fusions occur as a result of RNAse A or alkali hydrolysis, then, it should be possible to screen for matrix DAR associated DNA clones which bind to matrix gels from untreated nuclei but not from RNAse A or alkali treated nuclei and which fail to bind to untreated non-matrix gels but will bind to non-matrix gels derived from RNAse A or alkali treated nuclei. If this initial screening process were successful, then, it would be possible to repeat the experiments using matrix and non-matrix hydrolysed DAR's which have been subjected to ethanol precipitation, rinsed, and resuspended prior to gel electrophoresis. It is known that such a procedure results in a loss in both DNA and RNA label as compared to simple heating (Chapters V and VI). The RNA label loss is ASR and the DNA loss may be the DNA associated with the ASR. The loss of this DNA might be detectable by the loss of one or more Southern blot bands. If the matrix DAR associated DNA probe detects two or more bands in the gel containing electrophoresed matrix DAR which has been hydrolyzed but not subjected to ethanol precipitation and one or more of these bands is lost when the probe is hybridized against the gel containing electrophoresed matrix DAR which has been hydrolysed and ethanol precipitated (ARR), then, this indicates that both the matrix ASR and its associated DNA have been lost during ethanol precipitation as a result of the hydrolytic procedure. The ability of the DAR-associated DNA probe to bind to both the ARR and ASR associated DNA bands indicates that prior to hydrolysis, the ARR and ASR were connected together with DNA within the same nucleic acid structure. Direct evidence that the ASR associated DNA is clipped out during hydrolysis leaving the ARR

behind in an intact form of DNA could be derived by first subcloning the ARR and ASR DNA associated bands in order to obtain non-overlapping probes. Nuclei within gel inserts would then be subjected to hydrolysis followed by electroelution. The gel inserts could be digested in DNAse I and the digest subjected to electrophoresis. Both the electroeluted material and the digested electrophoresed gel insert material could be subjected to Southern blotting using ARR and ASR DNA associated probes. If all of the ASR associated DNA and only a part of the ARR associated DNA is removed from the gel insert, then, this is direct evidence that the ASR and its associated DNA is clipped out during hydrolysis leaving ARR and its associated DNA still intact and integrated into the main genome.

If the phosphate bridges exist only within the matrix DNA and a portion of the central DNA region is retained by Form IV ARR after alkali hydrolysis, then, the matrix DNA should be capable of reannealing faster than non-matrix DNA because of the presence of "snap-back" DNA. Therefore,the rate of reannealing of matrix ARR should be much faster than non-matrix ARR. A graph of the ratio of double stranded ARR to total ARR label applied to hydroxylapatite against reannealing time should reveal a much steeper slope for matrix ARR than for non-matrix ARR.

If the ARR complexes are long enough to be viewed under the electron microscope they can be compared as follows: Form I should be visible as two or more circles of DNA connected together. This form may have already been observed. Forms II and III should form X shaped patterns. Form IV should be half the length of Forms II and III. If Form IV has a "snap-back" region which has reannealed, then, it should appear as a tripartite set of linear strands. The non-complementary region should be split into two single strands and the "snap-back" region should be double stranded and observable as only one thick strand. If no "snap-back" region exists, then, Form IV should exist as a kinked single strand. The kink would be the ARR hairpin region.

As suggested by Dr. Ralph Stephens, it would be useful to probe the matrix DNA for Topoisomerase II sequence sites because such sites are known to be very close to the DNA attachment sites and located within the nuclear matrix (Cockerill and Garrad, 1986, Berrios et al, 1985; Earnshaw, 1985; Earnshaw et al, 1985). If the Topoisomerase II sites are contiguous with or overlap the DAR-rich DNA sites this would be evident from a Southern blot analysis.

Subhypothesis 5

As cells differentiate (age, etc.) the DNA superstructure is radically altered in at least three major ways relative to DNA domains. The DNA domains fuse together to form larger loops. The DNA domains become deleted. The DNA domains become amplified and the daughter loops either remain localized or undergo transposition to new areas. A fourth form of alteration involves exogenous infection of the cells by viral components which become incorporated into the genome as either DNA domains or linear segments.

It is hypothesized that loop fusions convert at least part of the phosphorylated ARR components into methylated ARR components which are released into the non-matrix DNA resulting in a partial loss of total ARR components within the matrix fraction and a concommitent increase in total non-matrix ARR. Loop deletions convert phosphorylated matrix ARR into methylated ARR with no loss of total matrix ARR. Loop amplifications, transpositions, and exogenous viral incorporations generate new matrix ARR phosphorylated components only if they result in the formation of new DNA domains and are not incorporated as simple linear segments.

The method of testing to be proposed here assumes that the DNA alterations within the developmental model to be used are primarily loop fusions and deletions. It is known that Reh human lymphoblastoma cells contain about twice as many DNA domains as human lymphocytes. This requires a reduction in loop number in lymphocytes which can occur either by loop fusions or deletions. The general assumption is that the Reh DNA domains have fused into larger DNA domains. However, DNA deletions are also known to occur during lymphocyte differentation. In either case the phosphate content of matrix ARR should be lower in lymphocytes than in Reh cells because of the removal of phosphorylated matrix ARR during loop fusions and its conversion to methyated ARR during loop deletions. The methyl content of matrix ARR should be higher in lymphocytes than in Reh cells because of loop deletions resulting in the conversion of phosphorylated ARR to methylated matrix ARR and because of loop fusions which remove matrix ARR thereby increasing the concentration of methylated matrix ARR to the remaining phosphorylated matrix ARR.

Methods of Testing

The matrix ARR M/U ratio should be larger in lymphocytes than in Reh cells and the matrix ARR P/U ratio should be smaller in lymphocytes than in Reh cells. Just the reverse situation should exist when non-matrix lymphocyte and Reh cell ARR ratios are compared. The ratios would be determined by triple labeling cells, removing DNA from the ARR, and subjecting the final product to column chromatography as described before under Subhypothesis 3.

Molecular composition and relative concentrations of samples can be determined by mass spectrometry with appropriate standards as previously described under Subhypothesis 3.

The recombinant DNA experiments described under Subhypothesis 4 regarding loop fusions by RNAse A or alkali hydrolyses can be repeated here assuming that similar loop fusions result from the process of differentiation.

Hypothesis II

The prokaryotic chromosome contains a single circular piece of DNA which is bound into independently supercoiled DNA domains by RNAse A/alkali sensitive RNA linkers. The origin of replication is attached to the cell membrane by a mesosome. The RNA linker associated with each independently supercoiled DNA domain constitutes a non-functional secondary origin of replication. The chromosome replicates as if it contained an outer major slave DNA replicon and an inner minor master replicon. Replication in the master replicon terminates much more rapidly than in the slave replicon, thereby establishing the RNA linkers for the daughter DNA replicons. Destruction of the secondary origin of replications by RNAse A or alkali results in the formation of a DNA fusion circle (see Appendix D) which is similar to an E. coli chromosome (Cairnes, 1963).

Methods of Testing

Most of the eukaryotic experiments described above can be applied to prokaryotic models as well. Changes in eukaryotic loop sizes,numbers, and locations during the course of cellular differentation may have a parallel in the prokaryotic system during the course of viral integrations and excisions. The ability to detect changes in prokaryotic loop numbers and sizes and in DNA domain attachment sites as a result of a

single viral rearrangment is probably not detectable using the above mentioned techniques. However, it has been possible to detect gross changes in prokaryotic loop sizes resulting from RNAse A treatments (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972; Pettijohn and Hecht, 1973; Worcel et al, 1973; Drlica and Worcel, 1975; Kavenoff and Ryder,1976; Hecht et al, 1977). It may be possible to separate prokaryotic "matrix" DNA from "non-matrix" DNA and check for differences in their respective P/U and M/U ratios. Following this, the nucleoids can be exposed to RNAse A at varying time intervals and then separated into prokaryotic matrix and non-matrix DNA fractions to determine the effect of increased loop fusions on these ratios. If phosphorylated ARR is responsible for the establishment of loop attachment sites in prokaryotes, then, following loop fusions the prokaryotic matrix and non-matrix fractions should experience decreases and increases in the P/U ratio respectively. An RNA processing activity has been discovered that debranches RNA lariats (Ruskin and Green, 1986). This 2' phosphodiesterase could be used to probe for the hypothetical prokaryotic ARR attachment sites. Detachment of ARR sites by a 2' phosphodiesterase should generate loop fusions but not loop deletions, the latter of which may occur in eukaryotes. If this occurred, it would elucidate one of the potentially critical differences in prokaryotic and eukaryotic DNA attachment sites.

The Evolutionary Origin, Structure, and Function of Eukaryotic DNA Domain Attachment Sites

This subject is best approached by restating the theoretical assumptions proposed in Chapter I and referring the reader to Appendix D for additional information:

1. The first true cell (progenote) was generated from a series of homologous viral replicon fusions coupled with transpositions, both of which increased the size of the genome. In both processes the viral replicons paired and fused together at their origins of replication. These origins of replication were preserved during homologous fusions but they were partially or completely deleted out during transpositions. Two alternative outcomes were possible depending upon which process was dominant: 1) the generation of a replicon with

multiple origins of replication and 2) the generation of a replicon with one origin of replication. The pairing up and connection of single origin replicons to a multiple origin replicon by flanking covalent RNA crosslinks generated a tetrameric base paired region containing paired origins of replication. This combination of crosslinked replicons and multiple origins of replication (a replicon cluster) constituted the chromosome of the progenote.

2. Origins of replication within replicon clusters can initiate DNA synthesis only when they are permanently attached to a membrane anchorage component such as a prokaryotic mesosome or the inner membrane of the nuclear envelope. All replicon clusters contain at least one membrane-bound origin of replication (master origin) which initiates DNA synthesis.

3. The evolution of the prokaryotic chromosome from the progenote is characterized by the fusion of all but one of the single origin replicons through the deletion of their origins. The inner multiple origin replicon did not fuse with these replicons and became the DNA attachment sites for the outer fusion replicon. The initiation of replication at the master origin involves both the inner replicon and the outer fusion replicon, the former completing a round of replication far more quickly than the latter because of its smaller size.

4. Mesokaryotes and eukaryotes evolved from the progenote by a series of cellular symbioses. Prokaryotic and mesokaryotic chromosomes have similar DNA super-structures and modes or replication. The evolution of the eukaryotic chromosome from the progenote is denoted by the presence of one or more membrane-bound origins of replication within a single replicon cluster. The initiation of DNA synthesis occurs at the master origin of replication which in turn initiates secondary rounds of replication in the membrane-bound slave origins of replication.

5. The replicon clusters of eukaryotes (unlike prokaryotes and mesokaryotes) have fused together to form a chromosome composed of a linear array of complex, interconnecting circles which are capable of extensive DNA rearrangements during

cellular differentiation. If intact DNA from a eukaryotic chromosome could be separated from all non-covalently bound materials in a manner non-destructive to the DNA, the linear array of DNA circles would remain intact because of the presence of 2' RNA covalent bonds flanking the tetrameric region. Therefore, any intact DNA isolated from a eukaryotic chromosome in such a manner would not exist as a simple, linear molecule.

6. During cellular differentiation, DNA domains may be fused, deleted, amplified, or transposed. Deletions and fusions result in the destruction of the tetrameric region at the DNA domain attachment sites of origins of replication and the inactivation of the ARR crosslinks by methylation at the 2' positions of the ribose sugars.

APPENDIX A

DATA AND INFORMATION RELATIVE TO NUCLEOID STUDIES

Nucleoid Theory

Measuring Supercoiling in DNA

The nucleoid technique measures supercoiling by using gradients which contain increasing amounts of ethidium bromide. The ethidium bromide intercalates between the DNA base pairs causing a reduction in the total amount of negative supercoiling which is normally present in the DNA following salt extraction.

As the ethidium bromide concentration is increased the supercoiling becomes fully relaxed. Still further increases in ethidium bromide concentration causes the DNA to become positively supercoiled. When DNA is centrifuged in sucrose gradients or electrophoresed in gels its migration rate is affected by the degree of supercoiling imposed upon the DNA. DNA which is more highly supercoiled is more cornpact in shape and hence travels faster through gradients and gels than DNA which is less supercoiled.

A graph of DNA migration in gradients and gels as a function of ethidium bromide concentration will contain a negative peak at the point at which the ethidium bromide has fully relaxed the DNA supercoiling. This is the point at which the DNA migration rate is minimal and at which all the supercoiling has been titered out by the ethidium bromide. Ethidium bromide concentrations which are greater or lesser than this amount will not completely titer out the supercoiling.

Changes in the total amount of supercoiling present within the DNA as a result of various outside treatments will shift the negative peak to the left when the total supercoiling is reduced and to the right if the total supercoiling has been increased. Figure 17 is a hypothetical graph illustrating differences in the average amount of supercoiling contained within two kinds of nucleoids.

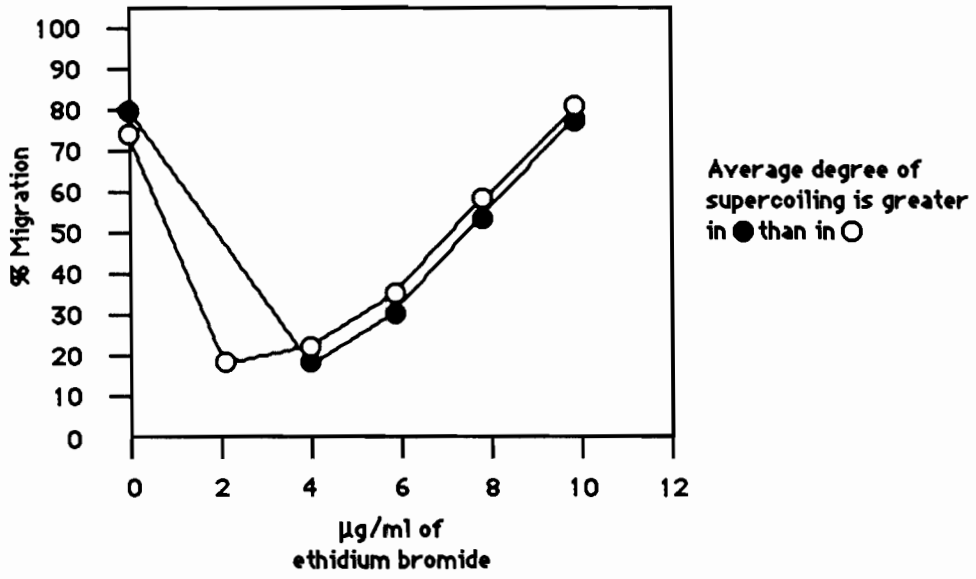


Figure 17

Graphical Illustration Depicting Differences in the Average Supercoiling of Two Kinds of Nucleoids

Differences in the average supercoiling of nucleoids could be attributed to cell type used or due to treatments with drugs like nalidixic acid which perturb supercoiling. The supercoiling of the nucleoids represented by white circles is titrated out at 2 µg/ml of ethidium bromide whereas the supercoiling of the nucleoids represented by the black circles requires twice as much ethidium bromide to be completely titrated out. Therefore, the average supercoiling of the first set of nucleoids is one-half that of the second set.

Measuring DNA Domain Sizes and Numbers

DNA domain sizes and numbers can be measured by X-ray or gamma ray titration. In this procedure the DNA is irradiated at low doses which produce primarily single strand nicks. Such nicks fully relax supercoiling in those DNA domains in which the nick occurred but have no effect on other DNA domains. The salt extracted nucleus referred to as a "nucleoid" has an average migration rate in a neutral sucrose gradient which is dependent upon the average supercoiling present within all its DNA domains. As the nucleoid is irradiated some of the DNA domains become relaxed which causes a reduction in the average migration rate of the nucleoid. A graph of nucleoid migration

as a function of increasing doses of X-rays appears as a straight line with a negative slope at low doses. At higher doses the slope of the line begins to decrease; probably because of an increase in double stranded nicks at higher doses of X-rays and/or because all the larger DNA domains have been titrated out leaving behind a large population of tiny DNA domains which require higher doses of X-rays to be titered out and which individually exert less of an effect on the average migration rate of the nucleoid when they are relaxed. Changes in the relative sizes of the larger DNA domains because of outside treatments can be measured in terms of changes in the initial slope of the line generated by X-ray titration. As the DNA domains become larger more of the DNA can be relaxed with smaller doses of X-rays. Therefore, the slope of the line becomes more steep as the average DNA domain size increases (see Figure 18). It is generally assumed that such increases in DNA domain size are the result of loop fusions. Therefore, cells containing equal amounts of DNA having different average DNA domain sizes also differ in the number of their DNA domains. In such cases the number of DNA domains drops as the DNA domain sizes increase.

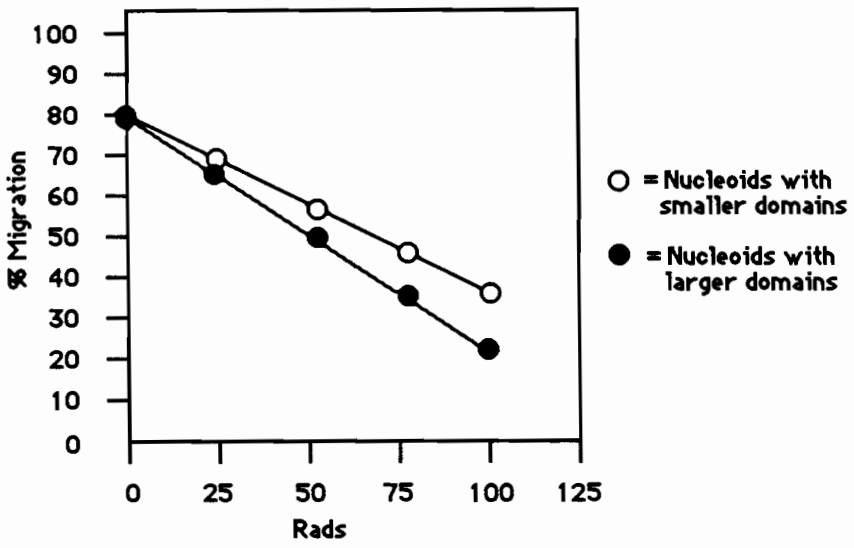


Figure 18

Graphical Illustration Depicting Differences in the Average Sizes of DNA Domains in Two Kinds of Nucleoids

Differences in the average DNA domain size of nucleoids could be attributed to cell type used or due to treatments with X-rays or gamma rays which damage individual DNA domains resulting in a partial loss of supercoiling. Assuming no change in the total amount of DNA, increases in the average DNA domain size must be accompanied by a loss in the total number of DNA domains present. Under appropriate conditions, each DNA domain has an equal probability of being hit by an X-ray resulting in a single stranded nick which relaxes the supercoiling in that domain only. Therefore, the more DNA domains that are present, the less the supercoiling is affected by X-ray titration. Nucleoids with small DNA domains require more X-ray titration to relax all the supercoiling than do nucleoids with larger DNA domains. In the graph above, the supercoiling of the nucleoids represented by white circles is titrated out less rapidly than the supercoiling of the nucleoids represented by the black circles as indicated by the more shallow negative slope of the curve. The ratio of the slopes of the two curves is a measure of the differences in the average DNA domain sizes of the two sets of nucleoids.

Preparation and Analysis of Nucleoids

The neutral sucrose gradients used in nucleoid analyses contain concentrations of as much as 2M sodium chloride. The solutions are generally buffered at pH 8.0 with Tris and EDTA is added to retard nuclease activity and clumping of cellular DNA by the

removal of divalent cations. A lysing solution is prepared containing the buffered salt solution together with a non-ionic detergent such as Triton X-100. This is layered over the sucrose gradient. Whole cells are gently pipetted over the lysing solution and mixed with it by a gentle rotation of the tube. The detergent in the lysing solution destroys the cell membrane and the outer membrane of the nuclear envelope. At the same time the high salt concentration extracts most of the histones from the nuclear DNA resulting in the production of independently supercoiled loops of DNA which are released from the vicinity of the nucleus. The resulting structure which is referred to as a "nucleoid" can be viewed under a U.V. fluoresent microscope using fluoresent dyes with an affinity for DNA such as acridine orange. It appears as a diffuse "halo" containing a small circular core. The "halo" is composed of everted supercoiled giant loops of DNA which have escaped from the nucleus but are still bound to the inner circular core which contains the remnant of the nuclear envelope. Figures 19 through 21 illustrate CHO cells, nuclei, and nucleoids respectively. Figure 22 is a diagrammatic representation of a nucleoid (a) together with a nucleus that has been subjected to a standard procedure of DNAse I digestion, salt extraction, and centrifugations used in the generation of matrix and non-matrix fractions (b).

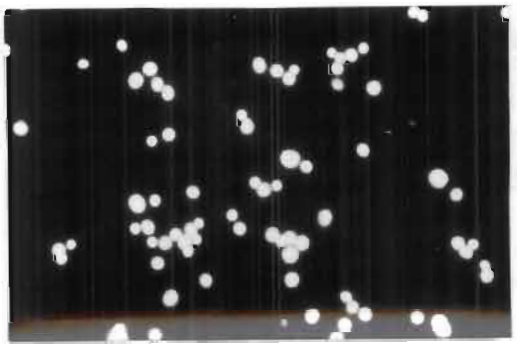


Figure 19

CHO Cells

Cells are visualized at a magnfication of 200X with acridine orange using U.V. fluorescent microscopy

Figure 20

CHO Nuclei

Nuclei are visualized at a magnification of 400X with acridine orange using U.V. fluorescent microscopy.

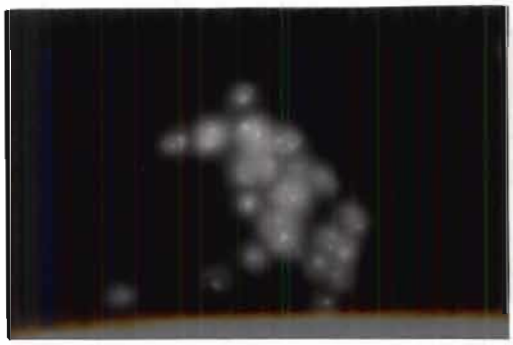


Figure 21

CHO Nucleoids

Nucleoids are visualized at a magnification of 400X with acridine orange using a U.V. fluorescent microscope.

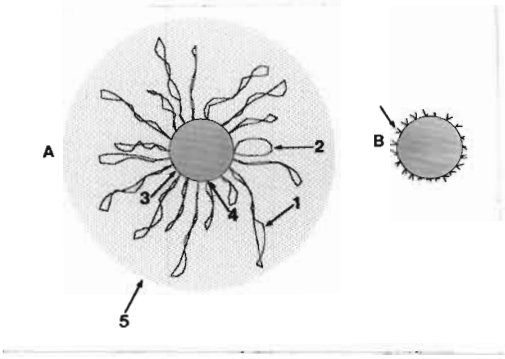


Figure 22

Diagramatic Representations of Nucleoids

Figure 22a represents an intact nucleoid (see Figure 21). Numbers 1, 2, 3, 4, and 5 indicate a supercoiled DNA loop, a relaxed DNA loop, the nuclear lamina, the approximate location of the DNA domain attachment sites, and the outer periphery of the nuclear "halo". Figure 21b represents the matrix DNA region of the nucleoid after digestion and extraction.

Figure 23 is a diagrammatic representation of the equipment used in the preparation and analysis of nucleoid gradients and Figures 24 and 25 are photographs of this equipment. In Figure 23 from the top (right to left) are the gradient maker, peristalic pump, and gradients. From the bottom (right to left) are the gradient fractionator, peristalic pump, DAPI dye flask, flow cell fluorimeter, signal analyzer, and chart recorder. Six gradient tubes at a time are filled from the bottom to the top by the gradient maker. Lysing solution is layered onto the gradients and cells are layered on top of the lysing solution. The cells are incubated for 15 minutes to form nucleoids and then centrifuged at the appropriate speed, time, and temperature. The gradients are then ready to be fractionated and analyzed. The gradient fractionator consists of a meniscus follower which drains the gradients from the top to the bottom through the action of the peristalic pump. The gradient solution is mixed with DAPI and this mixture enters the flow cell fluorimeter. Changes in the fluorescent intensity of the sample are detected by the signal analyzer and these changes are recorded as peaks by the chart recorder.

Examples of such recordings can be seen in Plates XVII, XVIII, and XIX which were used in obtaining the results described in Figures 1, 2, and Table 1. The large peak on

the left side of each graph is the result of the interaction of the dye with the Triton X-100 present at the upper end of the gradient. The DNA peaks, which can vary considerably in shape, size, and volume are generally found along the entire length of the gradient. The percent migration of the DNA within each gradient can be measured in two ways: The simplest method is to visually determine the midpoint of the DNA peak and divide this point by the total length of the graph. An alternative method which was used in most of these early studies involves a computerized program which takes into account the "tailing" of the nucleoids within the gradient. Once the migration rate has been determined for each recognizable DNA peak the triplicate sets can be averaged and standard errors determined.

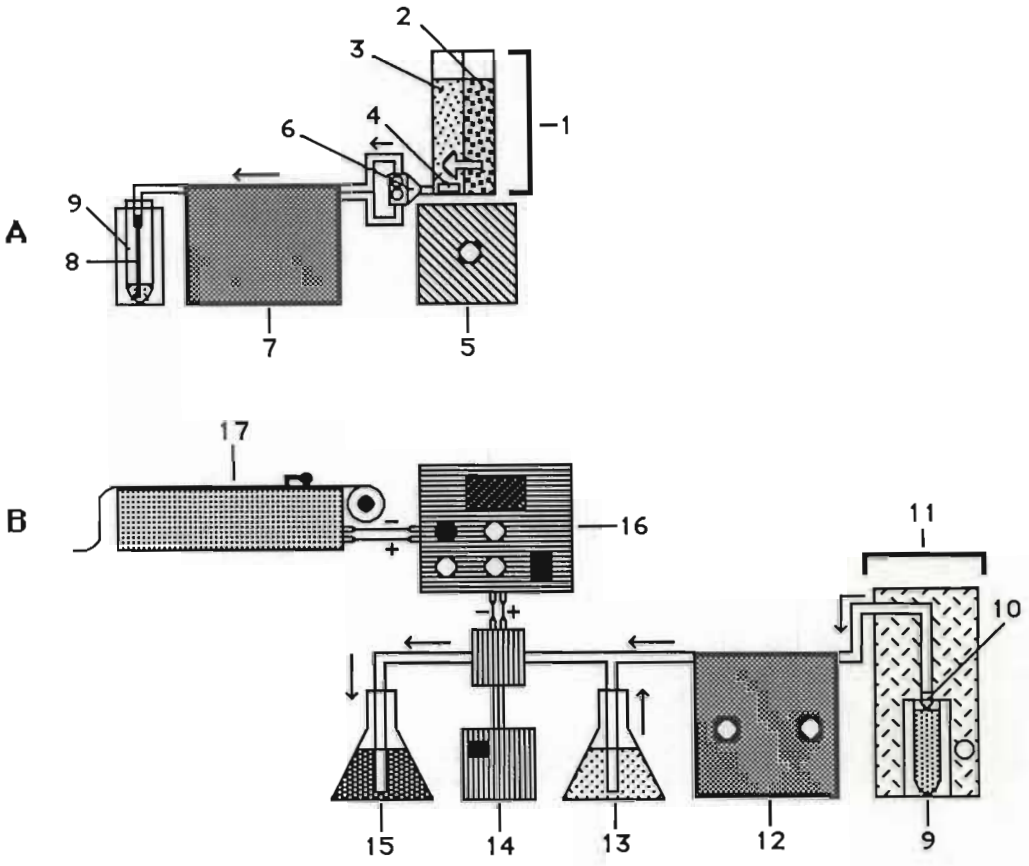


Figure 23

Flow Chart for Nucleoid Preparation and Analysis

In Figure 23a the gradient maker (1) is mixing 30% sucrose buffer (2) together with 15% sucrose buffer (3) by the mixing action of the stir bar (4) which is propelled by the mixer (5) to form a continuous sucrose gradient. This solution is partitioned into 6 tubes by the "spider" (6) through the peristalic action of the pump (7) where it exits through long needles (8) into 6 gradient tubes (9). In Figure 23b the gradient tubes (9) are being emptied from top to bottom by the meniscus follower (10) of the gradient fraction collector (11). The solution is pumped by the peristalic action of the pump (12) together with the fluorescent dye DAPI (13) so that the two solutions mix prior to entering the flow cell fluorimeter (14). From this point the mixture is discarded as waste into the flask (15). Changes in fluorescent intensity in the buffer-dye mixture due to the presence of DNA are detected by the signal analyzer (16) and recorded as "peaks" by the chart recorder (17).

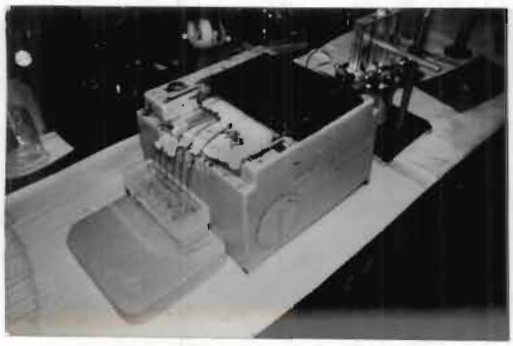


Figure 24

Nucleoid Gradient Maker

From right to left in the photograph are the gradient maker, the peristalic pump, and the gradients tubes which are filled from the bottom to the top. Six tubes are made simultaneously (see Figure 23a).

Figure 25

System for Fractionating and Analysing Nucleoid Gradients

From right to left are the nucleoid gradients, gradient fractionator, flask with DAPI dye, peristalic pump, flow cell fluorimeter, signal analyzer (back) and chart recorder (front), (see Figure 23b).

Plate XVII

Nucleoid Migration Graphs in Neutral Sucrose Gradients Containing 2M NaCl and 4M Urea.

Plates XVIIa and b represent triplicate assays for nucleoids which were derived from cells not exposed to X-rays or exposed to 100 rads of X-rays respectively. The results of this experiment are illustrated in Figure 1.

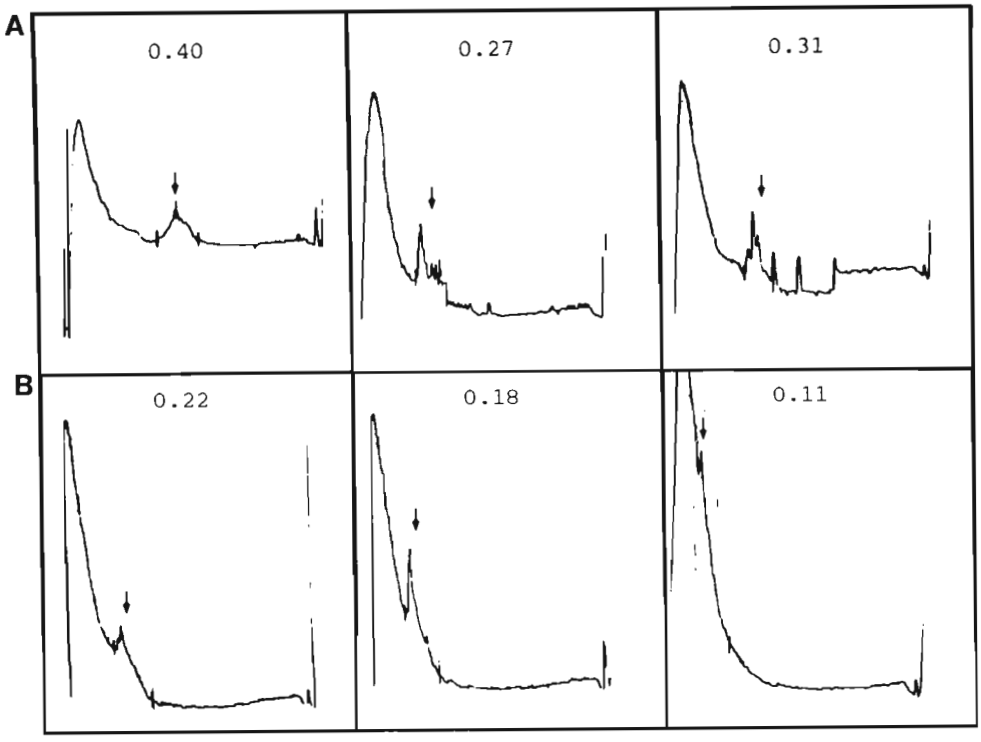


Plate XVIII

Nucleoid Migration Graphs in Neutral Sucrose Gradients Containing 2M NaCl With no Proteinase K.

Plates XVIIIa and b represent triplicate assays for nucleoids which were derived from cells not exposed to X-rays or exposed to 100 rads of X rays respectively. The results of this experiment are described in Table 1 (data row 4) and illustrated in Figure 2.

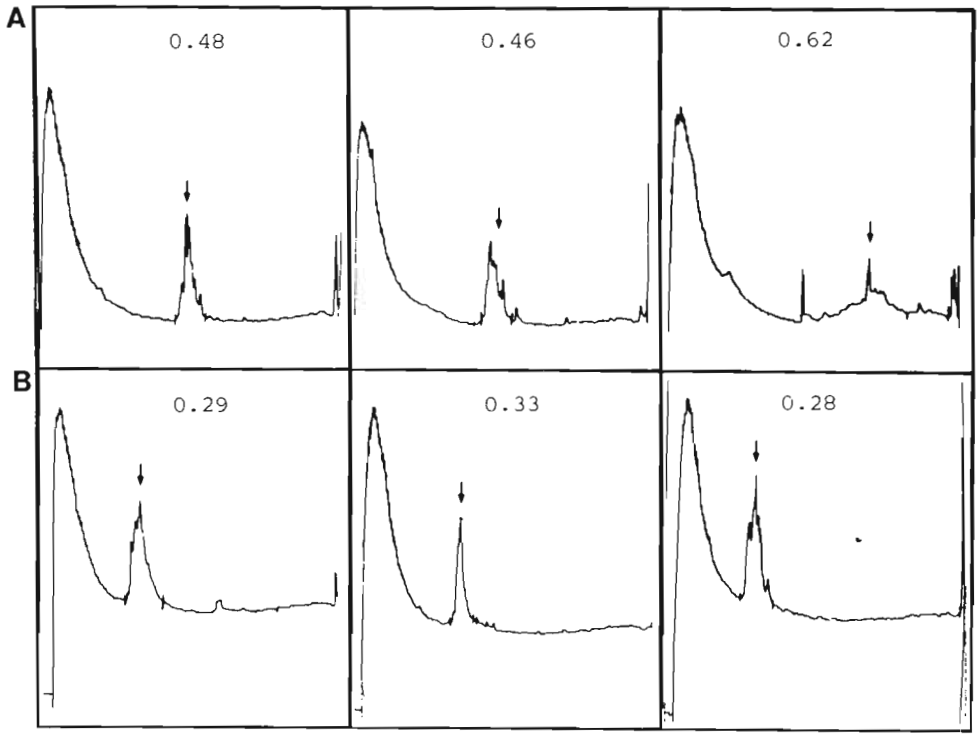
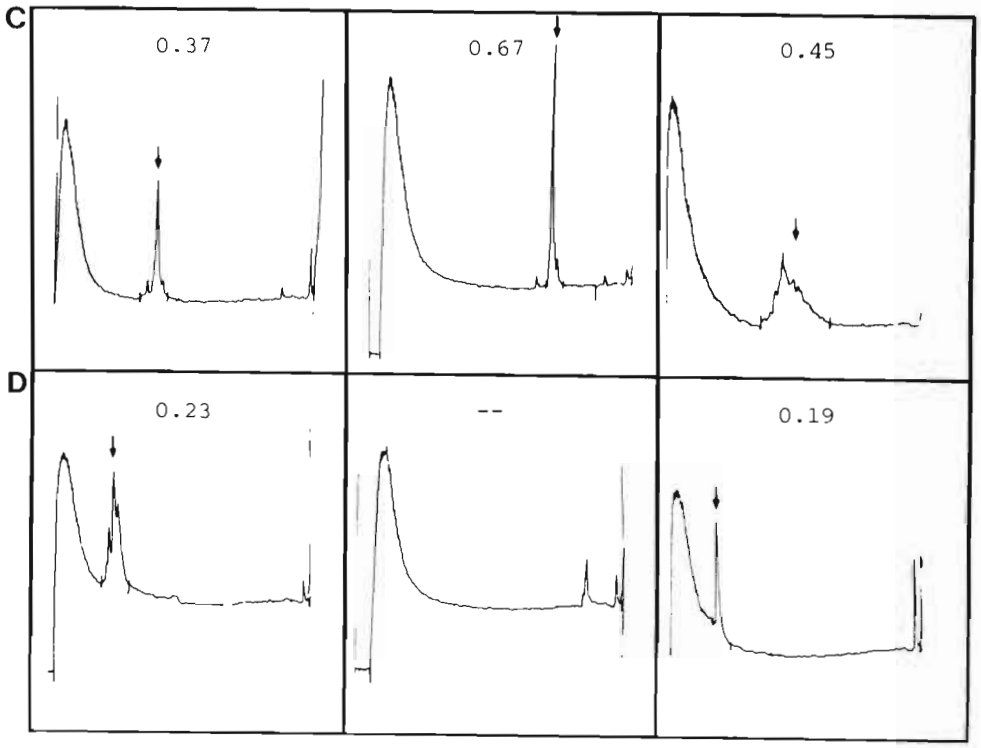


Plate XIX

Nucleoid Migration Graphs in Neutral Sucrose Gradients Containing 2M NaCl and Proteinase K.

Plates XIXc and d represent triplicate assays for nucleoids which were derived from cells not exposed to X-rays or exposed to 100 rads of X-rays respectively. The graphs in this plate are a continution of those in Plate XVIII. The results of this experiment are described in Table 1 (data row 4) and illustrated in Figure 2.



APPENDIX B

INFORMATION RELATIVE TO ALKALINE ELUTION STUDIES

Figure 26

Diagrammatic Representation of the Alkaline Elution Procedure

Figure 26a is an exploded view of the filter unit which is composed of the upper and lower filter housings (1 and 2), a rubber gasket (3), and a 0.22 micron polycarbonate filter (4). The assembled unit is shown in 23b. When large volumes of DNA sample (5) are assayed such as non-matrix fractions they are injected into the filter unit using the 30cc syringe (6) as shown in 23c and the DNA-free filtrate (7) is discarded. In 23d the opening of the lower filter housing is sealed with parafilm (8) so the unit can be filled with incubation solution (9). This solution may be alkali or buffer with or without RNAse A. It is added to the unit using a 3 or 5 ml syringe (10) attached to a truncated, bent needle (11). The needle is cut at a point which insures it will make no contact with the filter during filling. It is bent so the filling solution will flow along the inner wall of the upper filter housing and not directly impinge upon the sample. After the appropriate incubation period, the unit is flushed through with alkali solution using a 30 ml syringe. The unit is disassembled, the filter removed, neutralized, and counted along with a sample of neutralized alkaline elution solution. Total counts include those retained by the filter and those eluted into the alkaline solution. The relative retention is calculated by dividing filter counts by total counts for each respective filter.

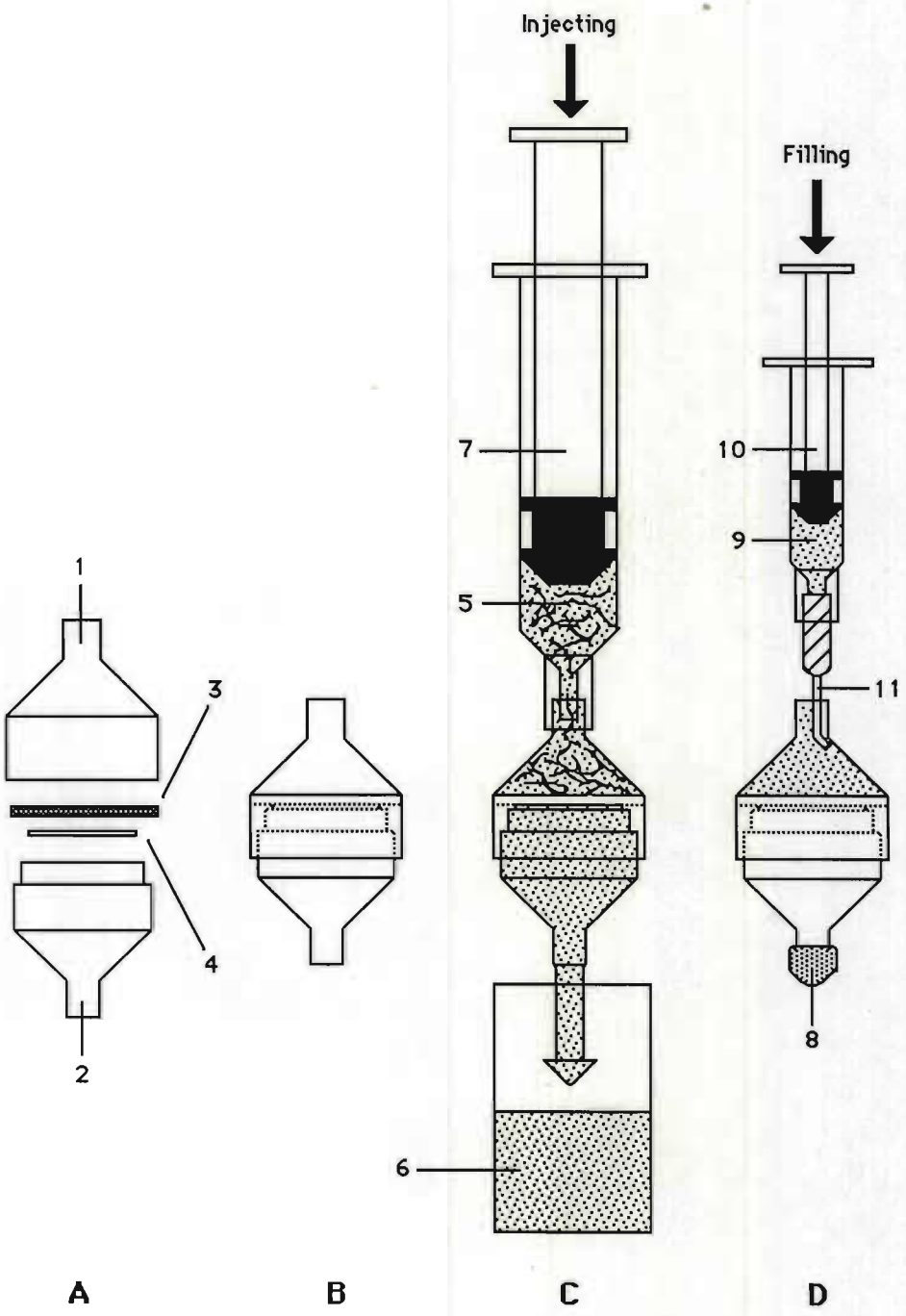


Figure 26

Figure 27

Alkaline Elution Apparatus

This consisted of a filter housing, 0.22 micron polycarbonate filter, and gasket seal. The large 30 cc syringe was used to inject large volumes of sample or alkaline elution solution through the filter unit. The smaller syringe with a truncated, bent needle was used to fill the unit when it was sealed on the bottom with parafilm for quiescent incubations with either alkaline solutions or buffer with or without RNAse A (see Figure 26).

APPENDIX C

DATA AND INFORMATION RELATIVE TO ELECTROELUTION STUDIES

General Procedures Used During Horizontal Tube Gel Electroelution Studies and in the Preparation of Gel Inserts

Figure 28 illustrates the tools and materials first used in the preparation of 10 µl gel inserts. From left to right are a pasteur pipette, cotton swab, 20 µl pipettor with a flattened plastic tip, flat metal spatula, gel insert pipettor, watch glass, cork borer, petri dish full of agarose plugged by the cork borer under which rest a piece of black paper for visualization of the free inserts. The agarose well holes in the petri dish were filled with buffer, then, 10 µl aliquots of liquid gel inserts were carefully pipetted underneath the buffer into each well. The dish was carefully placed in a horizontal position over a wet piece of black paper on ice to gel the inserts. The gelled inserts were plucked from their wells using the wide-mouthed gel insert pipettor which was a piece of glass tubing that was fire-polished on both ends. They were placed in a watch glass over a wet piece of black paper on ice. Excess fluid was frequently removed using the pasteur pipette. Great care was taken to avoid siphoning up the inserts during this process. The drained 10 µl inserts could be picked up with a damp cotton swab, a metal spatula, make-shift plastic forceps made from a weigh boat, or by using a plastic tip normally used with micropipettors which has been cut to a wide bore with a razor blade or scapel (not scissors) and flattened into a shape that accommodated the shape of the gel insert.

Figures 29, 30, and 31 are photographs of 10 µl gel inserts. Figure 32 is a photograph of a horizontal slab gel apparatus which was used exclusively during early studies with electrophoresis or electroelution but was replaced in most of the later studies by the horizontal tube gel electroelution technique. In many of these early studies a piece of U.V. transparent piece of plastic was sealed to the bottom of the gel bed with agarose in order to support the weak 0.15% LE agarose gels which were used at the time in order to provide as little resistance as possible to the electrophoresis of nucleoids. With careful manipulation the plastic film could be removed without destroying the overlying gel by using a frying pan spatula. The gel, supported on the plastic film could be manipulated during staining procedures and photography. In some instances the gels were dried and kept as permanent records. The photographs in Plates XXIc and d are examples of such gels although they were probably composed of 0.5% LE agarose.

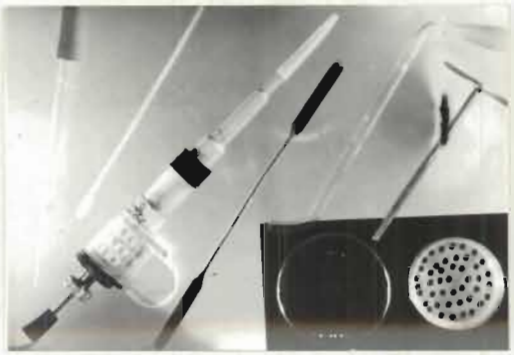


Figure 28

Tools and Materials First Used in the Production of Gel Inserts

From left to right are a pasteur pipette, cotton swab, 20 µl pipettor with a cut, flattened plastic tip , flat spatula, gel insert pipettor, watch glass, cork borer, and a 35 mm petri dish filled with agarose which has been plugged with the cork borer. The black paper under the dish aids in visualization of the freed inserts.

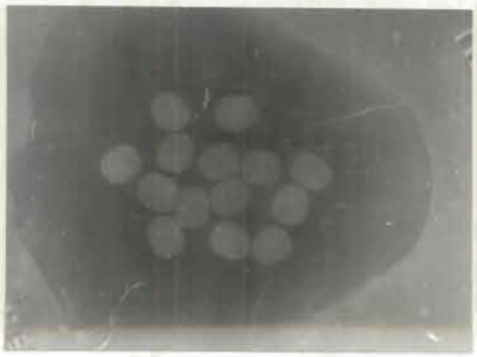


Figure 29

Ten µl Gel Inserts

The inserts are resting in a watch glass in an aqueous solution containing india ink for visualization

Figure 30

Ten μl Gel Inserts

The inserts are resting in a 1.5 ml microfuge tube in an aqueous solution containing india ink for visualization.

Figure 31

A Ten μl Gel Insert Containing CHO Cells

The insert has been stained in acridine orange and photographed at 100X magnification using U.V. fluorescent microscopy. The insert has been photographed at one edge. Cells are visible as tiny fluorescent spheres.

Figure 32

Agarose Slab Gel Apparatus

This device was used in many studies involving both gel electrophoresis and electroelution. The main gel bed straddles the two buffer trays. The well comb is in place and the DC current/voltage regulator is visible in the background.

Figure 33 is a flow chart indicating the procedures used and the flow of radiolabel in the DAR experiment described in Figure 15 and Table 20. Figures 34 through 39 show the sequential production, preparation, electroelution, and processing of large gel inserts (50 -250 µl) and tube gels.

Figure 33

Flow Chart Illustrating the Flow of Radiolabel During SDS Extraction and Horizontal Tube Gel Electroelution

In Figure 33a the radiolabeled cells or their nuclei are suspended in liquid agarose (1) which is used to make gel inserts (2). Total counts are obtained by mixing an aliquot of the cell or nuclei agarose suspension in aquasol (3) for liquid scintillation counting. In (b) the insert is extracted 3 hours or overnight in 1% SDS-TE buffer (4) and a sample of the buffer is counted to determine the extent of radiolabel extraction. In (c) the rinsed insert is placed between two tube gels (5) which are coupled together by rubber collars (6). The tube gels and insert are placed into an electrophoretic chamber (7) under buffer and subjected to electroelution. The tubes are dismantled and the gels are removed as shown in Figure 39. They are melted in vials, cooled, and aliquots removed and added to aquasol for counting. A sample of the electroelution buffer is also added to aquasol and counted to insure that most of the radiolabel remained within the tube gels. For DNA retention studies the electroeluted gel insert is melted and diluted in buffer and aquasol is added to it for counting. Total counts are considered to be those counts electroeluted into each tube gel together with the radiolabel still contained within their respective gel inserts. The DNA retention ratio is the ratio of the counts retained by each gel insert divided by the total counts obtained from that insert and its corresponding tube gel. In (d) the radiolabel within the insert is to be further characterized for DAR and/or ARR studies. The insert is incubated for 24 hours in DNAse I buffer (8), removed, melted, and counted in aquasol to determine the extent of radiolabel loss in the buffer. In (e) The DNAse I buffer is mixed with ethanol (9) and precipitated overnight at a temperature of -40^{o}C or below. The precipitated pellet (10) is recovered by centrifugation, followed by two ethanol rinses. It is then resuspended and counted directly in aquasol to determine DAR counts or subjected to alkali treatment to recover the ARR counts. The ARR counts may be counted directly or used in still other studies (Table 22).

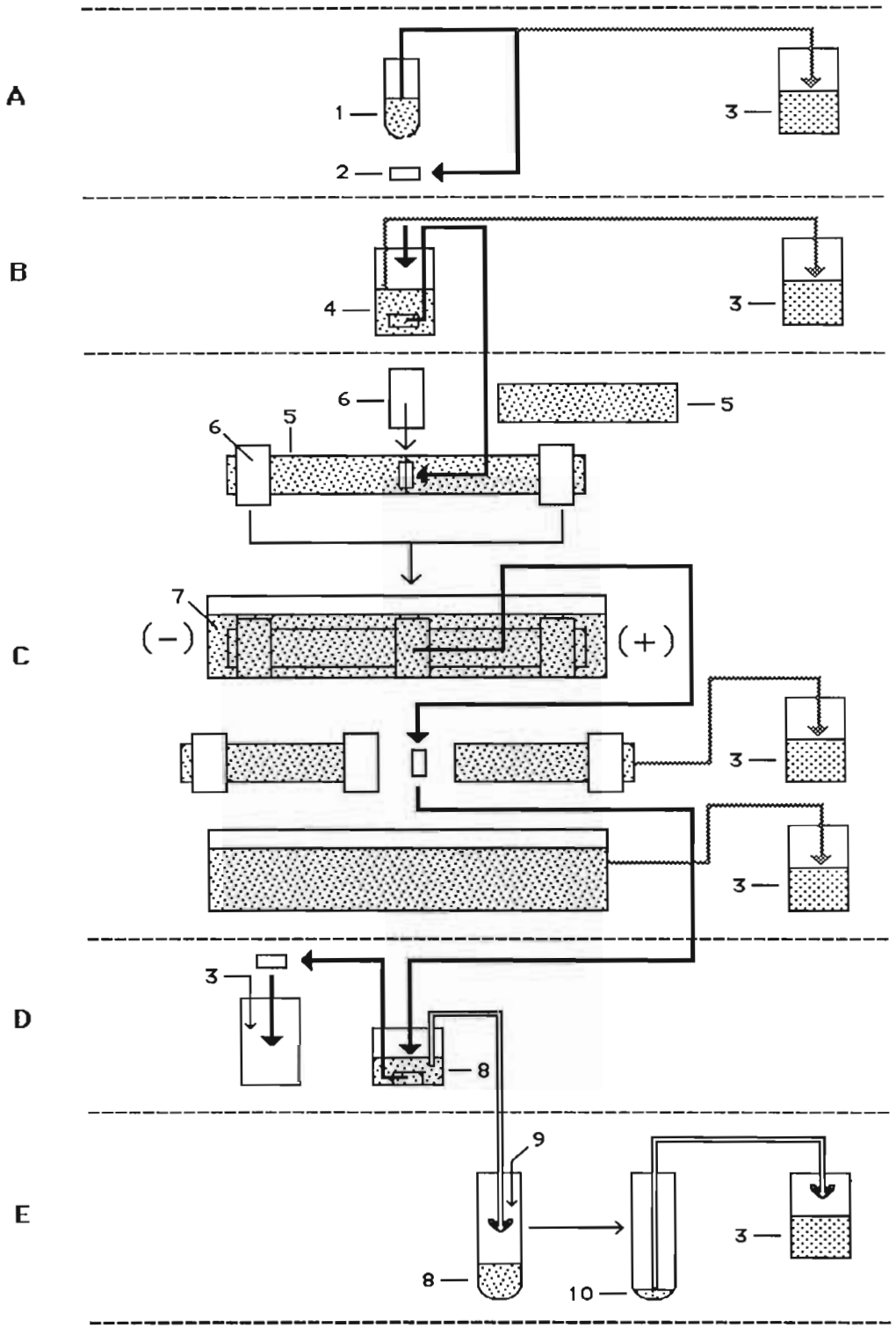


Figure 33

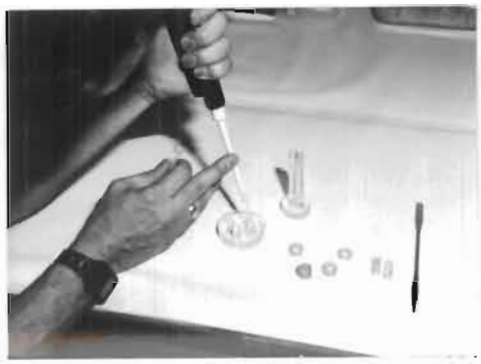


Figure 34

Preparation of Large Gel Inserts for Horizontal Tube Gel Electroelution

Liquid inserts are pipetted under buffer within 1 ml sections of 10 ml plastic pipettes which have been sealed into an agarose bed within a petri dish. After loading, the petri dish is chilled on ice to gel the inserts. In the background is a 4.5 ml tube gel which is sealed in an agarose bed and filled with agarose.

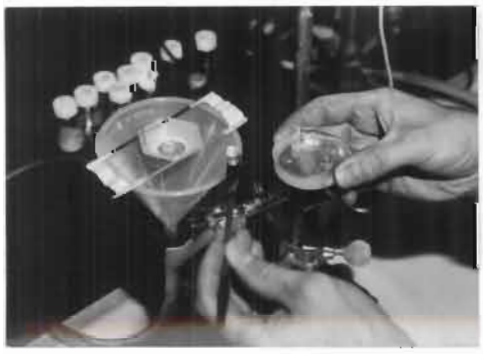


Figure 35

Removal and Rinsing of Gelled Inserts

The inserts are removed from the petri dish with a flat spatula. The spatula is also used to transfer the inserts to a colander which is used in rinsing the inserts.

Figure 36

Insertion of an Insert Back Into a 1 ml Pipette Section in Preparation for Electroelution

The insert is carefully inserted back into a clean pipette section using a flat spatula.

Figure 37

Coupling the Insert and 1 ml Pipette Section To a 4.5 ml Agarose Tube Gel Using a Rubber Collar

A second rubber collar is placed at the free end of the tube gel to balance it. Both pipette sections are immersed in the buffer prior to coupling to avoid trapping air bubbles.

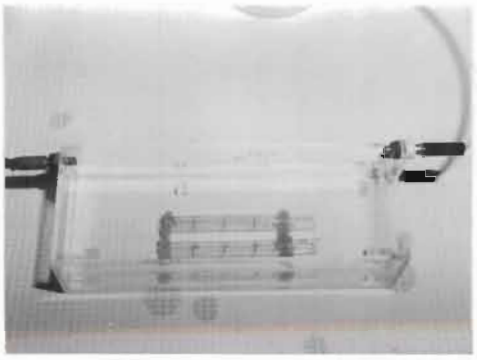


Figure 38

Immersion of the Connected Tube Gel and 1 ml Pipette Section in Preparation for Electroelution

The tube gel always faces the anode when DNA or RNA radiolabel are electroeluted. When protein radiolabel is electroeluted the insert is placed within a recessed portion of one tube gel and two tube gels are connected so radiolabel can be collected in both directions (see Figure 33).

Figure 39

Removal of Tube Gels for Melting and Counting.

Tube gels are removed from pipette sections using the rubber tip of a 3 ml syringe plunger connected to a 1 ml pipette or cotton swab. The gel is placed into a scintillation vial, melted, mixed, cooled to touch, and a 0.5 ml aliquot is removed and quickly mixed with 10 mls of aquasol for liquid scintillation counting.

Photographs of Examples of Results Obtained Throughout the Course of Electrophoretic and Electroelution Investigations

Examples of DNA Retention in Agarose Gel Wells During Gel Electrophoresis

In Plate XX photographs a, b, and c are of early studies involving agarose slab gel electrophoresis of nucleoids. The gel lanes are read from left to right. The main results of these studies were as follows: 1) when cells are lysed in the presence of TE buffer with 2M NaCl and Triton X-100 and subjected to slab gel electrophoresis, most of the DNA appears to resist electrophoresis, even in gels containing as little as 0.15% LE agarose. 2) The size and amount of the DNA released by cells during electrophoresis is dependent upon the cell type used, whether whole cells or just nuclei were used, and the type of lysing buffer employed. In these studies, long, prominent electrophoretic bands were released from all cell types tested except lymphocytes when the lysing solution was TE buffer with 2M NaCl and Triton X-100. When nuclei were used (Plate XXb) the bands were either greatly diminished, (lane 3), undetectable (lanes 4, 6, 8,12, and 16), or replaced by less prominent, slower moving bands (lanes 11 and 14); which suggested that the long bands were cytoplasmic in origin. 3) In Plate XXc when lymphocytes were exposed to either 4M urea (lanes 7,8, 10-13) or 0.1% SDS (lanes 15 and 16) instead of 2M NaCl and triton X-100; long, prominent, electrophoretic bands were released. Whether these bands were examples of new DNA release or non-specific staining because of the urea or SDS was not determined.

Enzyme Activity Assays in Agarose Wells and Gel Inserts

In Plate XXI the photographs a and b are of freshly electrophoresed slab gels which have been stained in ethidium bromide and photographed under U.V. light. Plates XXIc and d are light photographs of Plates XXIa and b respectively after staining in 0.2% amido black protein stain, destaining in 5% acetic acid, and air drying. These gels were used to assay the enzymatic activity of proteinase K against PM2 DNA and bovine serum albumin under varying kinds of conditions; one of which would simulate the conditions used when the enzyme was to be incorporated into nucleoid gradients (Table 1, Figure 2). The results of this assay suggested that the enzyme had no apparent effect on the ability of PM2 to migrate under electrophoresis, i.e., did not affect its

Plate XX

Examples of DNA Retention in Agarose Gel Wells During Gel Electrophoresis

Cells and nuclei were generally lysed in 2M NaCl-TE buffers containing 0.5% to 1% triton X-100. Lysis was done within the gel wells. The gels shown in Plates XXa, b, and c were 0.15%, 0.5% and 0.15% LE agarose gels respectively. Electrophoretic conditions varied. Samples were electrophoresed at least 1 or more hours at 70 volts. Gels were stained in 3 μg/ml of ethidium bromide, rinsed, and photographed under U.V. light. All lanes are read from left to right. Plate XXa includes the following kinds of samples: 1) lanes 1, 2, and 3, PM2 viral DNA, 2) lanes 4, 5, 6, 13, and 15, CHO Cells, and 3) lanes 8, 9 ,and 10, human lymphocytes. The tubular devices seen in lanes 3 and 13 were composed of 5 ml pipette sections glued to a piece of polycarbonate filter. They were tested to determine if they could be used for manipulating cells and nuclei following lysis. They were superseded in later studies by gel inserts. The cells used in lanes 6 and 10 had been exposed to 200 rads of X-rays. Plate XXb includes the following kinds of samples: 1) lane 1, PM2 viral DNA, 2) lanes 2,3,4,10,11, and 12, human lymphocyte cells or nuclei, 3) lanes 5, 6, 13, and 14, human fetal fibroblast cells or nuclei, and 4) lanes 7, 8, 15, and 16, human foreskin cells or nuclei. Nuclei were used in lanes 3, 4, 6, 8, 11, 12, 14, and 16. Nuclei used in lanes 4 and 12 had been exposed to Carnoy's fixative. Samples in lanes 10 through 16 were exposed to pancreatic protease for 15 minutes prior to electrophoresis. Samples in lanes 2 through 8 were sham incubated. Plate XXc includes the following kinds of samples: 1) lanes 1 and 2, PM2 viral DNA, 2) lane 3, PM2 DNA and lymphocytes, and 3) lanes 4, 5, 7, 8, and 10 through 16, human lymphocytes. Cells in the following lanes had been exposed to 100 rads of X-rays prior to lysis: 5, 8, 11, 13, and 16. Samples exposed to lysing buffer containing 0.5% triton X-100 without urea or SDS were in lanes 2, 3, 4 and 5. Cells lysed in buffers containing 4M urea were in lanes 7, 8, and 10, through 13. Cells lysed in buffer containing 0.1% SDS were in lanes 15 and 16.

Plate XX

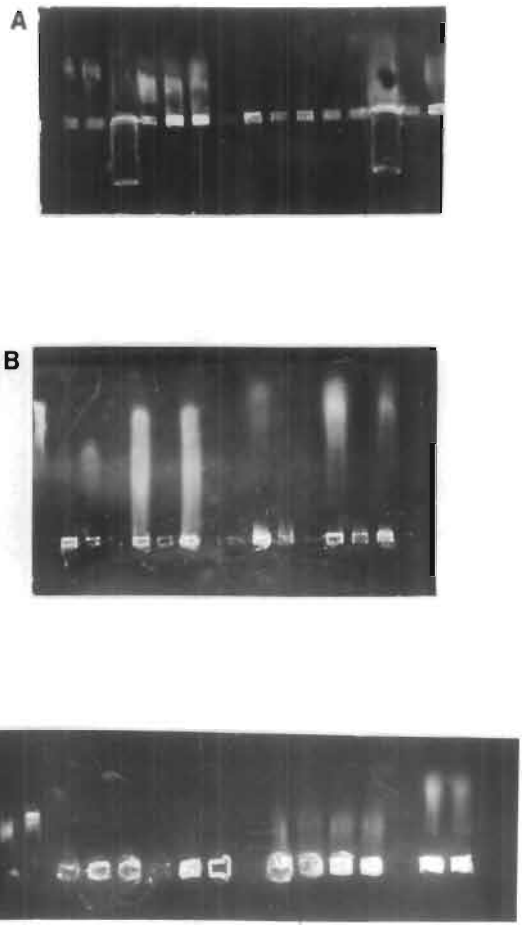
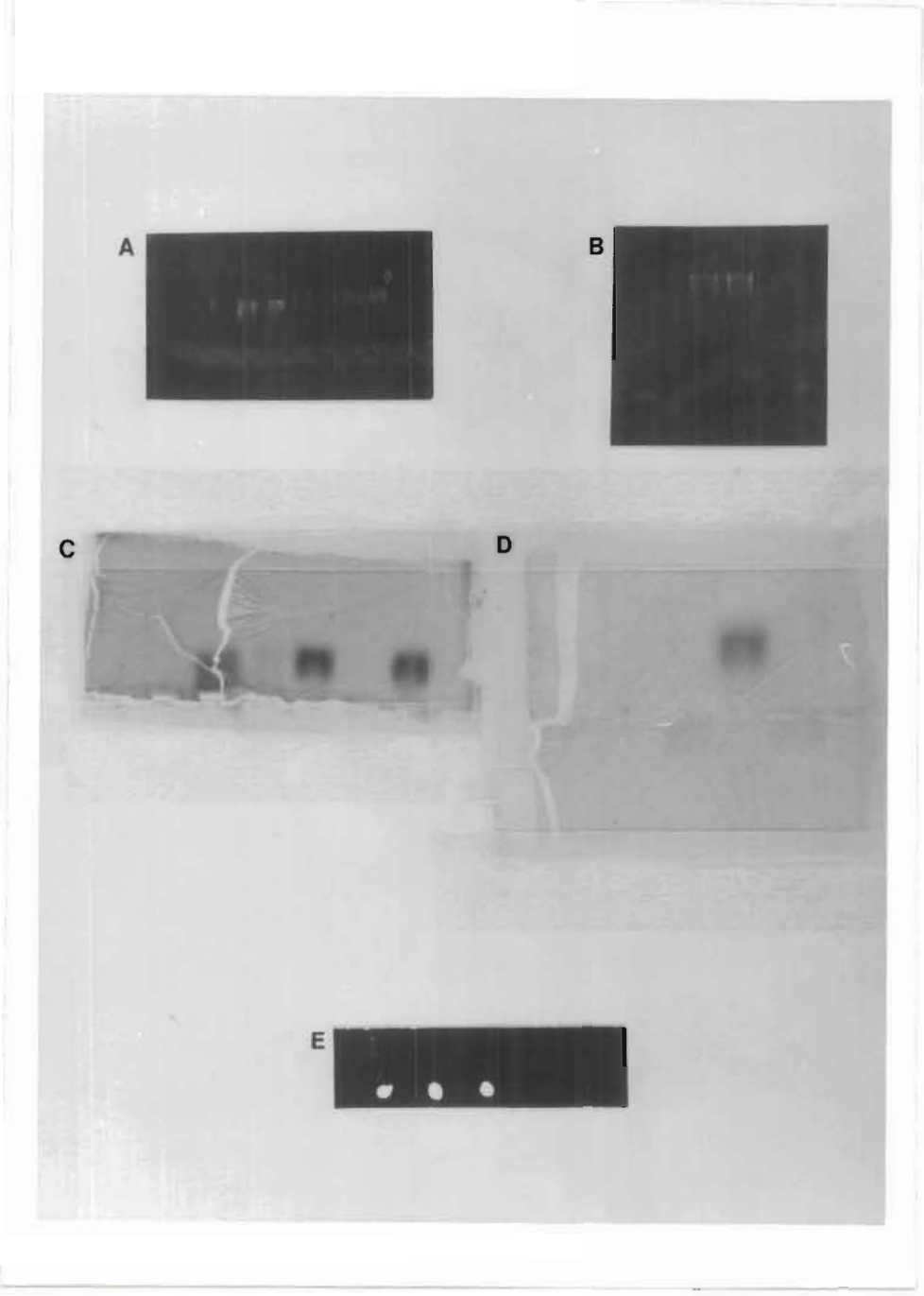


Plate XXI

Enzyme Activity Assays In Agarose Wells and Gel Inserts

Only the samples in Plates XXIa,b,c, and d were subjected to agarose gel electrophoresis. Gels were stained in 3 μg/ml of ethidium bromide, rinsed, and photographed under U.V. light. They were stained in 0.2% amido black protein stain and destained in 5% acetic acid. Plates XXIa and XXIb are photographs of material visualized under U.V. light with ethidium bromide. Plates XXIc and XXId are light photographs of material visualized by amido black protein stain. Plates XXIa and XXIc illustrate PM2 viral DNA at 62.5 ng/well and bovine serum albumin (BSA) at 10 μg/well respectively within the same gel. All lanes are read from left to right. PM2 samples were in lanes 3 through 8 and BSA samples were in lanes 9 through 14. They were incubated in the following buffers: 1) lanes 3,4, 9, and 10, TE buffer (40 mM Tris, 10 mM EDTA, pH 8.0) with 2M NaCl, 0.5% Triton X-100 and, 5% sucrose, 2) lanes 5, 6, 11, and 12, TE buffer with 0.15 M NaCl, 0.5% Triton X-100, and 5% sucrose, 3) lanes 7, 8, 13, and 14, 40mM Tris with 4mM magnesium and calcium salts, 0.5% Triton X-100, and 5% sucrose, pH 8.0. Incubation buffers used in lanes 3, 5, 7, 9, 11, and 13 contained 4 μg of proteinase K per well. Samples were incubated in the gel wells for 1 hour at 37°C prior to electrophoresis. Plates XXIb and XXId illustrate PM2 viral DNA and BSA within the same gel. PM2 samples were in lanes 3 and 4 and BSA samples were in lanes 5 and 6. All samples were incubated in TE buffer, 2M NaCl, 1% Triton X-100, and 5% sucrose. Samples incubated in the presence of 4 μg/well of proteinase K included lanes 4 and 6. They were incubated (R.T.) for 1 hour prior to electrophoresis. Plate XXIe illustrates 10 μl gel inserts (1% Sea Plaque) containing 200,000 mouse nuclei extracted in TE buffer (10 mMTris, 10mM EDTA, pH 8.0) with 1% SDS overnight (R.T.) followed by two thirty minutes rinses in TM buffer (10 mM Tris, 5 mM $MgCl_2$, pH 8.0). They were then incubated for 4 hours at 37°C in TM buffer with or without 20 units/ml of RNAse A and or DNAse I. Incubation conditions were as follows: From left to right the three visible inserts are as follows: insert 1 (sham), insert 2 (DNAse I), and insert 3 (RNAse A). The inserts which are not visible are insert 4 (RNAse A and DNAse I) and insert 5 which was a blank insert sham incubated to insure the absence of non-specific staining. After the incubation, the inserts were stained in 3 μg/ml of ethidium bromide, rinsed, and photographed under U.V. light.

Plate XXI



supercoiling status. However, the enzyme digested the bovine serum albumin in all cases below the limits of detection of the system used. This assay confirmed that the enzyme contained no detectable level of endonuclease activity and that its proteolytic activity was not significantly diminished in the presence of nucleoid gradient solutions or Triton X-100.

Plate XXIe is an enzymatic assay system used to monitor the ability of RNAse A and/or DNAse I to reduce the level of material in 10 µl 1% Sea Plaque gel inserts which is stainable by ethidium bromide. The gel inserts contained mouse nuclei extracted in 1% SDS-TE buffer. They were rinsed twice in 10mM Tris, 5 mM $MgCl_2$ (TM) buffer and then incubated in the buffer in the presence or absence of one or both enzymes but without the concommittent electrophoretic step. The results of this assay indicated that neither RNAse A nor DNAse I alone could remove all the material stainable by ethidium bromide. But when both enzymes were used together, all stainable material was removed. Such an observation confirmed that both enzymes were functioning in the TM buffer. Therefore, this buffer could be used in all forthcoming experiments in which these enzymes would be employed.

The Effects of Enzymatic and Alkali Treatments on "DNA"
Retention by Gel Inserts During Agarose Gel Electroelution

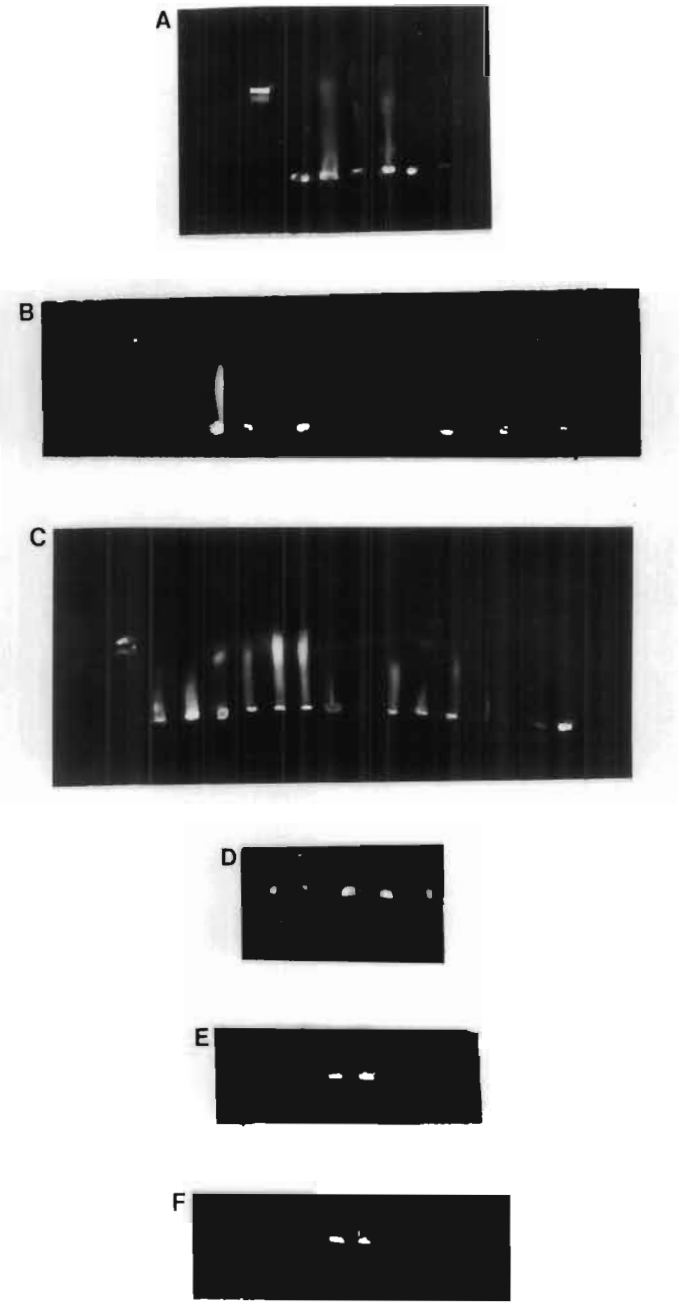
In Plate XXII four experiments are illustrated in a, b, c, and d through f in which 0.15% LE agarose slab gels were used in the electroelution of 10 µl, 0.15% LE gel inserts. The gel lanes are read from left to right. PM2 DNA markers and CHO cells were used in the first three experiments, CHO cell nuclei were used in the first and second experiments, and mouse nuclei were used in the last experiment. These experiments were quite complicated in terms of format. They were presented here to demonstrate the effects of alkali treatments followed by neutralization and various combinations of proteinase K, RNAse A, and DNAse I on the ability of gel inserts to retain DNA during electroelution. The results indicated that when CHO cell inserts were exposed to alkali and neutralized prior to electroelution, the ability of the inserts to retain DNA is (within the limits of detection) increased to 100% (Plates XXIIa, lanes 6 and 7, Plate XIIb, lanes 3, 12, 13, and 16, Plate XIIc, lanes 14-16). When cell inserts were not neutralized prior to electroelution, DNA retention was diminished

Plate XXII

The Effects of Enzymatic and Alkali Treatments on "DNA" Retention by Gel Inserts During Agarose Gel Electroelution

All photographs are of 0.15% LE agarose gels. All but Plate XXIId contain 10 µl, 0.15% LE agarose gel inserts within gel wells. Samples in Plates XXIIa, b, and c were electroeluted at 70 volts for 2 hours. Plates XXIIa and c were electroeluted in the cold. The gels and inserts were then stained in 3 µg/ml of ethidium bromide, rinsed, and photographed under U.V. light. Plates XXIId, e, and f illustrate parts of the same experiment. Plate XXIId illustrates a TM gel (minus the inserts) in which inserts which had been sham incubated or exposed to proteinase K were electroeluted for 3 hours at 70 volts in the cold. The gel was stained and photographed as described above. The inserts were then either sham incubated or exposed to RNAse A and/or DNAse I, stained in 3 µg/ml of ethidium bromide, rinsed, photographed in a TE gel (Plate XXIIe), electroeluted again in the cold for 3 hours at 70 volts, and re-photographed (Plate XXIIf). All lanes are read from left to right. Samples in Plate XXIIa are as follows: 1) lane 1, PM2 viral DNA, 2) lanes 2, 4, and 6, CHO nuclei, 3) lanes 3, 5, and 7, CHO cells. All CHO inserts were incubated outside the gel wells for 1 hour at 37°C in the following solutions: 1) lanes 2 and 3, 10mM Tris, 0.1M EDTA, pH, 8.0 (TE buffer) with 2M NaCl, 2) lanes 4 and 5, TE buffer and 0.1% SDS, and 3) lanes 6 and 7, TE buffer at pH 13.5. The samples in lanes 6 and 7 were removed and incubated an additional 30 minutes in TE buffer at pH 8.0. Samples in Plate XXIIb are as follows: 1) lanes 1, 2, and 3, PM2 viral DNA, 2) lanes 4 and 12, CHO cells, 3) lanes 5, 6, 7, and 13 through 16, CHO nuclei. Inserts were incubated under the following conditions: 1) lanes 3,12, and 13, 1 hour at 37°C in TE buffer, pH 13.5, then, 30 minutes (R.T.) in TE buffer, pH 8.0, then, placed on ice, 2) lane 16, 30 minutes (R.T.) in 0.1% SDS-TE buffer, pH 8.0, then, 30 minutes (R.T.) in TE buffer, pH 13.5, then 30 minutes (R.T.) in TE buffer, pH 8.0, then placed on ice, 3) lanes 2 and 15, 30 minutes (R.T.) in 0.1% SDS-TE buffer, pH 8.0, then 30 minutes (R.T.) in TE buffer, pH 8.0, then placed on ice, 4) lane 14, (R.T.) in TE buffer, pH 8.0 at all times, then placed on ice, 5) lanes 4 and 5, on ice at all times in 2M NaCl-TE buffer, pH 8.0, and 6) lanes 6 and 7, placed on ice for 30 minutes in 2M NaCl-TE buffer, pH 8.0, rinsed in PBS, 30 minutes on ice in PBS, then, 1 hour at 37°C in PBS with/without 8 mg/ml proteinase K, then placed on ice. The insert in Lane 7 was incubated in proteinase K. Samples in Plate XXIIc are as follows: 1) lane 1, PM2 viral DNA, 2) lanes 2 through 16, CHO cell inserts. All CHO cell inserts were incubated outside the gel wells for 30 minutes on ice in the following solutions: 1) lanes 2 and 3, 10mM Tris, 0.1M EDTA, 5% sucrose, pH 8.0 (TE buffer), 2) lanes 4 and 5, TE buffer and 2% Triton X-100, 3) lanes 6 through 16, TE buffer, 2% Triton X-100, and 2M NaCl. After this incubation period the following inserts were removed and placed into a second set of solutions as follows: 1) lanes 8, 9, and 10, TE buffer and 2M NaCl, and 2) lanes 11 through 16, TE buffer and 2M NaCl at pH 13.5. These inserts were allowed to incubate in the solutions for 1 hour at 37°C. At the end of this time these inserts were cooled to room temperature for 30 minutes. During this time the inserts in lanes 14 through 16 were incubated in TE buffer and 2M NaCl at pH 8.0. After this period all inserts were placed into their respective wells and electroeluted in the cold. Samples relating to Plates XXIId, e, and f are as follows: 1) lane 1, blank insert, and 2) lanes 2 through 5, mouse nuclei. All inserts were extracted in 10 mM Tris, 10 mM EDTA, pH 8.0 (TE buffer) with 1% SDS for 3 hours (R.T.), then, incubated for 30 minutes (R.T.) two times in TE buffer. Inserts 1 and 2 were kept on ice and the other inserts were incubated for 1 hour at 37°C in TE buffer with 20 units/ml of proteinase K. All inserts were electroeluted in a TM gel. Inserts 1 and 2 were removed and kept on ice in buffer. The others were removed and incubated for 1 hour at 37°C in TM buffer with 20 units/ml of RNAse A (3), DNAse I (4), or both enzymes (5). Inserts were photographed in a TE gel, electroeluted, and re-photographed in the gel.

Plate XXII



(Plate XXIIc, lanes 11-13). In the case of cell inserts, denaturation of the DNA followed by rapid renaturation may have generated a massive network of partially mismatched DNA which was impervious to electroelution. When CHO cell nuclei inserts were first extracted in SDS and then exposed to alkali followed by neutralization (Plate XXIIb, lane 16) a faint band of material was electroeluted from the insert. Whether this material was DNA or SDS was not confirmed.

In Plate XXIIb it is clear that when CHO nuclei are incubated in 2M NaCl-TE buffer in the presence of proteinase K (lane 7) the enzyme appears to cause a decrease in DNA retention as compared to an insert not exposed to the enzyme (lane 5).

Plates XXIId, e, and f are photographs of a TM gel without inserts after an initial electroelution (d), inserts within a TE gel prior to a second electroelution (e), and gel inserts within the unstained TE gel after the second electroelution (f). In this experiment the effects of proteinase K, RNAse A, and DNAse I treatments on the ability of SDS-extracted mouse nuclei inserts to retain DNA was tested. However, interpretation of some of the results is complicated by the incubation format used. It is not clear if proteinase K, RNAse A, or both enzymes are responsible for the loss of DNA which is occurring in the insert in lane 3 (Plate XXIIf). Earlier evidence suggested that neither proteinase K nor RNAse A affected the retention of intact DNA in inserts (Table 3). However, results from later experiments involving a more sensitive assay suggested that RNAse A treatment may have a slight affect on intact DNA retention (Table 20). Therefore, the effect of proteinase K on such retention cannot be ruled out without applying a similar assay. The ability of DNAse I to completely abolish DNA retention by the inserts under the conditions used was confirmed by the absence of any stainable materal in the inserts incubated in the enzyme prior to electroelution in the TM gel (Plate XXIIe, lanes 4 and 5).

<u>The Effects of DNAse I, Proteinase K, and U. V. Photography on the Retention of (M + N) and Non-matrix "DNA" by Gel Inserts After Electroelution and the Effect of Proteinase K on Electroleluted "DNA" Band Migration Rates</u>

Plates XXIIIa and b are of a 0.5% LE slab gel containing pre-stained 50 µl, 0.5% LE gel inserts photographed before (a) and after electroelution (b). The inserts were composed of mouse nuclei which had been incubated in the absence or presence of DNAse I prior to extraction in TE-buffer with 2M NaCl to form matrix and non-matrix

(M + N) and non-matrix fractions. The non-matrix fraction was separated from the matrix fraction which was discarded because of problems with clumping. The (M + N) sample was used as a substitute for the matrix fraction. This sample was different from the non-matrix sample only by the fact that the salt extracted nuclei had not been centrifuged to separate the matrix and non-matrix fractions. These samples were made into inserts which were then extracted in SDS buffer. Samples treated with DNAse I migrated completely out of the wells during electroelution (from left to right lanes 1 through 4 and 9 through 12). Those samples not treated with DNAse I exhibited a partial retention of the DNA. Treatment with proteinase K caused no discernable increase in the loss of the DNA during electroelution but it did appear to affect the migration rate of the DNA which was being released as compared to samples which had not been treated with the enzyme (lanes 5 through 8 and 13 through 16 respectively). This observation was mentioned earlier in Chapter III in relationship to the ability of proteinase K to relax supercoiling in nucleoids. Perturbations in the ability of inserts to retain DNA following electroelution based on differences in cell cycle kinetics, i.e., cells grown in log phase (LP) versus cells grown under conditions favorable for serum arrest (SA) were not observed.

Plates XXIIIc and d are of a 0.5% LE agarose slab gel containing 50 µl, 1% Sea Plaque gel inserts composed of mouse (M + N) and non-matrix samples, one-half of which were photographed before electroelution (c) and all of which were photographed after electroelution (d). This experiment was similar to the one illustrated by Plates XXIIIa and b except no proteinase K was used, the effect of pre-photography on insert DNA retention was being tested, and the non-matrix DNA had been purified with Sea Prep (Chapter II, Table 27). The label loss to the non-matrix fraction in DNA which had not been subjected to either DNAse I digestion or electroelution was about half as much in the sample isolated from serum arrested cells as compared to the sample isolated from log phase cells (Table 36, data rows 2 and 4). If these differences are significant, then, this could be an indication of the presence of a greater number of Okazaki DNA fragments within log phase cells as compared to serum arrested cells. Prior to electroelution all inserts photographed (lanes 9 through 16 were clearly visible (Plate XXIIIc). After electroelution, the only inserts which were clearly visible were three of the four (M + N) samples. All three samples demonstrated DNA retention by the inserts as well as band migration patterns (lanes 1, 9 and 11).

These results were further supported by the radiolabeled counts associated with these inserts. Twelve to 49 times more thymidine radiolabel was associated with (M + N) inserts than with non-matrix inserts following electroelution but this observation must be tempered by the fact that 11 to 21 times more DNA radiolabel was recovered in the matrix pellet than in the non-matrix supernatant in the absence of DNAse I digestion (Table 36, data column 2). The low initial non-matrix DNA concentration compared to the high (M + N) DNA concentration could be a factor in greater losses of non-matrix as compared to (M + N) radiolabel during electroelution (Table 36, data rows 2 and 4). However, in the previous experiment (Plates XXIIIa and b) in which Sea Prep underlayering was not used, DNA retention was visible in both (M + N) samples (lanes 6, 8, 14, and 16), and non-matrix samples (lanes 7 and 15). This combination of results supported the hypothesis that Sea Prep underlayering not only removed matrix DNA from non-matrix DNA but that in doing so it also removed that fraction of the DNA responsible for DNA retention by gel inserts during electroelution. No perturbations in DNA retention by gel inserts were noted because of differences in cell cycle kinetics (SA versus LP cells) or due to pre-photography.

Photographs of Agarose Gels and Inserts
Described in Figure 3 and Table 3

In Plate XXIV the protocol and the results of this experiment have been discussed in Chapter II, Table 3, Figure 3, and in the legend for Plate XXIV. These results suggested that proteinase K and RNAse A do not diminish the ability of mouse nuclei inserts to retain RNA radiolabel following electroelution whereas phospholipase C has a partial effect and DNAse I completely abolishes the retention. However, the ability of the assay system to accurately quantitate radiolabel retention of inserts was quite crude at this point in time and small losses of radiolabel as described in later studies may have gone undetected (Table 20).

Plate XXIII

The Effects of DNAse I, Proteinase K, and U. V. Photography on the Retention of (M + N) and Non-matrix "DNA' by Gel Inserts After Electroelution and the Effect of Proteinase K on Electroeluted "DNA" Band Migration Rates

Plate XXIIIa, b, c, and d illustrate 0.5% LE agarose gels containing 50 µl gel inserts pre-stained with 3 µg/ml of ethidium bromide and rinsed prior to insertion into gel wells. The gels were not stained. In Plate XXIIIa the inserts have been photographed under U.V. light prior to electroelution. In Plate XXIIIc only one-half of the inserts were photographed to determine the effects, if any, of pre-photography on DNA retention by gel inserts following electroelution or in the electroeluted DNA band migration rates. In Plates XXIIIb and d the inserts were subjected to 3 hours of electroelution at 70 volts. Plates XXIIIa and b illustrate one experiment and Plates XXIIIc and d illustrate a second experiment. Inserts in Plates XXIIIa and b were electroeluted at room temperature and were composed of 0.5% LE agarose. Inserts in Plates XXIIIc and d were electroeluted at 37°C and were composed of 1% Sea Plaque agarose. The samples used in these studies were derived from mouse cells which had been incubated either in log phase (LP) or under conditions favorable for serum arrest (SA) to determine the effects, if any, of cell cycle kinetics on DNA retention by gel inserts following electroelution. The nuclei were isolated and incubated in the presence or absence of 40 units/(mg of DNA) of DNAse I at 30°C for 5 minutes to observe the effects of DNAse I digestion on DNA retention by gel inserts following electroelution. They were extracted in 10 mM Tris, 10 mM EDTA, pH 8.0 (TE buffer) containing 2M NaCl. The extracted nuclei were separated into matrix and non-matrix (M + N) and non-matrix samples. The (M + N) samples were made into gel inserts and the non-matrix samples were centrifuged to generate a matrix pellet which was discarded and a non-matrix supernatant. In Plates XXIIIc and d the non-matrix supernatant was underlayered with Sea Prep, centrifuged, and chilled to gel the Sea Prep as described in Chapter II. Non-matrix supernatants were mixed with the appropriate agaroses to form non-matrix gel inserts. All inserts with incubated for at least 3 hours at room temperature in TE buffer with 1% SDS followed by a rinse. In Plates XXIIIa and b the extracted inserts were incubated in the presence or absence of 1 mg/ml of proteinase K prior to electroelution to determine the effect of this enzyme on DNA retention following electroelution. All lanes are read from left to right. The gel insert loading pattern for Plates XXIIIa, and b was as follows: 1) odd-numbered lanes contained non-matrix samples and even-numbered lanes contained (M + N) samples, 2) lanes containing samples derived from LP cells were 1,2, 5,6, 9,10, and 13, and 14 and those containing samples derived from SA cells were 3,4, 7, 8, 11, 12, 15, and 16, 3) lanes containing samples exposed to DNAse I were 1 through 4 and 9 through 12 and those containing samples not exposed to DNAse I were 5 through 8 and 13 through 16, and 4) lanes containing samples exposed to proteinase K were 1 through 8 and lanes containing samples not exposed to proteinase K were 9 through 16. The gel insert loading pattern for Plates XXIIIc and d was as follows: 1) odd-numbered lanes contained (M + N) samples and even-numbered lanes contained non-matrix samples, 2) lanes containing samples derived from SA cells were 1,2, 5,6, 9,10, and 13, and 14 and those containing samples derived from LP cells were 3,4, 7, 8, 11, 12, 15, and 16, 3) lanes containing samples not exposed to DNAse I were 1 through 4 and 9 through 12 and those containing samples exposed to DNAse I were 5 through 8 and 13 through 16, and 4) lanes containing samples which were not photographed prior to electroelution were 1 through 8 and lanes containing samples photographed prior to electroelution were 9 through 16.

Plate XXIII

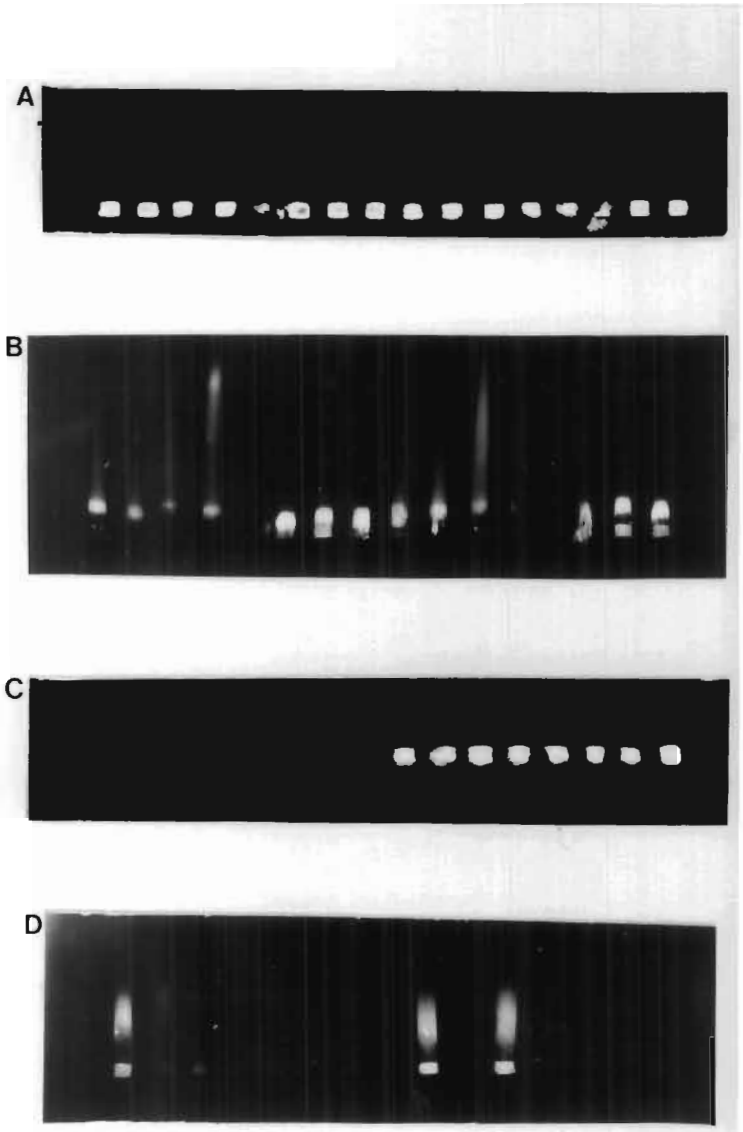


Table 36

[^{14}C]Thymidine Radiolabel Present in (M + N) and Non-matrix Gel Inserts in the Absence of DNAse I Digestion Before and After Gel Electroelution

Mouse nuclei were purified from cells which had been radiolabeled with [^{14}C]thymidine. Cells were grown in log phase (LP) or under conditions favorable to serum arrest (SA). The nuclei were processed into (M + N) and non-matrix samples in the absence of any DNAse I digestion, converted into 50 µl 1% Sea Plaque Gel inserts, and extracted in 1% SDS-TE buffer. The inserts were stained in ethidium bromide and one-half of them were inserted into gel wells and pre-photographed. The remaining inserts were inserted into the gel and all of them were electroeluted. The gel was photographed under U.V. light (Plates XXIIIc and d) and the inserts were removed and counted by liquid scintillation for 5 minutes per sample. The ratio of (M + N) to non-matrix counts is shown in data column 2.

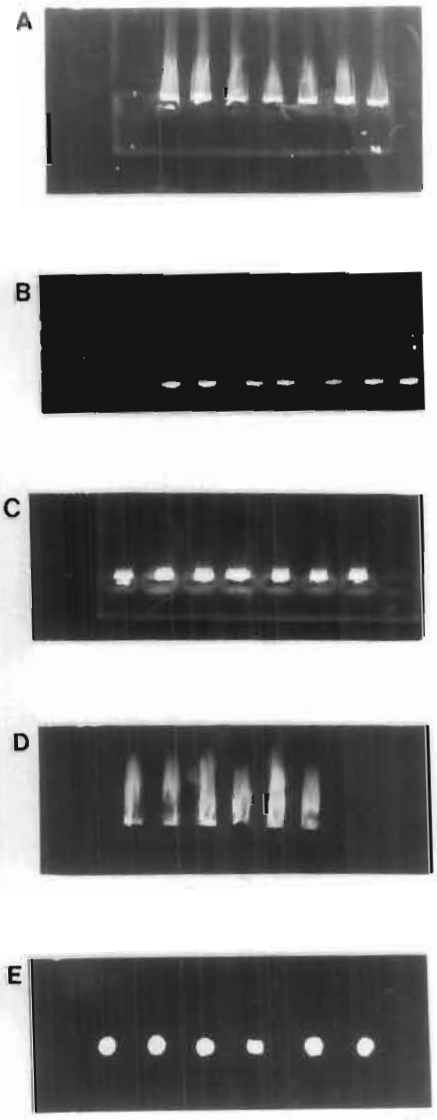
SA/LP	Nuclear Fraction	Pre-Photo Y/N)	CPM	(M + N) ÷ N
		No Electroelution		
SA	(M + N)	N	1652	21
"	N	"	80	--
LP	(M + N)	"	1627	11
"	N	"	145	--
		Electroelution		
SA	(M + N)	N	409	41
"	N	"	10	--
LP	(M + N)	"	65	13
"	N	"	5	--
SA	(M + N)	Y	260	12
"	N	"	22	--
LP	(M + N)	"	343	49
"	N	"	7	--

Plate XXIV

Photographs of Agarose Gels and Inserts Described in Figure 3 and Table 3

Gel inserts were removed from all gels prior to staining and photography. All gels were stained in 3 μg/ml of ethidium bromide, rinsed, and photographed under U.V. light. The inserts were pre-stained in 3 μg/ml of ethidium bromide prior to completion of the final electroleution step, rinsed, and electroeluted an additional 1.5 hours to remove non-specific stain. They were removed from the gel, photographed under U.V. light, melted in liquid scintillation vials, mixed with 10 mls of aquasol, and counted. All electroelutions were performed in the cold. Plate XXIVa is an agarose gel containing 40mM Tris and 10mM EDTA, pH 8.0 (TE gel) in which inserts extracted overnight at room temperature in 10 mM Tris, 10 mM EDTA, pH 8.0 with 1% SDS followed by rinsing were electroeluted for 3 hours at 70 volts. Plate XXIVb is an agarose gel containing 40mM Tris and 5mM $MgCl_2$, pH 8.0 (TM gel) in which inserts were electroeluted overnight at 25 volts following an 8 hour incubation of the inserts at 37°C in 10mM Tris, 5mM $MgCl_2$, pH 7.4 (TM buffer) with or without 40 units/ml of proteinase K. Plate XXIVc is a TM gel in which inserts were electroeluted for 3 hours at 70 volts following an 8 hour incubation at 37°C of the inserts in TM buffer with or without 40 units/ml of RNAse A, phospholipase C, or both enzymes. Plate XXIVd is a TE gel in which inserts were electroeluted for 9 hours at 70 volts following an 8 hour incubation at 37°C of inserts in TM buffer with or without 40 units/ml of DNAse I. The electroelution was interrupted after 8.5 hours to allow for staining of the inserts as described earlier. All samples were contained within 50 μl inserts composed of 1% Sea Plaque agarose. Plate XXIVe is a photograph of the inserts after their removal from the last gel just prior to melting for liquid scintillation. All lanes (and inserts) are read from left to right. The first visible insert on the left is insert 2. The sample in lane 1 was a blank insert. Inserts in lanes 2 through 8 contained 3 million mouse nuclei per insert. Inserts were processed as follows (see also Figure 3): 1) All inserts were incubated overnight in SDS and subjected to all four electroelution steps, 2) inserts 1 and 2 were sham treated in all four enzyme incubation steps, 3) inserts 3 through 8 were incubated in proteinase K during the first incubation step, 4) during the second incubation step insert 3 was sham incubated, 4 was exposed to RNAse A, 5 was exposed to phospholipase C, and, 6, 7, and 8 were exposed to both RNAse A and phospholipase C, 5) during the third incubation step inserts 3, 4, 5, 6, and 7 were sham incubated and 8 was exposed to DNAse I.

Plate XXIV



APPENDIX D

THEORETICAL CONCEPTS AND MODELS

Introduction

The focus of the body of this Dissertation has been on the following question: Do the nuclear matrix DNA domain attachment sites establish a DNA superstructure which is responsible for intact DNA retention during gel electroelution and for the preferential retention of matrix over non-matrix DNA during alkaline elution and gel electroelution? In Appendix D, the results of this work are used along with evidence from the literature to convey a generalized theory as clearly, simply, and concisely as possible for establishing evolutionary relationships between viruses, prokaryotes,. mesokaryotes, and eukaryotes. To accomplish this objective it is necessary to "hybridize" established phemenoma which cover a wide range of scientific biological endeavors. Drawing from such a wide body of knowledge precludes an extensive citation of references with the exception of models upon which some of the discussion will be based. Reviews can be obtained in most textbooks relating to the subject, e.g., (Strickberger, 1976; Karp, 1979; Swanson et al, 1981; Lewin, 1980). A series of papers on chromosomes and chromatin can be found in "Cold Spring Harbor Symposium of Quantitative Biology" in volumes 38 and 42 respectively. A recent review on eukaryotic chromosomes is available in "Research and Problems in Cellular Differentiation" edited by Henning. A good review on dinoflagellates is available in the book "DInoflagellates", edited by D. L. Spector and published by Academic Press, Inc., 1984. A series of older papers has been the subject of a review relating to the origin of eukaryotic cells (Dyer and Obar, 1985) and a recent publication on the importance of symbiosis in cellular evolution is also available (Margulis and Sagan, 1986).

The basic theme upon which the models to be presented here will be built is as follows: Prokaryotes co-evolved with pre-eukaryotes from viral constructions and both of these life forms together with viruses formed a series of endosymbiotic relationships that eventually gave rise to modern mesokaryotes and eukaryotes. The forces behind the divergence of these three major life forms is the subject of this discussion.

Novel Molecular Configurations in the Nuclear Matrix DNA

DNA Strand Tetrameric Base Pairing

The joint molecules so often described in electron micrographs of DNA undergoing recombination have been the subject of extensive research which will not be discussed here. Instead, a model is being presented in an attempt to explain how tetrameric base pairing may actually be accomplished. These models are presented here because of the importance of tetrameric base pairing in the development of the models to presented within this appendix. It is assumed that tetrameric base pairing is occurring between the origins of replication of two independently supercoiled circles of DNA as shown in Figure 40 at the level of the DNA domain attachment sites within the nuclear matrix. It is assumed here that the origins of replication for each circle are identical and at 180^{o} orientations relative to each other. Therefore, each nucleotide base has the option of pairing up either within daughter strands or non-daughter strands, resulting in a complex interaction of four DNA strands. Once tetrameric base pairing is completed replicon fusion is shown to occur in one of two ways: 1) by the deletion of most or all of the origins of replication (transposition, Figure 40d) or 2) by homologous recombination in which no base sequences are lost, (Figure 40e). In the latter case the origins of replication can serve as new binding sites for a second generation of replicons (See Figure 54). Any of the flanking tetrameric region which is retained following replicon fusion becomes palindromic sequences or inverted repeats (Figures 40e and f). Evidence that origins of replication may contain inverted repeats is available from studies with Alu DNA sequences. It is known that certain sub-populations of Alu sequences contain inverted repeats (Houck et al, 1979). Furthermore, Alu sequences can function as origins of replication (Johnson and Jelinek, 1986).

Figure 40

Tetrameric Base Pairing and Fusion Between Two Replicons at Their Origins of Replication

Illustrations are as follows: Figure 40a depicts two independent replicons (1) which are homologous only at their origins of replication. The orientation of the lower replicon is 180° to that of the upper replicon. The initiation of base pairing between them at their origins of replication (b) results in completed tetrameric base pairing (2) in which base pairing is either between daughter strands or non-daughter strands (c). The fusion of the two replicons at their origins of replication with the concommittent destruction of most of the origins is shown in (d) and fusion of the replicons at their origins of replication with no loss of origin sequences is shown in (e). In Figure 40e palindromic sequences (inverted repeats) are generated on both sides of the fusion joint. The palindromes to the left and right of the fusion joint are composed of the inverted repeats 3 and 4 and 5 and 6 respectively. In Figure 40f these palindromes are shown in an extended conformation in the final fusion replicon. The axes of symmetry for the inverted repeats 3 and 4 and 5 and 6 are shown by 7 and 8 respectively. Figures 40d and e represent forms of transposition and homologous recombination respectively. In the former the origins are destroyed but in the latter both origins are retained and serve as new binding sites for a second generation of replicons (see Figure 54).

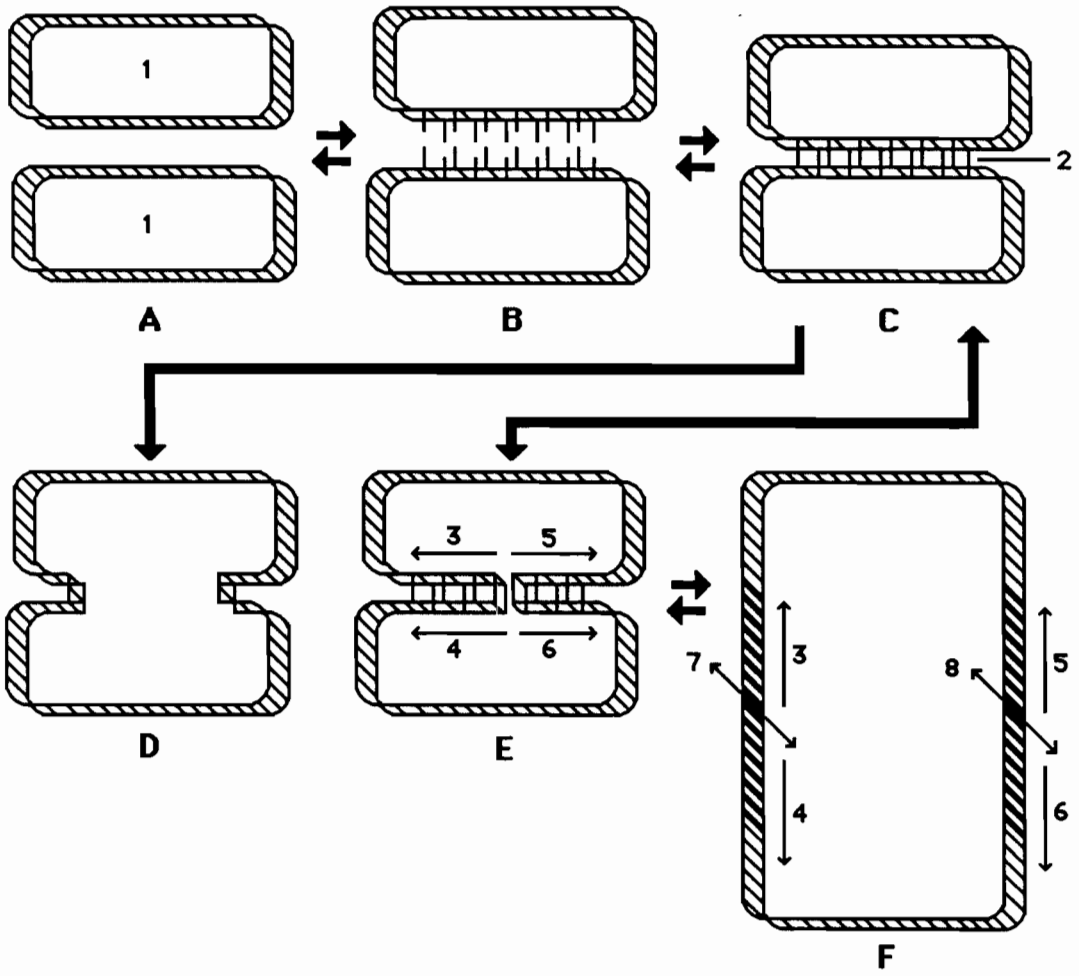


Figure 40

2' RNA Crosslinks

Prior to fusion, the tetrameric base pairing previously described is reversible. Two models are presented here to illustrate how such base pairing may become more permanently incorporated into the DNA superstructure using 2' RNA crosslinking between the non-daughter strands. These models assume that RNA exists within the origins of replication of the two joined DNA circles. The most probable origin of such RNA would be from Okazaki RNA primers used in the synthesis of DNA. It is assumed

that subsequent to the initiation of DNA synthesis a sequence signal within or near the origin of replications initiates a series of reactions that involve two major steps: 1) the conservation of the Okazaki RNA primers within the tetrameric region and 2) the crosslinking of part or all of the Okazaki RNA primers flanking this tetrameric region in a manner that results in covalent connections between paired non-daughter DNA strands from adjacent replicons. Evidence that RNA may be both retained and modified at 2' ribose positions at origins of replication is available (Birkenmeyer et al, 1987). The importance of such crosslinking will become more important as additional models are developed. Two principle forms of RNA crosslinking involving the 2' position of the ribose sugar include 2' RNA phosphotriester (Figure 41) and phosphodiester bonds (Figures 42 and 43). In the phosphotriester model the tetrameric region is crosslinked between non-daughter strands by a series of phosphotriester bonds between the phosphate group of one strand and the 2' hydroxyl position of the ribose moiety of the other strand (Figure 41a). In Figure 41b the isolated tetrameric region is shown together with attached DNA fragments following exhaustive DNAse I digestion. Possible damage to the central region by DNAse I is shown in more detail in Figures 44 ands 45. In the phosphodiester model (Figure 42) the tetrameric region is shown to be crosslinked by a phosphate bridge connecting the 2' RNA ribose moieties of adjacent, non-daughter strands. In this model the isolated tetrameric region and its associated DNA fragments are capable of denaturing into single strands which are connected together by the phosphate bridge (Figure 42c). Such a denatured molecule would represent an unusual form of "snap-back" RNA.

The phosphotriester model was tested and the results obtained were displayed in Tables 22 and 23. The rationale for testing RNA phosphotriesters as a possible mode of crosslinking was based on the following information: Alkali lability of RNA as compared to DNA is the result of a free 2' hydroxyl position located on the ribose sugar which is missing in deoxyribose (Michelson, 1963). When this hydroxyl position is covalently bound to another molecule such as phosphate (Wallace and Edmonds, 1983; Ruskin et al, 1984; Kiberstis et al, 1985) or methyl groups (Maden and Salim, 1974) the alkali lability of RNA is lost. At room temperature methyl and ethyl phosphotriesters of DNA are stabile in neutral solutions for at least 4 hours and for 90 minutes at 100 ^{o}C. But they can be completely hydrolyzed in 0.5 N NaOH after a one hour incubation

Figure 41

Phosphotriester Model for 2' RNA Crosslinks

Illustrations are as follows: Figure 41a depicts covalent bonding between two replicons (4) at their origins of replication by phosphotriester 2' RNA crosslinks (1). The presence of an alkali sensitive region is indicated (but not shown) which contains a combination of Okazaki DNA fragments connected together by alkali sensitive Okazaki RNA primers (2). Double stranded DNA within one replicon is shown by (3) and the tetrameric base paired strand region is illustrated by (5). Figure 41b illustrates exhaustive DNAse I digestion of the replicons leading to the isolation of the crosslinked tetrameric region which is resistant to denaturation by heat or formamide together with associated double stranded DNA fragments of varying lengths (6), the latter of which are responsible for the ability of the tetrameric to precipitate in the presence of ethanol or TCA. Figure 41c depicts the random destruction of phosphotriester linkages and the internal alkali-sensitive region leading to the concommittent secondary destruction of the resulting classic RNA bonds (d). The DNA fragments shown in Figure 41c are single stranded (7). The final product of alkali hydrolysis are RNA oligomers which are alkali resistant by virtue of 2' monophosphate bonds and which are tightly linked with surviving DNA (e). The removal of monophosphates by alkaline phosphate converts the RNA into an alkali sensitive form (f) and alkali hydolysis results in the complete loss of the RNA (g). If this model was correct, then, following treatment with alkaline phosphatase, ARR would be completely converted into alkali sensitive RNA by the removal of 2' monphosphate groups and totally destroyed by a second round of alkaline hydrolysis. This model was tested and the results displayed in Tables 22 and 23. These results indicated that ARR was not completely converted into an alkali sensitive form by alkaline phosphatase and, therefore, the phosphotriester crosslinking model was not supported by the evidence.

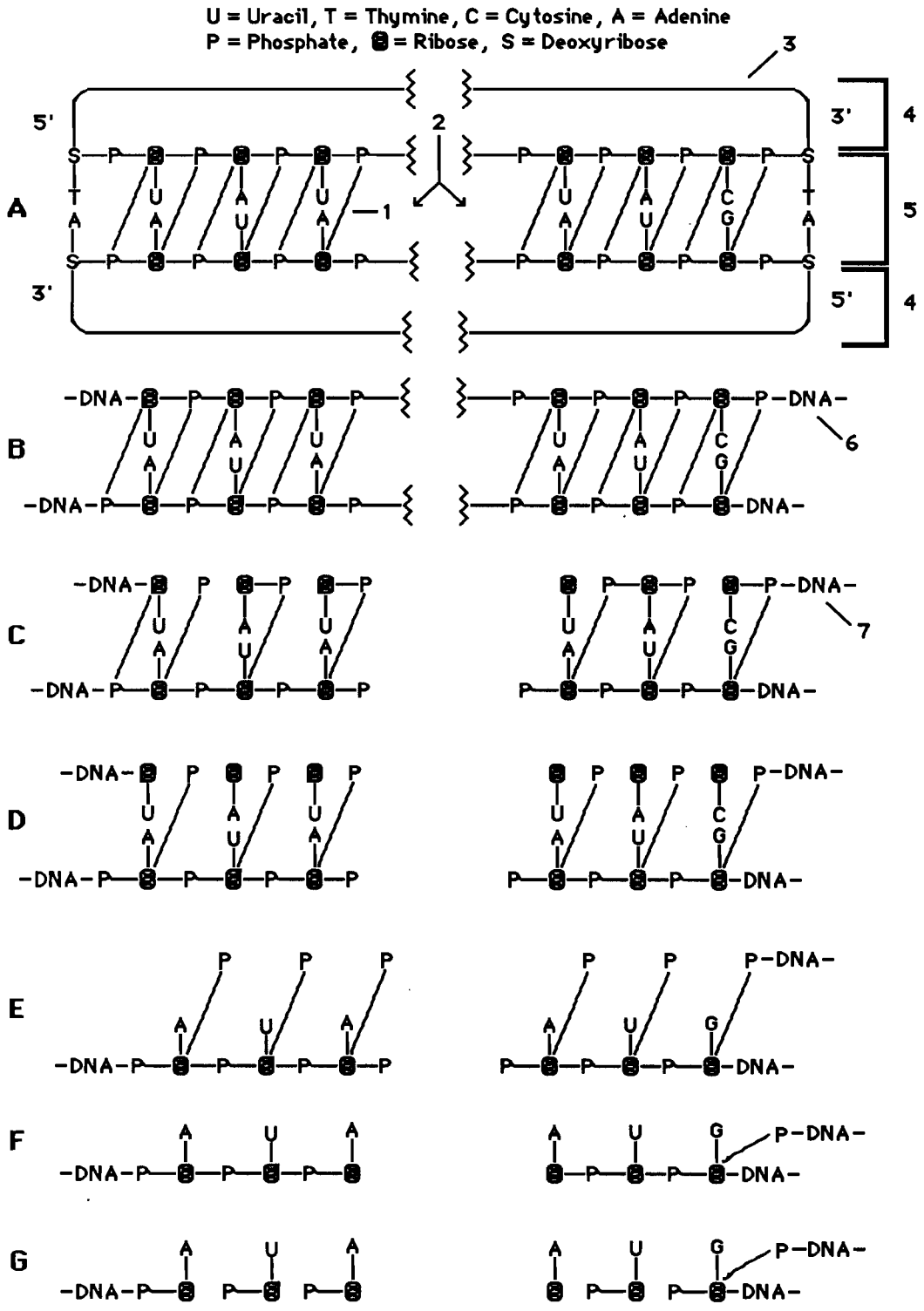

U = Uracil, T = Thymine, C = Cytosine, A = Adenine
P = Phosphate, = Ribose, S = Deoxyribose
A
B
C
D
E
F
G
5'
3'
1
2
3
4
5
6
7
-DNA-
P-DNA-

Figure 41

Figure 42

Phosphodiester Bridge Model for 2' RNA Crosslinks

Illustrations are as follows: Figure 42a depicts covalent bonding between two replicons (4) at their origins of replication by phosphodiester 2' RNA crosslinks (1). The presence of an alkali sensitive region is indicated (but not shown) which contains a combination of Okazaki DNA fragments connected together by alkali sensitive Okazaki RNA primers (2). Double stranded DNA within one replicon is shown by (3) and the tetrameric base paired strand region is illustrated by (5). Figure 42b depicts exhaustive DNAse I digestion of the replicons leading to the isolation of the crosslinked tetrameric region together with associated double stranded DNA fragments of varying lengths (6), the latter of which are responsible for the ability of the tetrameric to precipitate in the presence of ethanol or TCA. Figure 42c illustrates the denaturation of the tetrameric region by heating, resulting in the formation of two dimeric 2' RNA crosslinked regions (only one of which is shown) which exist in an unusual form of "snap-back" nucleic acid connected to single stranded DNA fragments (7). Figure 42d illustrates alkaline hydrolysis treatment resulting in the loss of the internal RNA/DNA region and Figure 42e depicts the removal of monophosphates by alkaline phosphatase which makes the end nucleotides susceptible to a second round of alkaline hydrolysis. In this model, treatment of ARR with alkaline phosphatase followed by a second round of alkaline hydrolysis should remove only the end nucleotides of the ARR. Evidence displayed in Tables 22 and 23 indicates that only a small portion of the ARR is responsive to alkaline phosphatase treatment but this response appears to occur even in the absence of any additional alkali treatment. If enough ARR were available, this model could be tested by subjecting it to a series of alternating treatments involving alkaline phosphatase and alkaline hydrolysis. Providing the ARR is not flanked on both ends by DNA, then, this series of treatments should progressively remove end groups from the ARR until all of the ARR is removed.

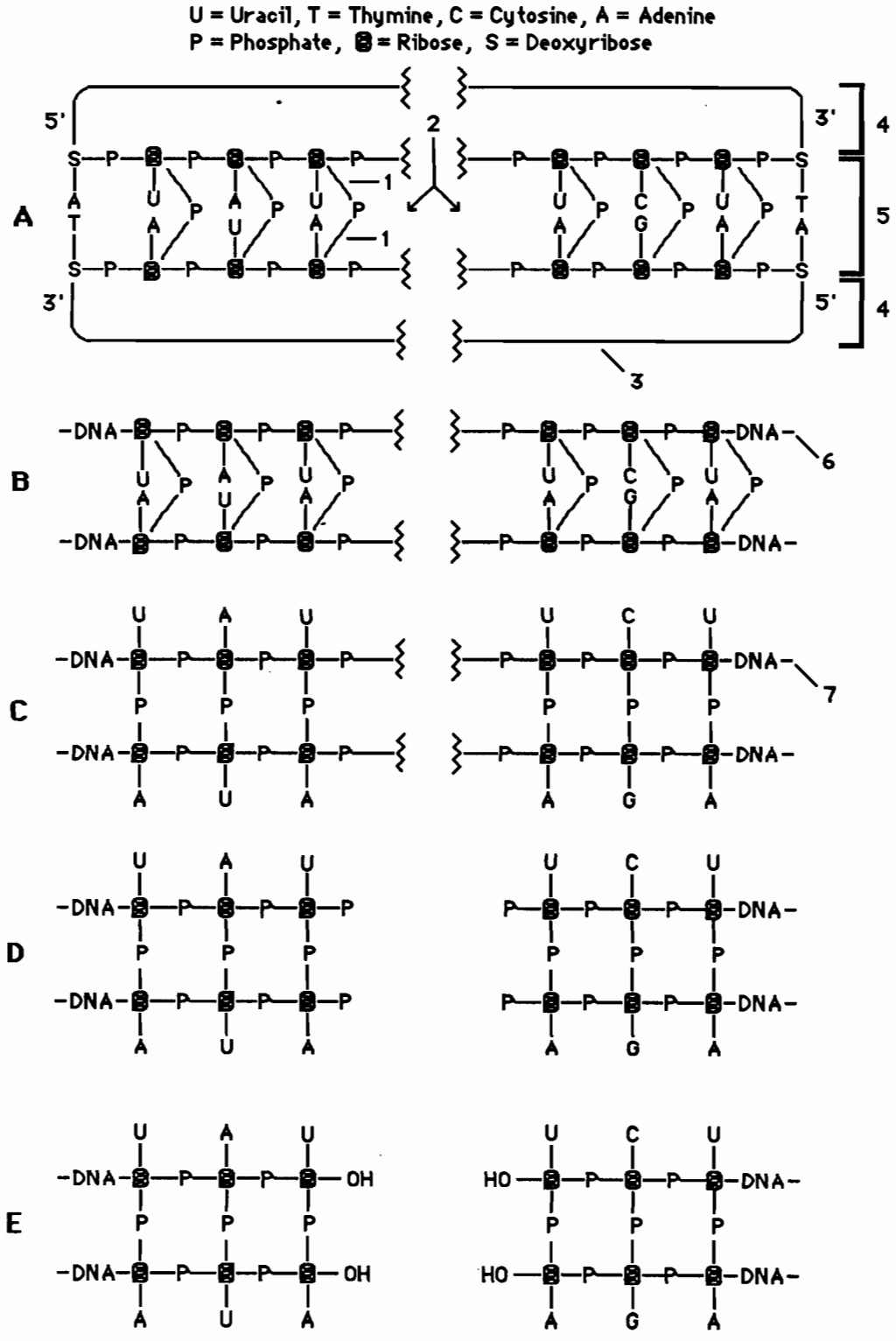

U = Uracil, T = Thymine, C = Cytosine, A = Adenine
P = Phosphate, = Ribose, S = Deoxyribose
A
B
C
D
E
5'
3'
-DNA-
OH
HO

Figure 42

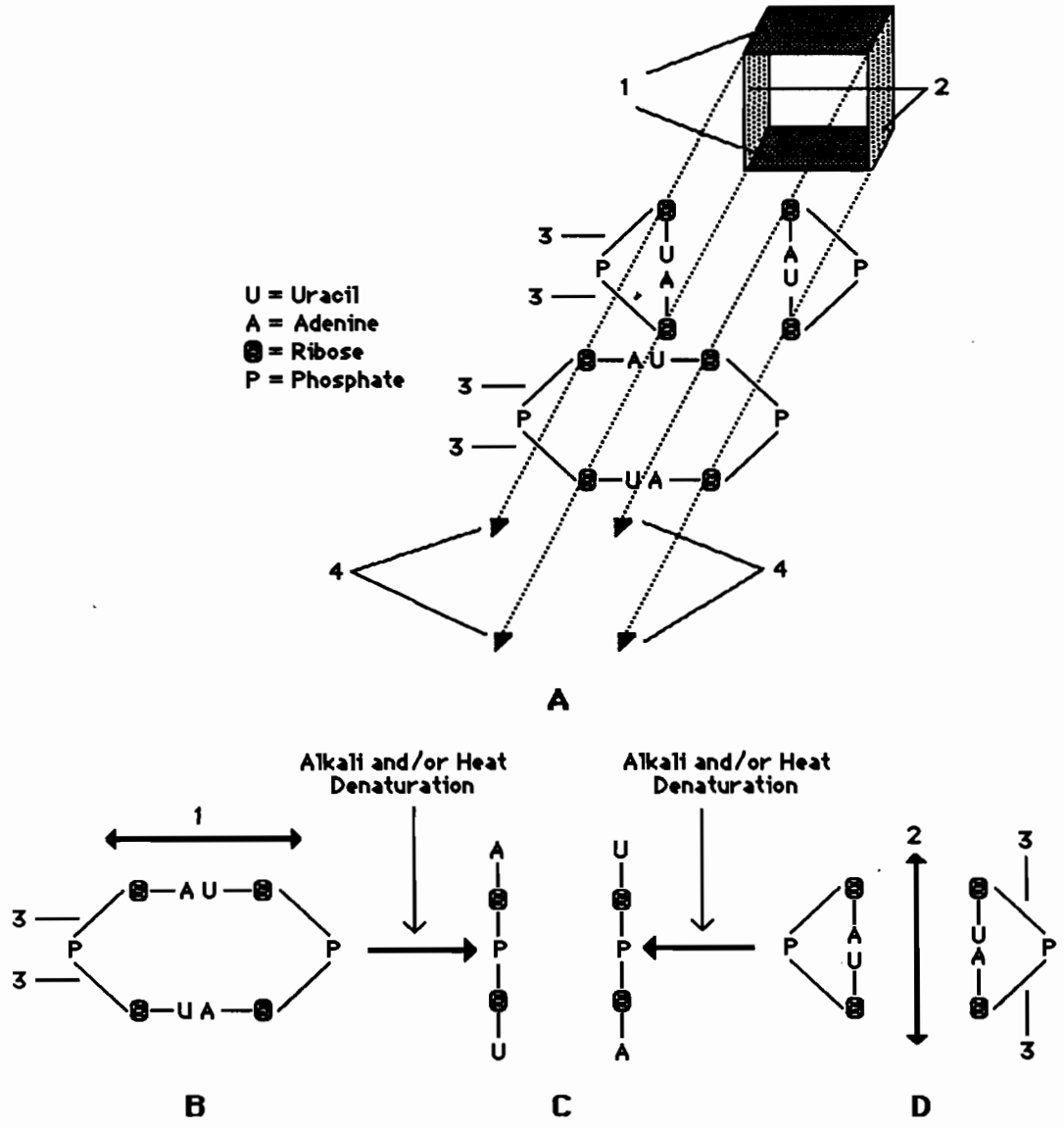


Figure 43

Three Dimensional and Cross-sectional Views of the Phosphodiester Bridge Model for 2' RNA Crosslinks

Figure 43a is a three dimensional view of the 2' RNA crosslinks within the tetrameric strand region previously discussed in Figure 42 and b through d are cross-sectional views. In (a) and (b) the plane of base pairing between daughter strands is shown by (1) and in (a) and (d) the plane of base pairing between non-daughter strands is shown by (2). The 2' RNA phosphodiester linkages (3) between the phosphate bridge and the two non-daughter strands are shown in a, b, and d. The axes (4) contain the 5', 3' phosphodiester backbones of all four strands. Denaturation of the tetrameric region in (b or d) generates the configurations in (c).

at 37°C (Shooter, 1976; Shooter and Merrifield, 1976). In contrast, phosphotriesters of RNA are very unstable, even at room temperature (Michelson, 1963). However, it is quite logical to assume that 2' RNA phosphotriesters should be as stable as DNA phosphotriesters since the triester bonding would eliminate an alkali labile hydroxyl position. Therefore, 2' RNA phosphotriesters were a possible candidate for crosslinking studies. In the studies involving alkaline phosphatase the following assumptions were made concerning the ability of ARR to survive the series of treatments imposed upon it. In order for ARR to be isolated from 2' RNA phosphotriesters after alkali hydrolysis the following set of reactions must have occurred (Figure 41). Figure 41a illustrates the tetrameric region crosslinked together with 2' RNA phosphotriester bonds. Figure 41b illustrates the isolated tetrameric region following DNAse I digestion. Figures 41c and d depict a two-step reaction that must occur during alkali hydrolysis of the triester bonds. Although the initial hydrolysis of the triester bonds is a random event only the reactions leading to an intact ARR oligomer are shown in Figure 41c. Once the initial attack is completed, the RNA is converted into a classical alkali labile form resulting in the obligatory second hydrolytic reaction shown in Figure 41d. Notice that the surviving ARR oligomer is protected by free phosphate groups bound to the 2' positions of each ribose. During the second hydrolytic step the free nucleosides are released yielding the structure shown in Figure 41e. If this structure is exposed to alkaline phosphatase, the resulting structure should be a simple RNA oligomer as shown in Figure 41f. Exposing this oligomer to a second round of alkali should result in its complete destruction as shown in Figure 41g. The results obtained in Tables 22 and 23 indicate that only 7% of the ARR label is lost as a result of exposure to alkaline phosphatase followed by a second round of alkali hydrolysis. Therefore, the 2' RNA phosphotriester model is not supported by this evidence.

Direct evidence for or against the 2' RNA phosphodiester model is not yet available. However, if it assumed that the circular networks described in Chapter VIII are covalently branched networks of DNA which are resistant to proteinase K, then, the 2' RNA phosphodiester model is a good candidate for explaining these molecular configurations. The models in Figures 42 and 43 are further elaborated by Models 1 and 2 in Figures 44 and 45 which are alternative interpretations of the results

described in Tables 20, 21, and 25. In both Figures 44 and 45 two replicons are connected together by 2' ARR phosphodiester bonds into the tetrameric base paired region. The tetrameric base region is shown to be composed of an alternating series of Okazaki RNA primers connected together with Okazaki DNA fragments. As indicated in the literature the Okazaki RNA primers and DNA fragments are about 10 bp and 100-150 bp in length respectively (Lehninger, 1977). The effects of RNAse A digestion, electroelution, and alkali and/or heat denaturation on the joined replicons is intepreted differently by the two models.

The Nature of the Tetrameric Core Region

In all of the models presented here it is inferred that the core region of the tetrameric strand site contains RNAse A and alkali sensitive sites. If such were the case, then, these treatments should completely destroy the non-linear DNA superstructure but the evidence described in this DIssertation suggests otherwise. Therefore, it is important to reiterate what was stated earlier in Hypothesis I in Chapter IX. These core regions may exist as either RNAse A/alkali resistant or sensitive forms. The resistant form may be found in small DNA circular networks as described in Chapter VIII and may be structural DNA's which comprise the main body of such structures as centromeres. The sensitive form may reside in functional DNA's that are capable of generating replicon fusions as described in the body of Appendix D.

Figure 44

Model 1 for Explaining Results in Tables 20, 21, and 25

Figure 44a illustrates upper and lower replicons (1) bound together into the tetrameric region (2) by ARR crosslinks (3) located within a partially alkali resistant Okazaki RNA primer region. The tetrameric region is divided up into alternating sections of Okazaki RNA primers (4) and Okazaki DNA fragments the latter of which are flanked by (5) or flanking (6) the ARR crosslinks. The RNA primers which are flanked by ARR are not removed following DNA replication but all primers outside the ARR sites are removed, including those originally associated with the Okazaki DNA fragments (6). RNAse A digestion (b) destroys the RNA primers except for the ARR section (3). During gel electroelution of "intact" DNA (c) the Okazaki DNA fragments still associated with Okazaki RNA primers are removed but the fused replicon DNA remains behind together with the palindromic DNA sequences (6). The gel inserts are then digested in DNAse I (d) which destroys the replicons leaving only assymetric fragments of DNA connected to the ARR hairpins. The association of the DNA fragments to the ARR hairpins allows the ARR to be precipitated with ethanol or TCA. The loss of the Okazaki DNA fragments following RNAse A digestion and electroelution results in the loss of DNA radiolabel in ethanol or TCA precipitated pellets (Table 20). A similar loss occurs when DAR pellets are subjected to alkaline hydrolysis (Table 21). When the DNA/ARR fragments are denatured by heat or alkali (e), small single stranded fragments of DNA generated by DNAse I action may be released from the ARR/DNA fragments and lost, generating a base sequence assymetry in the remaining single stranded DNA fragments (7) which is reflected by the low thymine/purine ratios seen in Table 25. Any palindromic sequence still associated with ARR crosslinks is "snap-back" DNA. Model 2 is described in Figure 45.

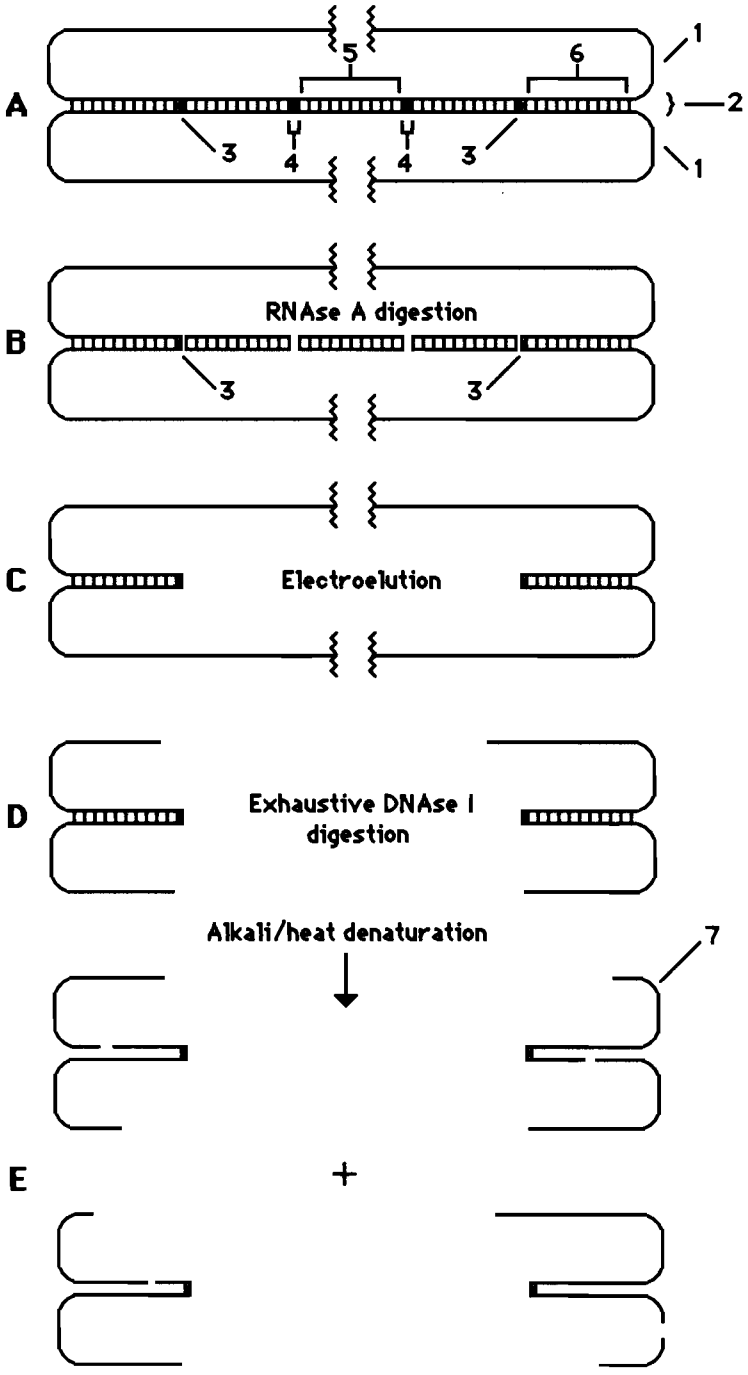

A
5
6
1
2
3
4
4
3
1
B
RNAse A digestion
3
3
C
Electroelution
D
Exhaustive DNAse I
digestion
Alkali/heat denaturation
7
E
+

Figure 44

Figure 45

Model 2 for Explaining Results in Tables 20, 21, and 25

Figure 45a illustrates upper and lower replicons (1) bound together into the tetrameric region (2) by ARR crosslinks located within a completely alkali resistant Okazaki RNA primer region (3). The tetrameric region is divided up into alternating sections of Okazaki RNA primers (4) and Okazaki DNA fragments the latter of which are flanked by (5) or flanking (6) the ARR crosslinks. The RNA primers which are flanked by ARR are not removed following DNA replication but all primers outside the ARR sites are removed, including those originally associated with the Okazaki DNA fragments (6). RNAse A digestion (b) destroys the RNA primers except for the ARR section (3). During gel electroelution of "intact" DNA (c) the Okazaki DNA fragments still associated with Okazaki RNA primers are removed but the fused replicon DNA remains behind together with the palindromic DNA sequences (6 and 7). The gel inserts are then digested in DNAse I (d) which destroys the replicons leaving only assymetric fragments of DNA connected to the ARR hairpins. The association of the DNA fragments to the ARR hairpins allows the ARR to be precipitated with ethanol or TCA. The loss of the Okazaki DNA fragments following RNAse A digestion and electroelution results in the loss of DNA radiolabel in ethanol or TCA precipitated pellets (Table 20). A similar loss occurs when DAR pellets are subjected to alkaline hydrolysis (Table 21). When the DNA/ARR fragments are denatured by heat or alkali (e), small single stranded fragments of DNA generated by DNAse I action may be released from the ARR/DNA fragments and lost, generating a base sequence assymetry in the remaining single stranded DNA fragments (8) which is reflected by the low thymine/purine ratios seen in Table 25. Any palindromic sequence still associated with ARR crosslinks is "snap-back" DNA.

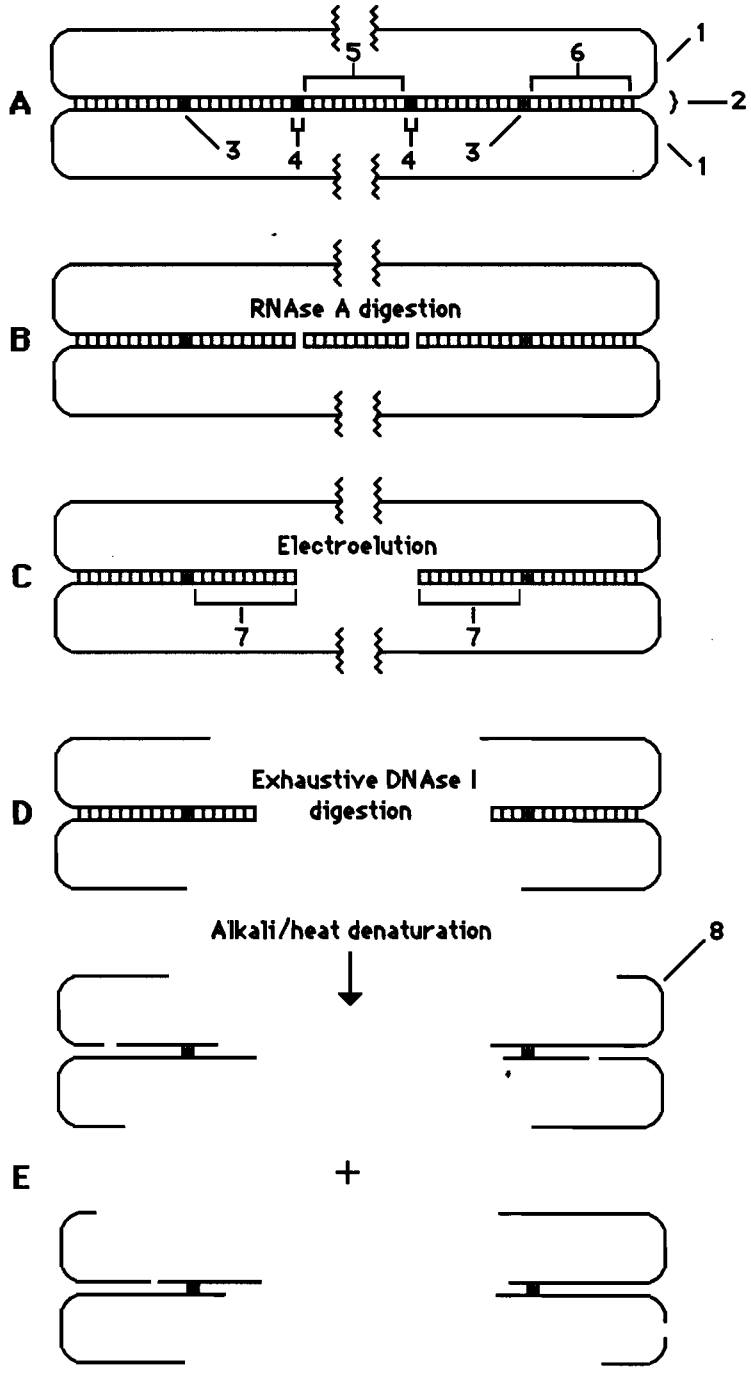

A
5
6
1
2
3
4
4
3
1
B
RNAse A digestion
C
Electroelution
7
7
D
Exhaustive DNAse I
digestion
Alkali/heat denaturation
8
E
+

Figure 45

Replicon Clusters in Eukaryotes

Origins of replication in Drosophila appear to be clustered together in chromomere sized (30 kb) arrays that initiate DNA synthesis at approximately the same time (Blumenthal, et al, 1973). Similar clustering has been observed in Physarum (Funderud, et al, 1978). Newly synthesized DNA appears as small radiolabeled spots over nuclei which contain enough DNA to account for many replicons (Huberman, 1987). These observations suggest that the cluster is initiated into DNA replication by a master "switch". It is postulated here that each replicon in the cluster is activated into replication by a master origin of replication. The DNA domain attachment sites may represent the connection of the replicon cluster to the nuclear matrix by the master replicon. The DAR regions were postulated to be within each origin of replication. A signal sequence within each DAR could be responsible for achieving three things following the intitiation of DNA replication: 1) preventing the loss of the Okazaki RNA primer region which constituted the DAR, 2) establishing the diphosphate ARR bridges which may be flanking alkali-labile tetrameric regions, and 3) establishing the attachment site of the replicon cluster to the nuclear matrix.

Eukaryotic DNA Rearrangements During Cellular Differentiation

Loop Fusions and Deletions

During the course of cellular differentiation in lymphocytes there may be a decrease in the total number of DNA domains. Such a decrease could be the result of two different phenomena: 1) the DNA domains may be fusing together to make larger domains and 2) some of the DNA domains may be removed entirely from the cellular genome and lost as intact circles of DNA. The former case is suggested by the fact that the DNA domains in leukemic lymphoblasts are about one-half the size of their counterparts in normal lymphocytes (Hartwig, 1982) and during the development of frog embryos the average nucleoid loop size apparently increases in later stages of development (Flickinger et al, 1986). The latter case is suggested by the releasing of large numbers of small circular DNA's which are heterogenous in size during the course of development of embryonic chicken bursa lymphocytes (Delap and Rush,

1978) and during the aging of fibroblasts in culture (Icard-Liepkalns et al, 1986). In the latter case, new gene sequences were found associated with the circular DNA's of older fibroblasts that were not present in the circles of the younger fibroblasts. I speculate that such loop fusions and deletions are the result of changes in the DAR attachment sites which establish replicon cluster formation. The central tetrameric regions binding two origins together could be hydrolyzed resulting in loop fusions; this would inactivate the origins of replication for both replicons. Therefore, as loop fusions progressed during the course of cellular differentiation the number of active replicons would diminish. Evidence that less differentiated cells contain more replicons than more highly differentiated cells has been shown in Drosophilia studies (Blumenthal et al, 1973). The model in Figure 40 illustrates how DNA replicons may be removed by deletion (c to b) or how their origins of replication may be inactivated by fusions (c to d) resulting in deletion of critical sites within the tetrameric based paired region. The model in Figure 46 illustrates how loop fusions within a replicon cluster can result in the formation of a large single replicon. The model in Figure 47 was derived from an older concept relating to the use of branched DNA pathways in establishing alternative transcriptional pathways during immunoglobulin gene rearrangements (Smithies, 1970). The latter model is identical in design to the recently reviewed disproportionate replication model of chorion gene amplification (Spradling, 1987). The model in Figure 47 may apply to other clustered genes as well including heat shock genes (Wright, 1987) and chorion genes (Spradling, 1987). It illustrates how loop fusions and deletions may be used to rearrange genomic DNA into one irreversible pathway for transcribing RNA. The original loops containing exons are evolutionary mosiacs. Most of the loop is viral and may be considered as an intron but the exon portion is pre-eukaryotic in origin. The origins of replication connecting the loops together constitute highly repetitive DNA. This DNA is lost during the gene rearrangement process. Evidence that repetitive DNA sequences can serve as origins of replication has been demonstrated with human Alu sequences using recombinant DNA technology (Johnson and Jelinek, 1986). The viral portions of each loop represent moderately repetitive DNA and portions of this are transcribed into introns. The pre-eukaryotic exon is single copy DNA.

Figure 46

Model of a Eukaryotic Replicon Cluster

The replicon cluster in (a) is composed of a master DNA replicon (1), slave replicons (2), the master origin of replication (3), double slave origins or replication (4), and ARR cross links (5). The initiation of replication of the replicon cluster is hypothesized to occur at the master origin of replication (see Figure 50 for details). The double slave origins are equivalent to the tetrameric base paired regions previously discussed in Figures 40c, 41a, 42a , 43, 44a, and 45a. In Figure 46b the replicon cluster double slave origins have deleted their alkali sensitive regions either through developmental processes (See Figures 47 and 49) or by treatment with alkali, RNAse A or other procedures (see Figures 40d, 41c, 42d, 44b, and 45b). Figure 46c demonstrates how these treatments could lead to the ultimate fusion of the replicon cluster into one single replicon of DNA containing a single intact and functional origin of replication. Losses in replicon number and hence active origins of replication during cellular differentiation are a hallmark of embryogenesis in such organisms as Drosophila (Blumenthal et al, 1973).

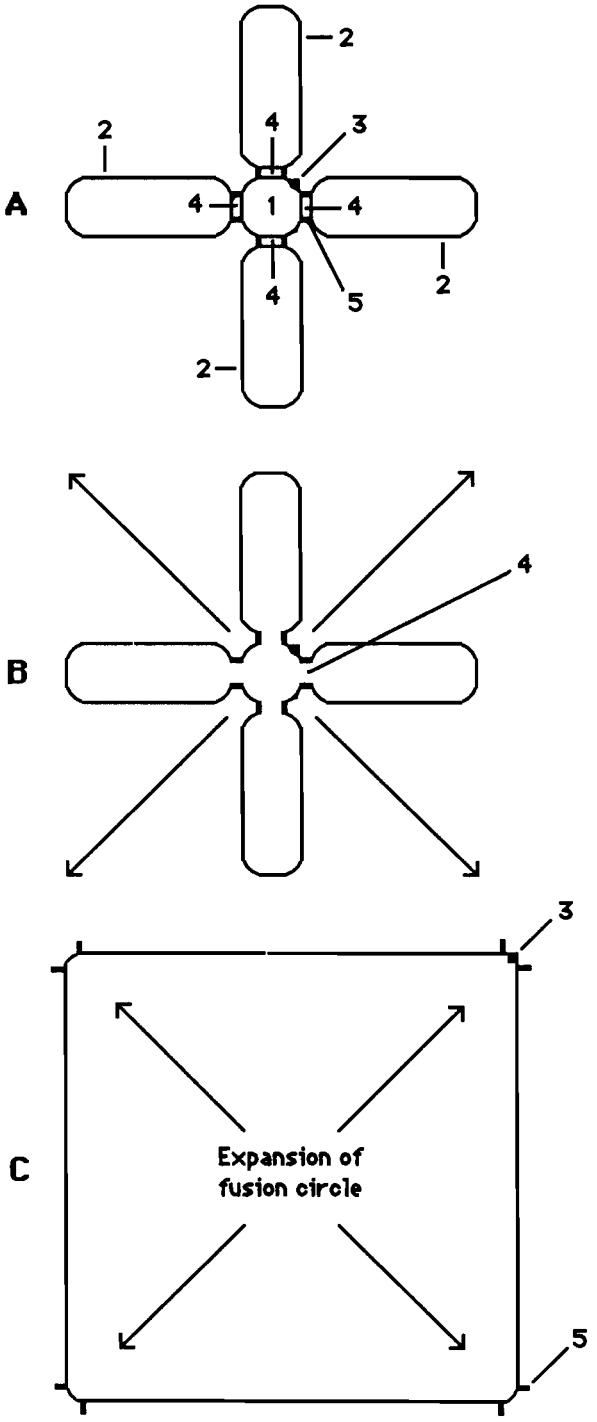


Figure 46

Figure 47

Model of a Immunoglobulin Complex Replicon Cluster

This model was developed from an older concept describing how branched DNA pathways may be used in providing alternative transcriptional pathways for immunoglobulin gene rearrangements during lymphocyte development (Smithies, 1970). The model focuses on replicon fusions and deletions as the principle means for generating DNA rearrangements, but other phenomena such as transposition, gene amplification, and integration of exogenous viral DNA are also involved. Figure 47a depicts a complex replicon cluster (see also Figure 52) composed of 1 extra large replicon (1) containing the master origin of replication (2), 4 large replicons (3), 8 medium sized replicons (4), and 12 small replicons (5). The size of the master replicon is exaggerated in order to save space in the illustration by placing slave replicons parallel to it. All the replicons are interconnected by tetrameric regions or double slave origins of replication (6). The medium-sized and large replicons are depicted as mosiac DNA in which most of the replicon is viral in origin (7) and can be considered as an intron containing a small segment of pre-eukaryotic DNA (8) which is the exon (see text on the evolution of introns and exons). One of many potential heterogenous RNA transcriptional pathways (9) is denoted in Figure 47b. In Figure 47c this pathway is irreversibly fixed by the deletion of 2 large replicons, 6 medium-sized replicons, and 8 small replicons coupled with the fusion of the extra large replicon, 2 large replicons, 2 medium-sized replicons, and 4 small replicons. The deleted replicons are shown in Figure 47d together with the fusion replicon (10). The fusion replicon is composed of a non-transcribed section which is composed of contiguous viral introns and a transcribed section which is a combination of 5 introns (I1-5) and 4 exons (E1-4) from which heterogenous RNA (11) is transcribed. Note that during this process of gene rearrangement the number of functional double origins of replication (5) decreases from 24 to zero leaving only the master origin of replication intact.

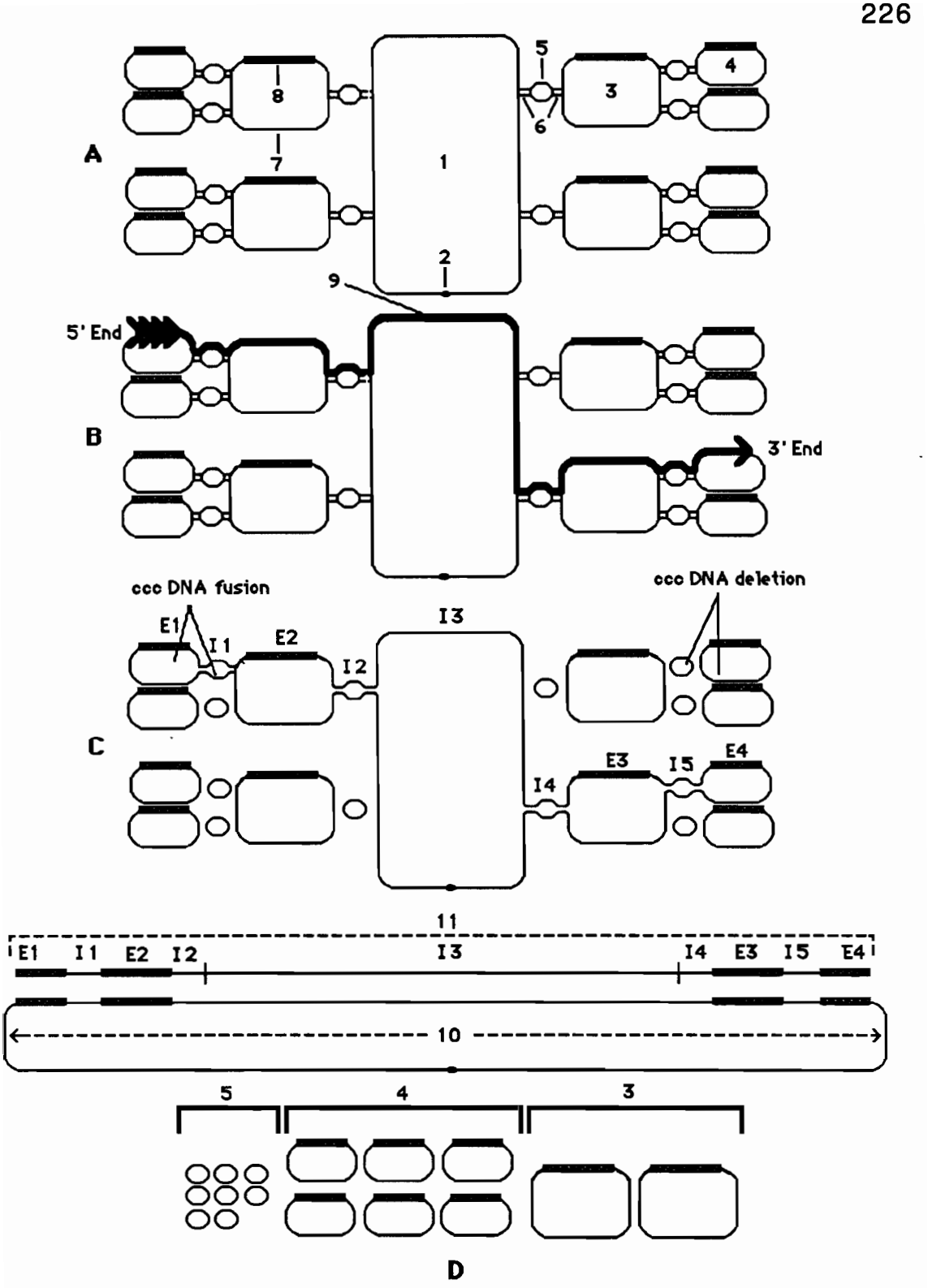

A
B
C
D
5' End
3' End
ccc DNA fusion
ccc DNA deletion
E1
I1
E2
I2
I3
I4
E3
I5
E4
1
2
3
4
5
6
7
8
9
10
11

Figure 47

If the fusion model is correct, then, in the absence of DNA replication alkali/RNAse A sensitive RNA should exist only within the tetrameric region of the matrix DNA. Alkali treatment should destroy this RNA resulting in a concommittent loss of tetrameric DNA associated with it. No such loss of DNA should occur when non-matrix DNA is treated with alkali. However, alkali treatment results in DNA loss in both matrix and non-matrix samples as compared to controls not treated with alkali (see Tables 26 and 28). Therefore, the losses in non-matrix DNA attributable to alkali treatment currently cannot be explained by the present models.

The Role of Methylation in DNA Rearrangements During Differentation

It has been hypothesized that methylation of DNA at cytosine residues may be playing a developmental role in gene regulation (for review see Lewin, 1980). Methylation could also play a developmental role in the inactivation of ARR crosslinking sequences. Following a fusion or deletion of a replicon the flanking diphosphate ARR bridges could be inactivated during the next round of replication by substituting methylation for phosphorylation at the 2' hydroxyl position of the ribose (Figure 48). The diphosphate bridge which is immediately adjacent to the hydrolyzed section would not be methylated and would maintain strand continuity. As loop fusions continued to progress, more and more of the ARR sites would be inactivated by methylation and released into the non-matrix region of the DNA (Figure 49). This could account for the difference in methylation content which has been observed for matrix and non-matrix ARR's (Table 32). During loop deletions the domains would be released as intact circles of DNA leaving behind a "scar" of DAR within the replicon cluster. The regions responsible for ARR formation in this "scar" could be inactivated by methylation. Once loop fusions and deletions were completed, the replicon cluster would be reduced to one or more DNA domains with an average size of about 100-300 kb depending on cell type resulting in a 1:1 correlation between DNA domain numbers and alkali sensitive sites.

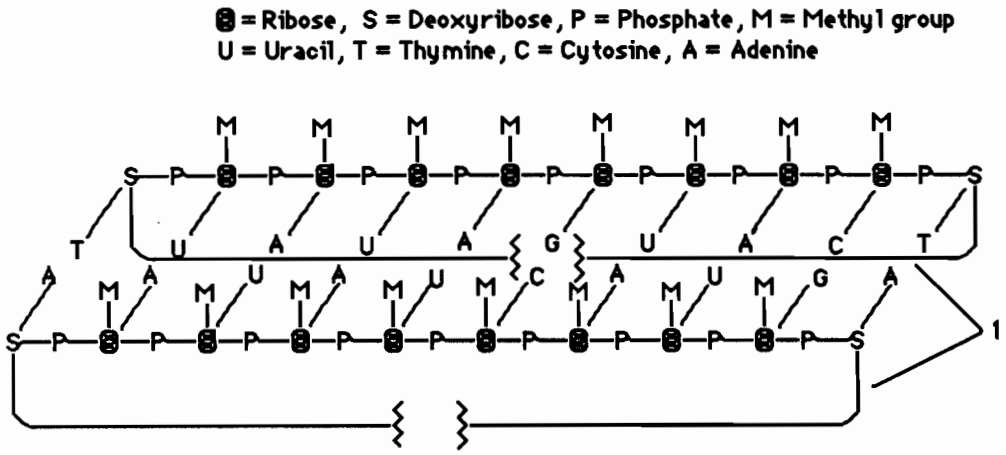


Figure 48

The Inactivation of ARR Sites by Methylation

Figure 48 is an illustration of a single replicon (1) in which the 2' RNA positions of the ARR sites have been methylated instead of phophorylated. It is hypothesized that methylation at these sites prevents ARR crosslinks and, therefore, constitutes a form of cellular differentiation. This is analagous to the hypothesis that the methylation of cytosine residues in DNA is responsible for at least one form of developmental gene regulation (for review see Lewin, 1980).

Figure 49

The Methylation and Releasing of DNA From Nuclear Matrix Attachment Sites During Cellular Differentiation

Figure 49 is an interpretation of the results described in Tables 31 and 32. Figure 49a illustrates the master replicon (1), the slave replicons (2), the master origin of replication (3), the slave origins of replication (4), and the ARR attachment sites (5). Most of the DNA within the slave replicons is within the non-matrix region (6) and the master replicon and all the origins of replication are within the matrix region (7). The master replicon is presumed to lie within the plane of the nuclear envelope (see Figure 56) and all origins of replication constitute DNA attachment sites. During cellular differentiation (b) the slave loop origins are deleted and the ARR sites are methylated during the next round of replication. Once these sites are methylated, loop fusion begins to occur. Figures 49c, d, and e illustrate the releasing of the DNA attachments from the nuclear matrix into the non-matrix region. Note in 47e that the ratio of non-matrix methylated ARR to matrix methylated ARR is 2.0.

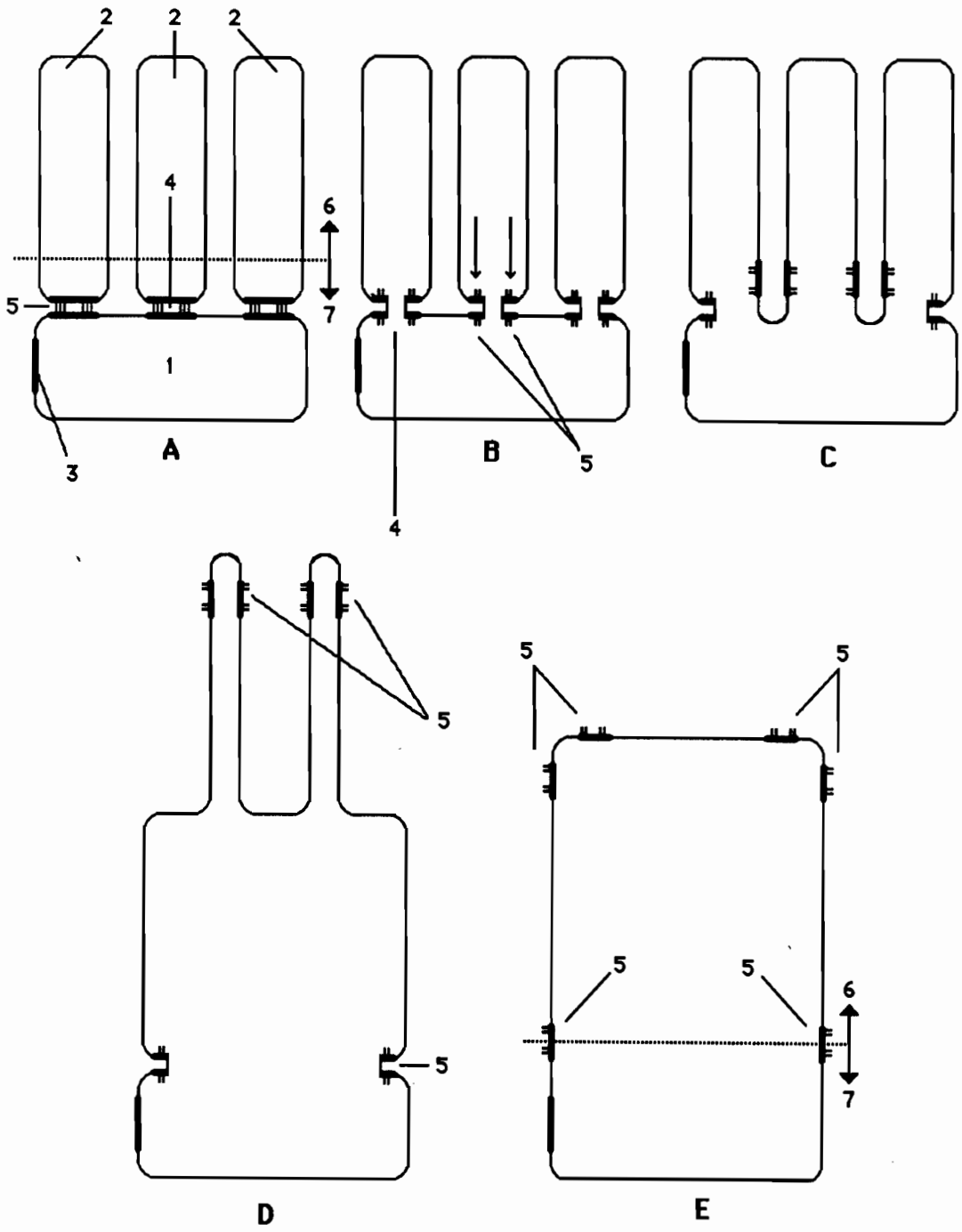


Figure 49

Other DNA Rearrangements

Other forms of DNA rearrangements besides loop fusions and deletions which may be occurring during cellular differentiation include DNA domain additions, amplifications, and transpositions. In this discussion emphasis was placed on loop fusions and deletions. During the discussion on evolution, additional emphasis will be placed on loop additions, amplification, and transpositions as vehicles for increasing the size of the genome.

The Relationship of DNA Superstructure to DNA Replication in Prokaryotes and Eukaryotes

A Comparison of the Mode of Replication of a Eukaryotic Replicon Cluster to a Prokaryotic Chromosome

The term slave loop was originally used to describe the descrepancies in gene number and DNA content within such structures as polytene chromosomal "puffs" and lampbrush chromosomal loops. The hypothesis was that master DNA loops amplify themselves many times to form slave loops (Callan, 1967). The term is used here in a different context to describe the same phenomeon in terms of replicon clusters. Figure 50 is a comparison of DNA replication between a prokaryotic chromosome and a eukaryotic replicon cluster. In both models the initiation of bidirectional replication begins at a master origin which is composed of two individual origins of replication base-paired together into a tetrameric arrangement referred to as a double origin. A total of two sets of replication forks is generated. One set replicates the inner, core replicon and the other set replicates the surrounding single replicon in prokaryotes or the slave replicon adjacent to the master origin in eukaryotes. Once the initiation of DNA synthesis has occurred in the prokaryote, replication proceeds independently within both replicons until one of the replicons is completely replicated. Since the inner replicon is much smaller than the outer fusion replicon it can began a new round of DNA synthesis before the fusion replicon has completed replication. When this occurs, a second round of replication is initiated simultaneously in both replicons. In contrast to this, the eukaryotic replicon cluster is depicted as initiating secondary and tertiary rounds of replication before the master origin of replication initiates a

Figure 50

A Comparison Between the Modes of Replication of a Prokaryotic Chromosome Versus a Hypothetical Eukaryotic Replicon Cluster

Figures 50a and e illustrate a prokaryotic chromosome and a eukaryotic replicon cluster respectively. In 50a is illustrated a small RNA-rich replicon (1P), a larger DNA replicon (2P) which is the fusion product of four replicons, the master double origin of replication (3P) which is attached to a mesosome, single slave origins (4P) which are non-functional and unattached to mesosomes, and regions of tetrameric base pairing (5). No ARR sites are present. In 50e is illustrated the master replicon (1E), slave replicons (2E), the master double origin of replication (3E) which is attached to the nuclear envelope, the slave double origins of replication (4E) which are also attached to the nuclear envelope, and the ARR crosslinks (6). See Figure 56 for additional information on membrane attachments sites. In the prokaryotic model the origins of replication within the four fusion replicons have been deleted. Their counterparts within the smaller RNA-rich replicon have been retained as single, non-functional slave origins and function as RNA-rich linkers for the four fusion replicons. In these models double origins of replication are required to generate highly repetitive palindromic sequences (see Figures 40, 44 and 45). In the prokaryotic model only the master origin of replication fits these requirements but it is not connected together by ARR crosslinks. During DNA extraction the RNA-rich replicon is destroyed, resulting in the loss of all highly repetitive DNA. In both models replication begins bidirectionally from the master origin of replication. In the prokaryotic model, once DNA replication is initiated the two outer and inner replicons proceed with DNA replication in an independent manner (50b). This process is continued in Figure 50c so that only two replication forks are ever present during any single round of replication. In the eukaryotic model (50d) DNA replication proceeds halfway into each double slave origin and in doing so activates the slave origins of replication within the slave loops (see also Figure 53). This secondary round of replication results in the formation of four replication forks, two of which replicate the slave loop and two of which continue replicating the master DNA circle. In Figure 50e a third round of replication has occurred resulting in the formation of 2 additional replication forks. The first two replication forks have fused and are no longer visible. If replication rates were identical between this prokaryotic chromosome and the eukaryotic replicon cluster the former would take longer to complete replication than the latter (compare 50c and f). In reality, prokaryotic DNA replication is much faster than eukaryotic replication. The Okazaki RNA primers and DNA fragments within eukaryotes are about 10 times smaller and, therefore, 10 times more numerous than they are in prokaryotes (for review see Lehninger, 1977). This means that the continuity of DNA replication is interrupted 10 times more often in eukaryotes than in prokaryotes and these discontinuities no doubt contribute to the inertia of eukaryotic DNA replication.

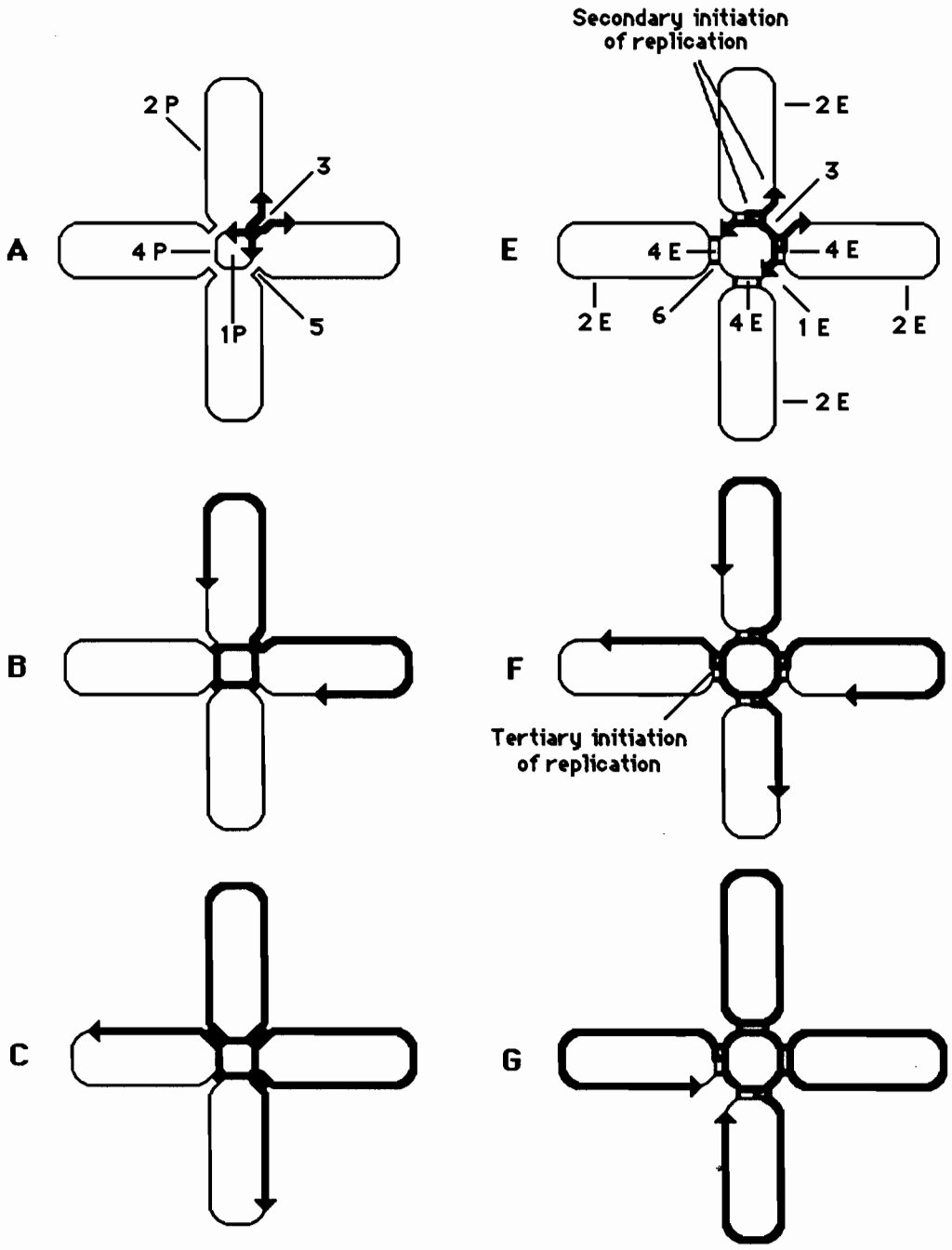

A
2 P
3
4 P
1P
5
B
C
E
Secondary initiation of replication
2 E
3
4 E
4 E
2 E
6
4 E
1 E
2 E
2 E
F
Tertiary initiation of replication
G

Figure 50

second round of replication. This occurs whenever DNA synthesis within the master replicon enters a double slave origin region. It is assumed that only single, slave origins exist in the prokaryotic model and that the origins of the outer replicons have been deleted to form a single, outer fusion chromosome. The loss of these origins is considered to be at least one factor in preventing the initiation of secondary rounds of DNA synthesis within individual prokaryotic DNA domains. It is also assumed that these single origins have no mesosomal attachment sites, whereas both the slave and master origins are membrane-bound in the eukaryotic model (see Figure 56). Evidence that eukaryotic cellular origins of replication are permanently attached to a substrate is available (McCready et al, 1980; Vogelstein et al, 1980; Wanka, 1982; Aelen et al, 1983; Smith, 1984; Bekers et al, 1986; Dijkwel et al, 1986; Jackson and Cook, 1986; Carri et al, 1986; Razin et al, 1986). Evidence that such attachment sites may be composed of membranes is suggested by the mesosomal attachment sites of prokaryotic chromosomes to their cellular membranes (Swanson, 1981). Cis-acting origin "silencers" are known to exist within plasmids (Patel and Bastia, 1986). The relationship of these plasmid origin silencers (if any) to the regulation of the initiation of replication in prokaryotic and eukaryotic origins is uncertain.

Note that the DNA appears to replicate much faster in the replicon cluster than in the prokaryotic chromosome. In reality prokaryotes replicate much faster than eukaryotes (for review see Lehninger, 1977). The increase in the rate may be the result of less interruptions of prokaryotic DNA synthesis by Okazaki DNA fragments and RNA primers. Okazaki RNA primers and DNA fragments are 100 and 3000 base pairs long respectively in prokaryotes but only 10 and 100-150 base pairs long respectively in eukaryotes (Lehninger, 1977) which means 10 times less interruptions in DNA synthesis in prokaryotes than in eukaryotes. Less interruptions in DNA synthesis in prokaryotes should increase its efficiency which in turn should increase the overall rate of synthesis.

The Relationship of Different Hierachies of Eukaryotic DNA Superstructure to DNA Replication

In the Preface of this Dissertation I mentioned that eukaryotic DNA superstructure could be viewed in a different light other than as different hierachies of supercoiling (Bak and Zeuthen, 1978; Sedat and Manuelidis, 1978) and that models involving

chains of circles (Stahl,1962) or radial loops (Paulson and Laemmli, 1977) were more appropriate. Current studies suggest that chromosome structure may be more complex than can be accounted for by either helical or radial loop models (Belmont et al, 1987). In spite of this apparent complexity it is generally believed that the DNA within the eukaryotic chromosome is a continuous linear molecule. Evidence supporting this idea is available from studies with lampbrush chromosomes in which lampbrush loops were pulled apart by micromanipulation and fused into a continuous chromatin thread. The fusion phenomenon appeared to be the result of a transverse fission within the chromomere from which the loops emerged (Callan, 1955). This has been interpreted by a model which depicts the DNA within a chromomere as a highly convoluted continuous thread (Gall, 1955). The model in Figure 51 challenges the concept that the DNA within a eukaryotic chromosome is a continuous linear molecule. Instead it suggests that the transverse fission which occurs within lampbrush chromomeres following micromanipulations is the result of damage to the tetrameric region which results in the loop fusions observed by these early investigators.

The model presented in Figure 52 represents different hierarchies of DNA superstructure below the level of a complete chromosome in which the DNA is arranged as a complex linear array of loops interconnected by covalent bonds. The different levels of DNA superstructure include the following: 1) the DNA replicon (viral size range), 2) the DNA replicon cluster (prokaryotic chromosome size range) 3) subchromatid, (a single giant circle of DNA to which is attached the replicon clusters), 4) the chromatid, which is comprised of one or more subchromatids attached together at origins of replication by tetrameric bonding and 2' RNA crosslinks, 5) the chromosome, which includes the chromatids which are attached to the centromere by tetrameric bonding and 2' RNA crosslinks, the latter of which is composed of many amplified redundant circles of DNA networked together at their origins of replication by tetrameric bonding and 2' RNA crosslinks, and 6) the entire genome. It is quite possible that most of the circular networks observed during electron microscopy originated from the centromeric regions of chromosomes or chromatin. Huge replicon clusters would have been destroyed by the initial DNAse I digestion used in separating matrix from non-matrix DNA. The replicon clusters are shown to exist as simple "daisy-like" radial arrays as seen in Plates VI and VII or in more complex forms. A

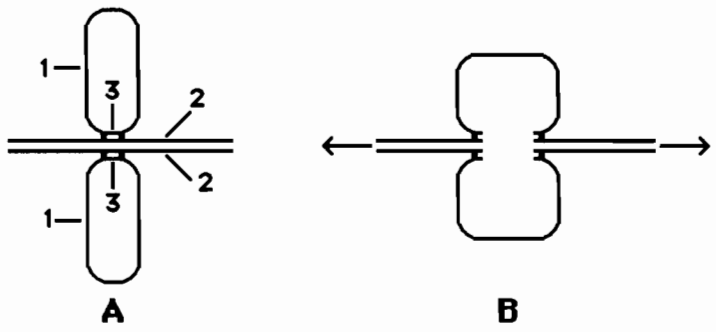


Figure 51

Interpretation of Callan's Lampbrush Chromosome Loop Bridge Experiment

In Figure 51a are represented the lampbrush slave loops (1), sister chromonema (2), and the tetrameric base paired region crossed linked together with ARR (3). The slave loops and chromonema are heavily protected with protein. The least protected and, hence, weakest physical region in the structure is within the tetrameric region. When the chromonema are drawn apart as shown in Figure 51b the tetrameric region fractures, allowing the formation of lampbrush loop bridges (Callan, 1955).

complex replicon cluster was illustrated in Figure 47 as an example of how loop fusions and deletions within the cluster may be responsible for the development of permanent transcriptional pathways. Units of recombination are displayed as the linear distance between replicon clusters along the chromosome. It is hypothesized that the physical manifestation of this linear arrangement of replicon clusters is seen as chromomeres during the leptotene stage of meioisis. The paired chromonemata on which the chromomeres are attached are considered to be opposite sides of the collapsed subchromatid DNA circle. Units of translocation are displayed as the linear distance between subchromatids along the chromosome. The junctions between subchromatids are also referred to as translocation "hot spots". They are considered here as centromeric vestiges (secondary constrictions?) which still associate with the nuclear envelope in the form of heterochromatin similar to centromeres but have lost their ability to associate with mitotic spindles (see monocentric chromosomes under the section on intracellular symbiosis). These junctions are easily disturbed by

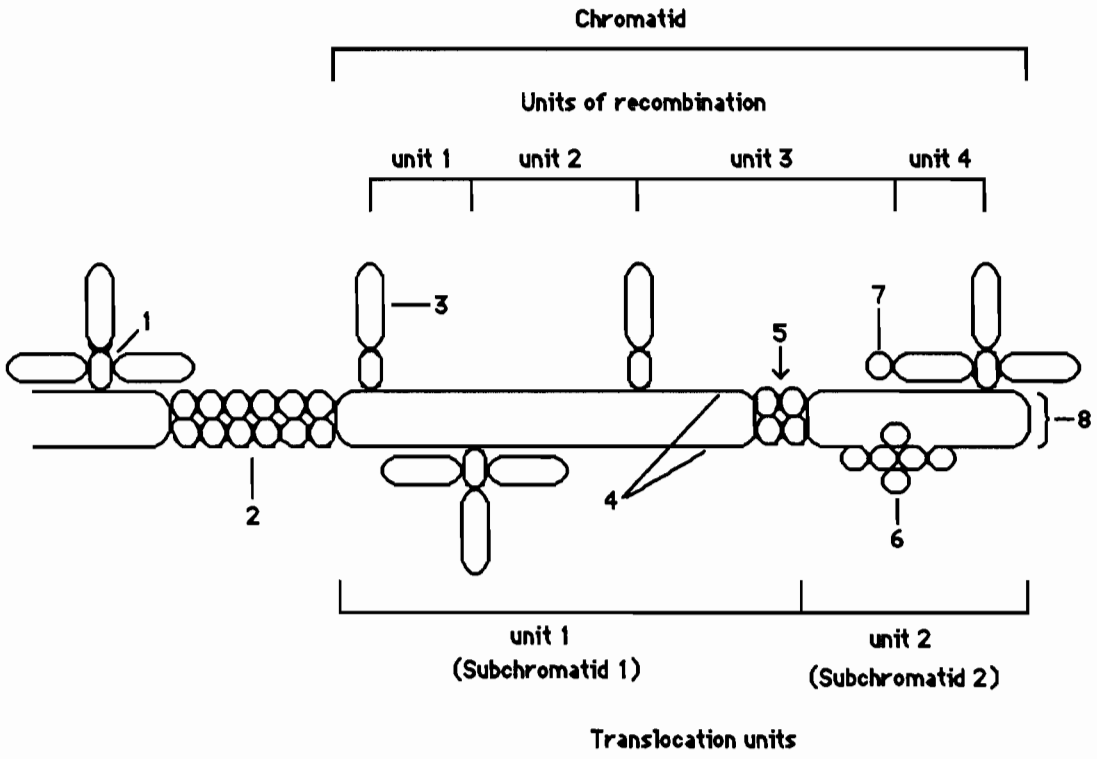


Figure 52

Different Hierarchies of Eukaryotic DNA Superstructure

Figure 52 is a hypothetical illustration of different hierachies of DNA superstructure within a typical metacentric chromosome. Illustrated are a simple replicon cluster (1), the centromere (2), single slave loop replicon clusters (3), sister chromonema (4), a vestigial centromere (5), a complex replicon cluster (6), a slave replicon which is not attached to the nuclear matrix (7), and the telomere (8). The centromere and the vestigial centromere are also considered to be complex replicon clusters. The different levels of DNA superstructure starting with replicons and ending with the chromosome are as follows: 1) the replicon, 2) the replicon cluster, 3) the subchromatid, 4) the chromatid, and 5) the chromosome. A chromomere is considered to be the physical manifestation of a replicon cluster. The replicon clusters are attached to one side of a giant circle of DNA, the subchromatid, which is a fusion product of replicon clusters (see Figure 55). The arrangement of the replicon clusters along the subchromatid is similar to "beads on a string" in that the subchromatid circle is collapsed into two parallel sister chromonema. Such an arrangement allows for the paradox of circular chromosomes behaving as linear chromosomes. In this model a telomere is simply a DNA hairpin connecting the two sister chromonema together within the giant collapsed circle of DNA. Chromatids may be composed of two or more subchromatids connected together by vestigial centromeres (secondary constrictions?) which no longer attach to spindle fibers. It is assumed that subchromatids were originally independent chromosomes that have fused together at centromeres and eventually lost their centromeric connections (see Figures 58 and 59). These vestigial centromeres may be sensitive to radiomimetic drugs and, therefore, translocation "hot spots".

radiomimetic drugs or other treatments known to enhance the frequency of occurrence of such translocations. The process of sister chromatid exchange may also be occurring in these areas as well.

Bidirectional replication of DNA from replicon clusters along the length of a subchromatid is illustrated in Figure 53 at the bottom together with a diagrammatic interpretation of a pulse chased autoradiograph.

The Evolution of the Progenotic Chromosome

The term "progenote" has been coined for a hypothetical ancestor for prokaryotes and eukaryotes (Woese and Fox, 1977). I currently share the contention that prokaryotes and eukaryotes evolved from a common progenotic ancestor and I have described the hypothetical evolution of this progenotic ancestor in Figure 54. In Figures 54a-k it can be seen that viral chromosomes are undergoing a series of recombinations in which they pair up and recombine at their origins of replication resulting in the retention of both origins of replication. These daughter origins serve as sites of recombination for still other replicons, and so on until a large integrated chromosome is generated containing many origins of replication. These multiple origins of replication are forerunners to the highly repetitive DNA observed in eukaryotes because they must be quite similar in base sequence for base pairing to occur. The remainder of the viral integrants are less conserved in base sequence and constitute the forerunners of moderately repetitive DNA in eukaryotes. Transposition and deletion of origins of replication is shown to occur in Figure 54l. If transposition was the dominant force, then, the final outcome would be the generation of a large replicon containing a single origin of replication (Figure 54m). If viral integration was occurring at a faster rate than transposition, the ultimate outcome would the the generation of a large replicon with many functional origins of replication (Figure 54n). The pairing and integration of replicons with single origins to a replicon containing many origins of replication could have generated the progenotic chromosome as described in the "Theoretical Assumptions" of this Dissertation.

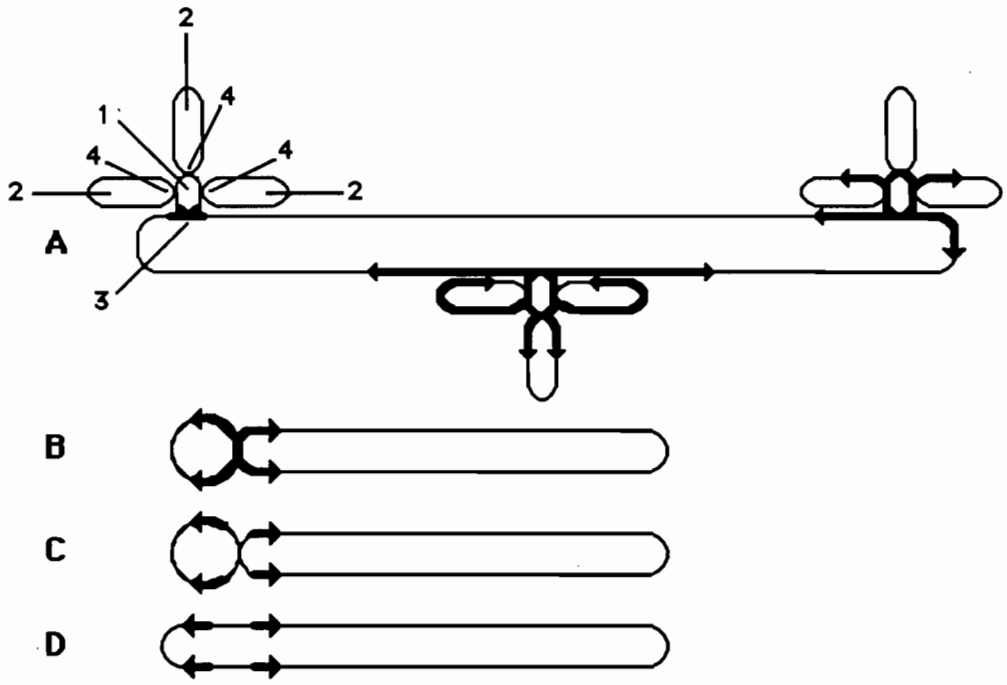


Figure 53

Patterns of DNA Replication in a Subchromatid

Figure 53a illustrates the pattern of bidirectional DNA replication within a subchromatid. Illustrated are a replicon cluster or chromomere containing a master replicon (1), slave replicons (2), a master origin of replication (3), and slave origins of replication (4). Varying degrees of initiation of DNA synthesis are shown from the earliest (center) to the latest going in a counterclockwise from the center replicon cluster. Figure 53b depicts a continuous radiolabel grain pattern within the master DNA replicon and a slave replicon, (c) illustrates a pulse-chased grain pattern between the two replicons, and (d) illustrates the pulse-chase pattern when the two replicons have fused during the isolation procedure.

Figure 54

The Evolution of the Progenotic Chromosome

Figures 54a-k depict the build-up of the progenotic genome from a series of viral integrations. Each unit viral replicon (1) and their corresponding origins of replication (2) are ilustrated in Figure 54a. As each virus integrates, no DNA is lost and the origins of replication are preserved and serve as new sites for the addition of more viral genomes. The junction at Figure 54g and 54n indicates that a master DNA replicon evolved with multiple origins of replication. The junction between 54g and 54h indicates a continuance in the build-up of the genome. At 54l the process of transposition is occurring and is competing with the normal genome build up cycle for access to origins of replication. However, transposition results in the destruction of the two paired origins. If transposition outpaces the genome integration process, eventually the origins are deleted out until only a single origin remains. Such a process could give rise to pre-slave replicons (54m) which may integrate with the master replicon (54n) via ARR covalent attachments to form the progenotic chromosome or replicon cluster.

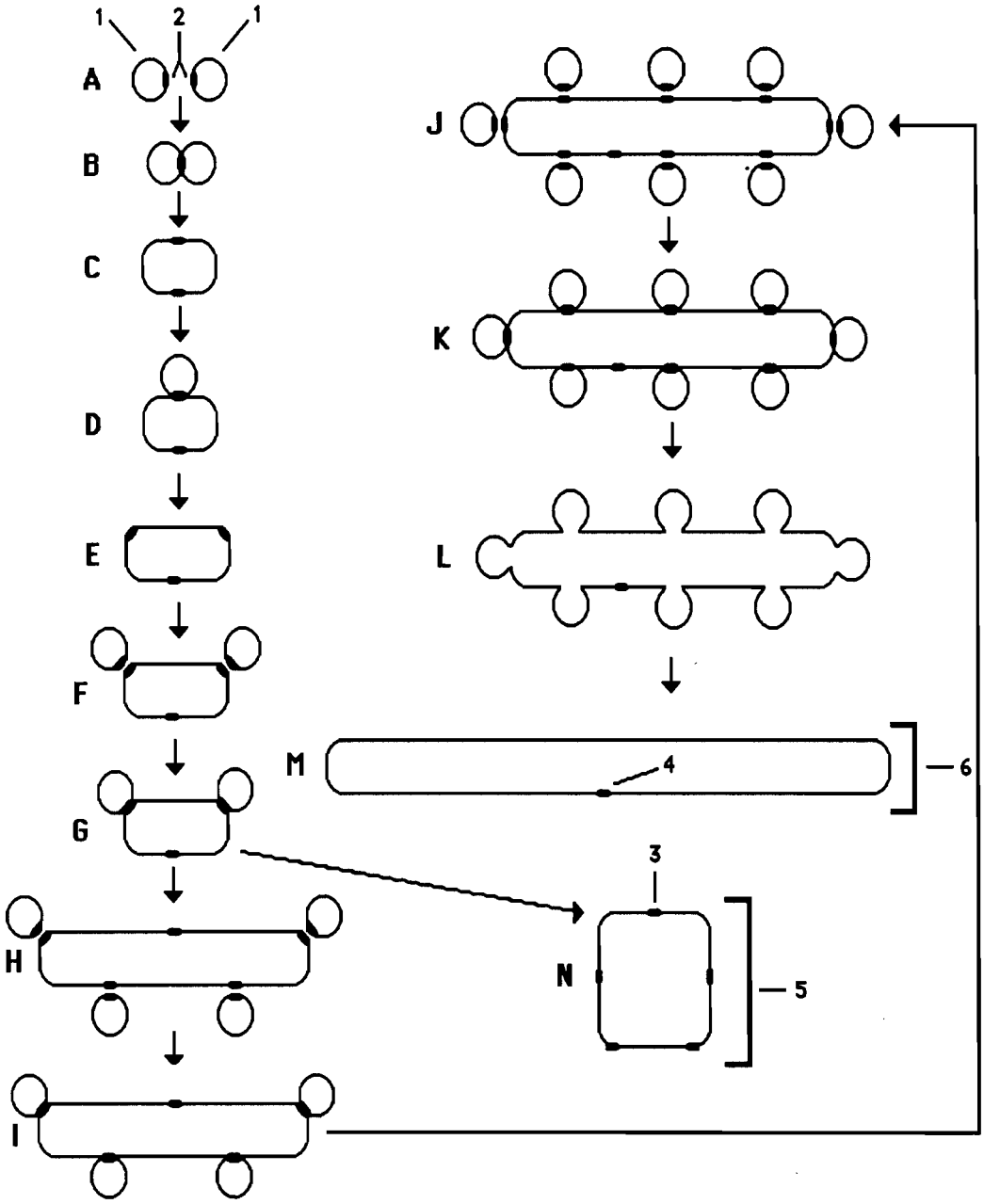


Figure 54

The Evolution of the Prokaryotic Chromosome

The evolution of the single origin prokaryotic chromosome is typified in the "Theoretical Assumptions". The chromosome is hypothesized to consist of an inner replicon tetramerically base-paired to fusion replicons at sites flanking the areas originally occupied by their origins of replication and at the master double origin. All of the inner replicon slave single origins are non-functional with respect to the initiation of secondary rounds of DNA synthesis because of the lack of mesosomal attachment sites (Figures 50, and 56).

Sophisicated forms of bacteria such as Myxobacteria are capable of cellular differentiation in which one set of cells develops into a fruiting body and another set develops into spores (Frobisher, 1974). Some of the slave origins may exist as double origins and could be involved in gene rearrangements during terminal differentiation in those cells which generate fruiting bodies and, therefore, are not associated with the maintenance of the germ line. It is possible that such double origins may also have mesosomal attachment sites.

The Evolution of the Mesokaryotic Chromosome

The evolution of the mesokaryotic chromosome has been discussed in the "Theoretical Assumptions". Basically the assumption is that mesokaryotic chromosome evolved from prokaryotic chromosomes containing single origins of replication. The replicon clusters have not undergone fusions into subchromatids (Figures 57, 58, and 59). Evidence of similiarities of mesokaryotic chromosomes to both prokaryotes and eukaryotes have been reviewed (Swanson, 1981; Spector, 1984).

The Evolution of the Eukaryotic Chromosome

The Evolution of Introns and Exons

It has been speculated that prokaryotes evolved from early eukaryotes by the progressive loss of introns (Senapathy, 1986). It is my contention that introns

evolved after the divergence of prokaryotes and pre-eukaryotes by a process of "reverse" transduction" in which viruses containing a piece of eukaryotic DNA combine at their origins of replication to homologous origins of replication within the eukaryotic genome, becoming established as mosiac slave loops containing a piece of pre-eukaryotic DNA. The exon is the unique sequence and the intron is the viral sequence which is composed of at least one origin of replication (highly repetitive DNA). The remainder of the DNA would be viral (moderately repetitive DNA) and would represent the majority of the DNA. Polydisperse circular DNA's containing highly repetitive, moderately repetitive, and unique DNA sequences have been discovered in mouse 3T6 cells and they are enriched in moderately repetitive sequences (Sunnerhagen et al, 1986). The process by which these repetitive DNA sequences may have become established throughout the genome was illustrated in Figure 54 and their hypothetical role in gene rearrangements during cellular differentiation has been illustrated in Figure 47.

The Evolution of Subchromatids and Chromatids

Figure 55 illustrates how replicon clusters may have integrated together in a manner reminiscent of the development of the pre-eukaryote. Such a process would lead to the formation of a super replicon cluster or the subchromatid. The replicon clusters within the subchromatid are analagous to the slave loops of a simple replicon cluster and the giant subchromatid circle (double chromonemata) is analagous to the master replicon. Again there is an interplay between the forces of transposition which delete origins of replication and homologus integration which preserves the origins of replication. Integration and/or fusion of subchromatids to form chromatids occurs in a similar manner. However, when subchromatids form fluid loops instead of recombining or fusing, the junction between them becomes a translocation "hot spot".

Nuclear Pores and the Initiation of DNA Replication

Two basic models relating to the protein composition of nuclear pores have been the subject of review (Aaronson et al, 1982). Nuclear pores appear to be composed of interconnecting rings of chromatin (Schel and Wanka, 1973). Furthermore, these chromatin-like rings are very resistant to extensive treatments with trypsin and to mechanical shear and it has been suggested they are composed of circular rings of

Figure 55

The Evolution of the Subchromatid From Replicon Clusters

Figure 55a depicts two replicons clusters (1) and origins of replication (2) located on the periphery of pre-slave replicons. The pre-slave replicons are distinguished from slave replicons because they have more than one origin of replication. The two replicon clusters in (a) pair up together at the peripheral origins of replication (b) and fuse by homologous integration, regenerating the two origins of replication in the partially fused integrant (c). The process is repeated again with a third replicon cluster (d) which fuses by homologous integration to generate the large circular subchromatid (3) seen in (e).

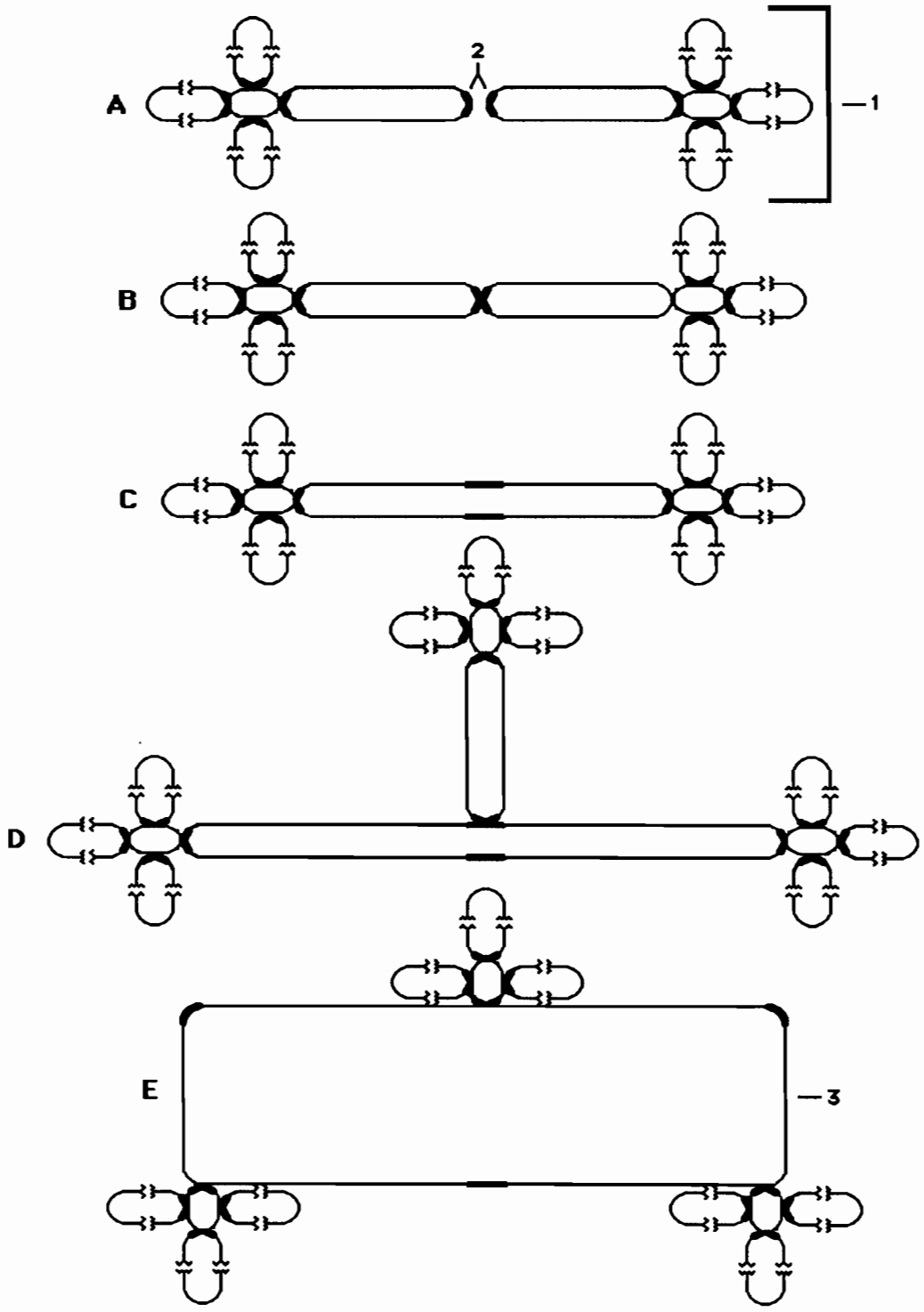


Figure 55

DNA (Troncale et al, 1972). Additionally, nuclear pores are absent from those mesokaryotic nuclei which contain telocentric prokaryotic-like chromosomes which are attached at one end to the inner nuclear envelope (Swanson, 1981; Spector,1984). I wish to elaborate further on these observations and the DNA hypothesis by suggesting that these nuclear pore complexes may be composed of circles of DNA (replicon clusters) which are networked together by chromonema in a covalent fashion. The daisy-like arrays of denatured "DNA" observed in Plates VI and VII may be examples of this kind of superstructure. The absence of nuclear pores in mesokaryotic nuclei containing prokaryotic-like chromosomes may be an indication of the absence of multiple DNA attachment sites (Figure 56). In Figure 56a a eukaryotic nuclear envelope model is depicted containing an array of nuclear pore complexes (replicon clusters) attached to each other by a single chromonema as shown in Figures 52, 53, and 55. Such an arrangement suggests the following: 1) the initiation of DNA replication occurs within the nuclear envelope and not the nucleus proper, 2) most or all of the DNA attachment sites are within the nuclear envelope in the form of replicon clusters, and 3) eukaryotic DNA replication is similar to prokaryotic replication in that both systems require membrane attachment sites. The distinction between the nuclear envelope and the nuclear "proper" may be a "gray" area in that the nuclear envelope may convolute into the inner recesses of the nucleus in a manner analagous to convolutions of cell membrane into endoplasmic reticulum. The concept of nuclear compartmentalization has been suggested; (Bouteille et al, 1983) together with evidence (Bourgeois et al, 1979). Evidence for chromosomal compartmentalization within the nucleus has also been demonstrated (Saumweber, 1987). This compartmentalization may be indicative of the evolution of the nuclear envelope from individual pre-eukaryotic cell membranes as suggested in Figure 58. If topoisomerase II sites are associated with DNA domain attachment sites at origins of replication (Earnshaw et al, 1985; Earnshaw and Heck, 1985; Cockerill and Garrad, 1986), then, the scattering of topoisomerase II sites throughout the nucleus (Berrios et al, 1985) may be indicative of this compartmentalization phenomenon.

The mode of replication of the replicon clusters shown in Figure 56 is assumed to occur by the "reeling in" of the DNA through permanent attachment sites within the nuclear matrix (Wanka et al, 1982). Evidence for such a model is available (McCready et al, 1980; Vogelstein et al, 1980). It is conceivable that complex

Figure 56

A Comparison of Replicon Cluster Membrane Attachment Sites Between Prokaryotes, Mesokaryotes, and Eukaryotes

Figure 56a depicts the nuclear and cytoplasmic sides (1 and 2 respectively) of the spherical or convoluted plane of the eukaryotic nuclear envelope (3). Three replicon clusters (analagous to nuclear pore complexes) (4) are bound within the nuclear envelope such that the slave replicons (5) protrude up into the core of the nucleus while the master replicons (6) are embedded parallel to the plane of the nuclear envelope and is the annulus of a nuclear pore. The slave replicon are 65 kb in size and typify DNA domains. The master replicons are about 0.38 kb in size and are almost completely composed of origins of replication. The master replicons are connected in tandem by a chromonema (7) which attaches to the nuclear envelope at nuclear pores. The origins of replication (not shown) for both master and slave replicons lie in the plane of the nuclear envelope and are permanently attached there during periods of non-replication. The satellite replicon (8) indicates the possibility that all replicons may not be attached directly to the nuclear matrix. Although the origin of such a replicon may be incapable of initiating replication it would replicate as oridinary DNA once it was "reeled" into the nuclear matrix as described in the text. Figure 56b depicts either the nuclear (1) and cytoplasmic (2) sides of a mesokaryotic nucleus (3) or the inner (1) and outer (2) side of the cellular membrane (3) of a prokaryote. The inner replicon and the outer fusion replicon are illustrated by 4 and 5 respectively. The master and slave origins are illustrated by 6 and 7 respectively. Note that the only connection to the nuclear envelope or cellular membrane is at the master origin of replication (6). It is hypothesized that the lack of connections between the inner replicon single slave origins of prokaryotes and mesokaryotes (7) and membranes attachment sites is responsible for the inability of these origins to initiate secondary rounds of DNA synthesis. For additional information on replication see Figures 50 and 53.

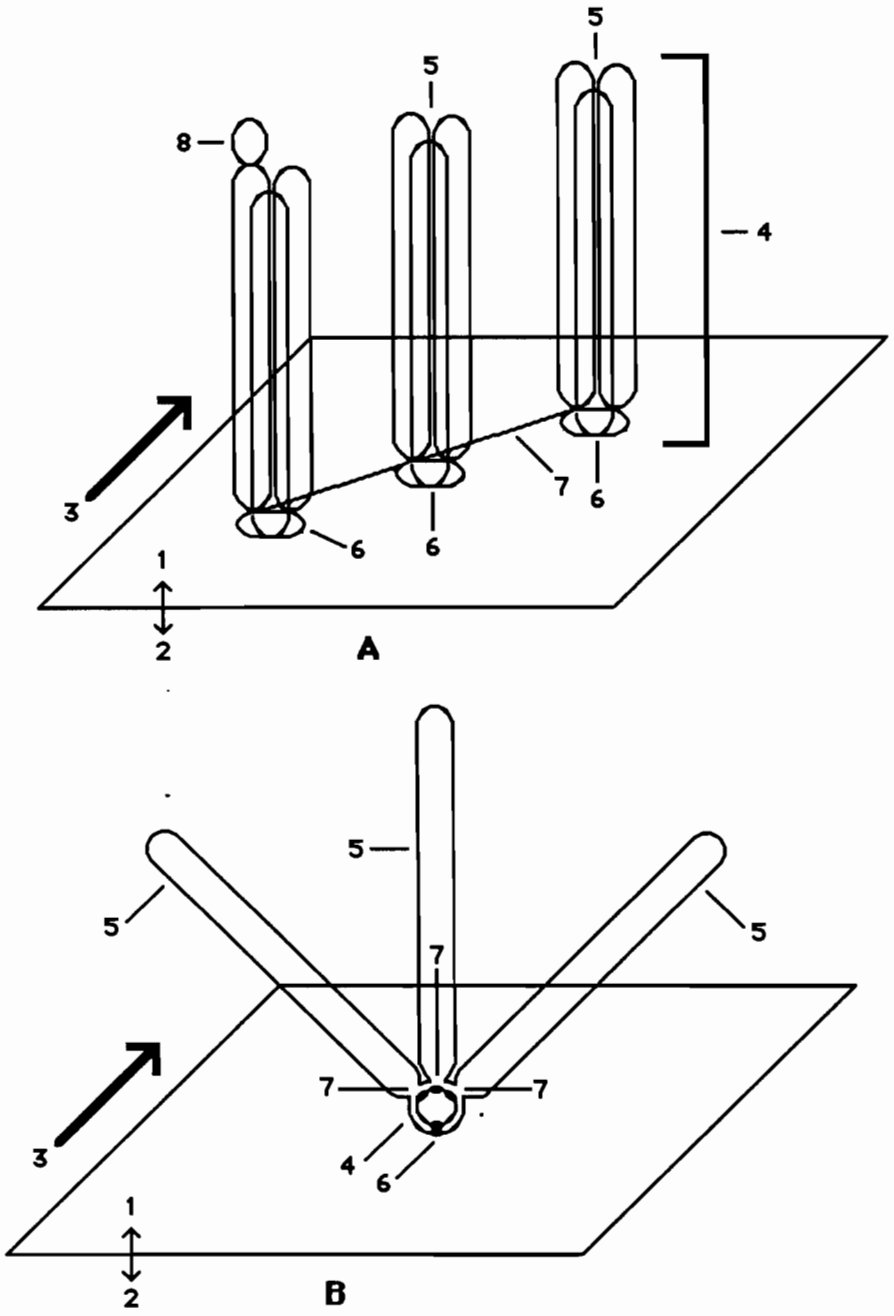


Figure 56

replicon clusters may consist of replicons which are not permanently attached to the nuclear matrix (Figure 56a, 8). If so, such replicons could be reeled down to the nuclear matrix and their origins of replication activated at that point. In Figure 56b the replicon cluster is attached to the membrane at only one site, the master origin of replication. It is hypothesized that the lack of additional membrane attachment sites in this kind of replicon cluster is responsible for preventing the initiation of DNA synthesis at slave origins of replication. The association of heterochromatin with nuclear pores and with centromeres suggests to me that many of these nuclear pore complexes are associated with centromeres and perhaps with telomeres and secondary constrictions within the spherical or convoluted plane of the nuclear envelope. The interconnecting rings of "DNA" discussed and illustrated in Chapter VIII could be remnants of centromeric DNA (Figure 52). These associations may be quite important during synaptic events in meiosis by restricting the space homologs must pass through in order to "find" each other to within the confines of a spherical or convoluted plane.

Heterochromatin and Cellular Differentiation

Heterochromatin is broadly classified as being either constitutive or facultative. The former is considered to be composed of structural elements as found in centromeres and is never transcribed. The latter is considered to be euchromatin which has been converted into non-transcribed heterochromatin as a result of cellular differentiation. Whether facultative heterochromatin can be converted back into fully functional euchromatin is a matter of debate. It is conceivable that dedifferentiation never occurs within individual cells and instead a population of pro-stem cells exists that is stimulated to divide and differentiate into different kinds of stem cells during such phemonema as limb bud regeneration in amphibians.

It is possible that some or all of the acidic proteins associated with facultative heterochromatin may have been derived from viral capsids. In an earlier section in Appendix D I discussed the possibility that eukaryotic replicon clusters may be composed of mosiac loops of transducing viruses and progenotic DNA. If such were the case, then, it is conceivable that many of the viral genes (introns) are still intact and functioning. The recent flood of evidence in the field of recombinant DNA technology suggests that eukaryotic introns are not simply "dead" pieces of DNA but appear to be

quite active in gene regulation. One possible function of the viral genes could be in the production of capsomeres and viral capsids. These capsids could encapsulate the entire replicon and interrupt the transcription of the progenotic exon, converting the entire mosiac loop into facultative heterochromatin; if so, then, some or all of the acidic proteins associated with facultative heterochromatin could actually be viral capsids.

The Role of Cellular Symbiosis in the Evolution of Mesokaryotes and Eukaryotes

Introduction

It is assumed here that the principle driving force in the evolution of mesokaryotes and eukaryotes is cellular symbiosis. The importance of intercellular symbiosis in the evolution of multicellular organisms is quite obvious. However, the importance of intracellular symbiosis in the development of both unicellular and multicellular organisms is not as obvious. Intracellular symbiosis can be divided into two categories: 1) cytoplasmic intracellular symbiosis, and 2) nuclear intracellular symbiosis. In recent years the importance of cutoplasmic intracellular symbiosis in the evolution of chloroplasts and mitochondria from prokaryotes has become more accepted (Dyer and Obar, 1985); especially in light of the fact that intracellular symbiosis between infecting bacteria and amoebae has actually been observed under laboratory conditions (Lorch and Jeon, 1985). The concept of intracellular symbiosis has been elaborated to incorporate basal bodies as well. It has been hypothesed that basal bodies evolved from spirochaetes (Margulis, 1970, 1981, Sagan 1985). The concept that nuclear DNA evolved by a series of DNA amplifications of prokaryotic DNA attached to the inner membrane of the nuclear envelope has been suggested (Margulis, 1970). A build up of the eukaryotic genome from prokaryotic DNA (Goksoyr, 1985) or plasmids (Sonea, 1985) has also been proposed.

I have taken these concepts along with other pieces of information and integrated them into a still larger picture. The diagrams in Figure 57, 58, and 59 illustrate the step-wise evolution of mitotic cells (57), a mesokaryotic nucleus containing small, telocentric chromosomes (58), and a modern eukaryotic nucleus containing a large monocentric chromosome (59).

Cellular Symbiosis

The beginnings of endosymbiosis leading up to the evolution of eukaryotes could have originated with the development of cytoplasmic relationships between the following kinds of prokaryotes (see Figure 57): piliated bacteria (1), spirochaetes (2), and mycoplasma-like phagocytes (5). The piliated bacteria is envisioned here as having developed a conjugation tube from a sex pilus which connected it to a spirochaete. As suggested by Margulis and Sagan such a relationship may be mutually beneficial in that the piliated bacteria serves as a food processing plant for the spirochaete which provides the means of locomotion for procuring this food. The connection between the two symbionts may be further enhanced by chromosomal fusions made possible by the conjugation tube, thereby generating a hybrid chromosome and a new organism (3). This primary symbiotic event could be further enhanced by the incorporation of this new hybrid organism onto the cell surface of the phagocytic prokaryote (5) resulting in the formation of a complex symbiotic hybrid (6). The original phagocyte (5) would more efficient at gathering food than the hybrid (3) but would be rendered still more efficient by the addition of the hybrid and a more efficient means of locomotion. Evidence for the development of such a relationship is exemplified by the termite protozoan Mxyotricha paradoxa (Margulis, 1970).

This scenario can be taken one step further by assuming that the development of a cytopharnyx and axopodia for trapping prey further increased the efficiency of the symbiotic hybrid in collecting food resulting in the still more complex symbiotic hybrid (8). Again this relationship would be enhanced by the fusion of the original individual chromosomes together to form a "hybrid" organelle (7). During cell division the contractile organelle (7) and accompanying axopodia would divide in a manner reminiscent of a mitotic spindle apparatus without chromosomes (Figures 57g-j). The formation of a predatory hybrid of this nature paves the way for still another scenario in which some of the bacterial prey being engulfed by the symbiotic hybrid are not digested but instead become an essential part of the genetic machinery of still a new hybrid (13) because of their ability to co-replicate and divide together with the contractile organelle and the main body of the original phagocyte (Figures 57k-n). This is accomplished by the connections of axopodia derived from sex pili to a specific membrane location within the endosymbiotes where the origin of replication is attached. Therefore, the axopodia could be serving a dual function in that genetically

Figure 57

The Evolution of Mitosis by Intracellular Symbiosis

Figures 57a illustrates the piliated bacteria (1) and the spirochaetes (2) which will undergo an semi-intracellular symbiotic relationship in which their chromosomes become joined through a sex pilus forming the motile cellular hybrid (3) illustrated in 57b. This hybrid then joins together with the phagocyte (5) to form the complex intercellular hybrid (6) depicted in 57c. Evidence for the feasibility of such an arrangement is suggested by the situation observed in the protozoan Myxotricha paradoxa (Margulis, 1970). The prokaryotic chromosome (4) of the original phagocyte is shown in all relevant figures. In 57d a set of 9 spirochaetes have joined chromosomes through sex pili with one piliated bacterium to form a subcellular hybrid (7) which "upgrades" the intercellular phagocytic hybrid (6) into the more complex symbiotic hybrid (8). The subcellular hybrid (7) is a contractile apparatus which is the forerunner to the centriole. It is anchored within the cell by a "rope" of microtubules (9) and is used to trap bacterial or pre-eukaryotic prey (10) with axopodia (11). In 57e the prey is drawn into the cytopharnyx (not shown) by the rotation and contraction of the microtubules associated with the contractile apparatus. Figure 57f is a cross section of (8) and the contractile apparatus showing the relationship between the original piliated bacterium and the 9 spirochaetes. Figures 57g-j depict the phagocytic hybrid (8) in the process of cellular division. In 57h the axopodia (12) have formed and the contractile apparatus has replicated and divided. In 57i the contractile apparatus is being partitioned into daughter cells. The axopodia (11) differ from (12) in that the former are used in attaching to prey while the latter are used in cytokinesis. In 57j the daughter cells are formed and the daughter axopodia have separated. In 57k the phagocytic hybrid (8) has engulfed and retained either bacterial or pre-eukaryotic endosymbionts (10) upgrading the phagocytic hybrid (8) into the complex hybrid (13). The cellular descendents of the bacterial and pre-eukaryotic endosymbionts will be mesokaryotes and eukaryotes respectively. In 57l the axopodia (11) and (12) have formed and the endosymbionts are lined up on the equatorial pole by the daughter axopodia (11) in preparation for cell division. The separated daughter endosymbionts are shown in 57m and n. The development of true nuclei from these ancestral endosymbionts is depicted in Figure 58.

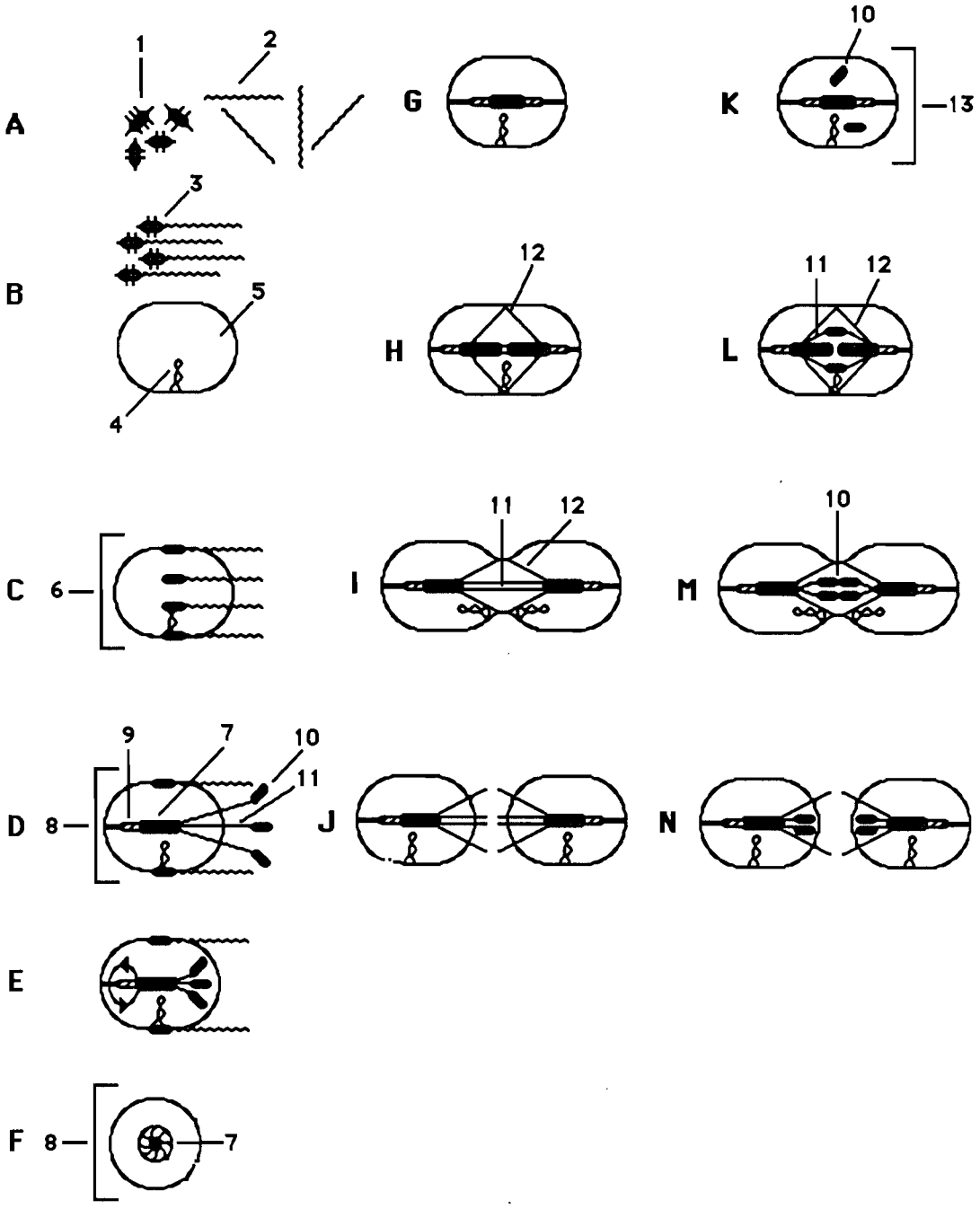


Figure 57

desirable prey are retained as endosymbionts whereas other cells are merely phagocytised. Such a connection could be the forerunner to the evolution of telocentric chromosomes.

The cytopharnyx would be the forerunner to centrioles and basal bodies and the axopodia would be forerunners to the mitotic spindle apparatus. The ability of the phagocyte to engulf endosymbionts without digesting them coupled by the synchronous replication and separation of the endosymbionts together with the phagocyte would represent a quantum leap in evolution. This point in time would mark the beginning of the development of mesokaryotes and eukaryotes (see Figure 58). It would also mark the demise of the progenote as a cell containing a single replicon cluster. As prokaryotic, mesokaryotic, and eukaryotic cells rapidly evolved to fill available ecolgical niches the progenotic cell would be unable to compete effectively with any group, thereby becoming a "missing" link" which not only became extinct but was unable to flourish to a point where it could be recovered from the fossil record.

The Centriole Paradox

One of the biggest long standing mysteries in cellular biology today is the presence of centrioles in animals cells and their absence in plant cells. If centrioles are as important in eukaryotic cellular division as they seem to be, why are they missing from plant cells? My conjecture on this point is that the DNA of the original endosymbiotic hybrid from which the centriole evolved has been incorporated into the nuclear genome; at least in plants. From this vantage point the centriolar DNA is transcribed and the proteins necessary for the mitotic event are generated. In this regard the presence of centrioles in animal cells would represent the primitive condition from which the plant cells evolved. The original eukaryotic cell was probably an autotroph (similar to a euglena) which contained centrioles and from which plant and animal cells evolved.

The Formation of the Nucleus

Once the endosymbionts became established as part of the intracellular genetic machinery they began to lose their cell walls or other protective coatings, exposing their cell membranes to the vacuolar membrane surrounding them (Figure 58). The outer vacuolar membrane and the inner endosymbiont cell membrane became the

Figure 58

Mesokaryotic Nuclear Development

Figures 58a-f illustrate the degeneration of the endosymbionts into membrane-covered chromosomes which have congregated into a vesiculated pseudonucleus (1A). In 58a are illustrated the telocentric chromosomes (2A) attached at their telocentric ends to individual nuclear vesicles (3) in which they are compartmentalized, the individual vesicular envelope (4), and the centrioles (5). The outer membrane of the vesicular envelope is derived from the cell vacuole surrounding each vesicle and the inner membrane constitutes the original endosymbiotic cell membrane. In 58b the centrioles have divided and are migrating towards opposite poles. In 58c each chromosome set is surrounded by a membrane pellicle (6) which is derived from the vesicular envelope. In 58c-e the pellicle and daughter chromosomes are surrounded by non-chromosomal spindle fibers (7) and in 58d and e are being pulled apart by the chromosomal spindle fibers (8). The daughter cells are illustrated in 58f. Evidence for this form of nuclear division is available in coccid insects during the course of meiosis (Swanson, 1981). Figures 58g-l illustrate a true nucleus (1B) in which the membranes surrounding each chromosome have fused allowing the chromosomes (2B) to make direct contact with each other. During cell division the spindle fibers attach to each telocentric chromosome (58i) and the nucleus is pulled apart as the daughter chromosomes separate (58j-l). This kind of cell division is seen in mesokaryotes (Swanson, 1981; Spector, 1984) and in protists like Barbulonympha (Swanson, 1981).

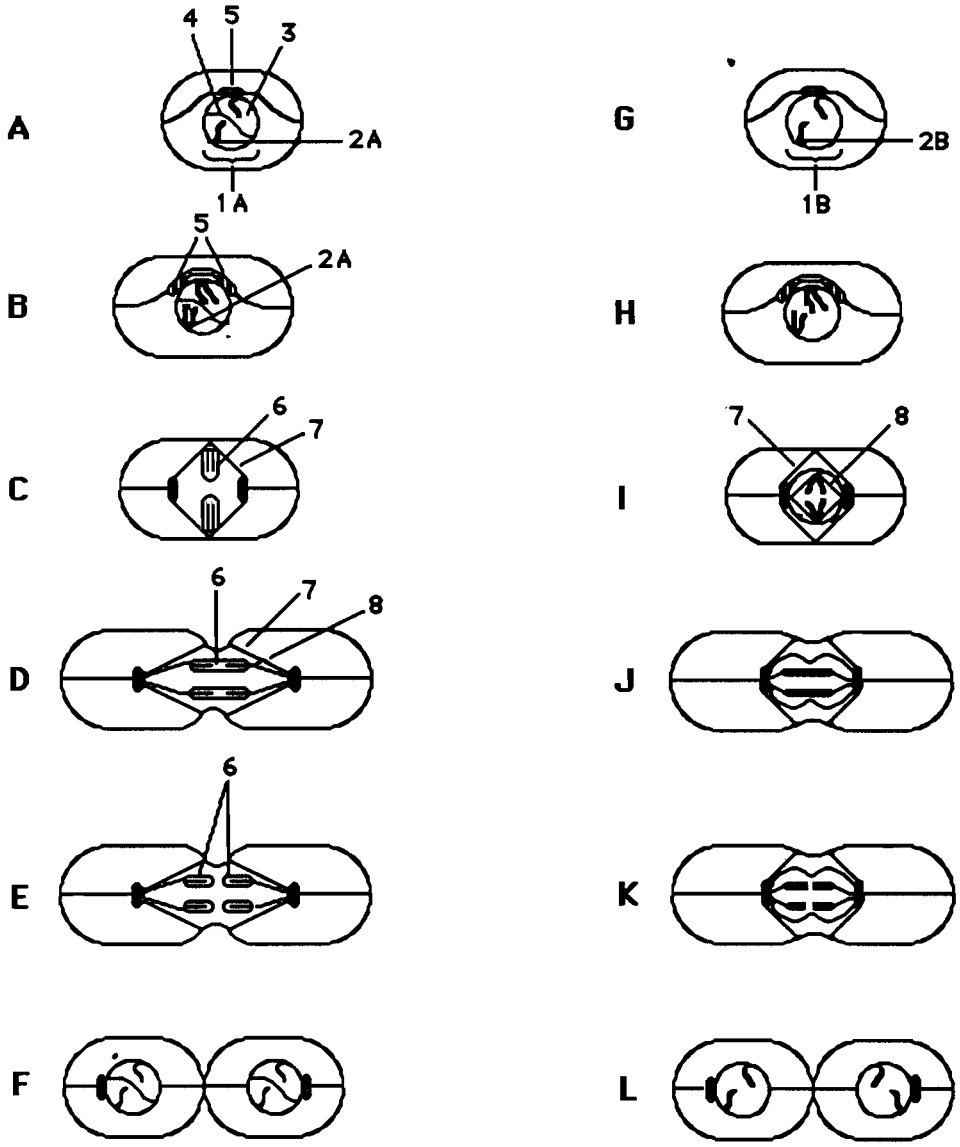


Figure 58

forerunners to the outer and inner membranes of the nuclear envelope. As evolution proceeded, the requirement for endosymbiotic cytoplasm became less and less necessary because only the genetic machinery of the endosymbiont and the membrane components associated with the telomere were required to establish the endosymbiotic

relationship. This is in contrast to mitochondria and chloroplasts which require cytoplasm not only to produce their products but also to insure that "cytokinesis" can occur within the organelle since the mitotic apparatus of the cell has little if any effect on replication and separation of daughter mitochondria or chloroplasts. As evolution continued, the prenuclear endosymbionts became little more than chromosomes surrounded by double membranes. In effect, they were transformed into micronuclei. Eventually these micronuclei became clustered together into a vesiculated nucleus which fused together (Figure 58a). Evidence for chromosomal domains in interphase nuclei was discovered over 100 years ago by Rabl and still more data is being presented today (Saumweber, 1987). Evidence that chromosomes may be encapsulated into individual "nuclear envelopes" is illustrated during meioisis in coccid insects (Swanson, 1981). Vesiculated nuclei can be seen in whitefish embryonic cells shortly after the formation of daughter cells (data not shown).

The Divergence of Mesokaryotes and Eukaryotes

Mesokaryotes appear to be intermediate between prokaryotes and eukaryotes relative to the composition of their nuclei (Swanson, 1981; Spector, 1984). The driving force separating mesokaryotes from eukaryotes and relegating the nuclei of the former, in most cases, to little more than "glorified" prokaryotes may have been the absence of replicon cluster fusions in mesokaryotes together with degenerate prokaryotic-like DNA domains which somehow inhibited the complex gene rearrangements necessary for cellular differentiation in higher eukaryotes. Nuclear fusion probably had little impact on the continued evolution of mesokaryotes and they continued to replicate in a prokaryotic-like fashion (Figures 58g-l). This kind of replication mode is also observed in the protist Barbulonympha (Swanson et al, 1981). The small telocentric chromosomes are always visible and connected by one end to the inner membrane of the nuclear envelope, similar to a prokaryotic mesosomal connection. During division mitotic spindle fibers attach to the telocentric ends of each chromosome and the nucleus (which never disappears) is simply pulled apart.

The Formation of the Eukaryotic Monocentric Chromosome

Once the individual telocentric pre-eukaryotic chromosomes were confined within a single nucleus the process of subchromatid, chromatid, and monocentric chromosomal

formation began (Figure 55). During early chromosomal fusion events each pre-eukaryotic chromosome retained its individual telocentric membrane attachment site to the mitotic spindle resulting in the formation of a diffuse centromere (Figures 59a-e). Evidence for diffuse centromeres in the nematode worm Ascaris and in the genus Sciara in Dipteran insects (Strickberger, 1976, Swanson et al, 1981). The giant chromosome eventually breaks up into smaller chromosomes, (replicon clusters?) most of which are lost during embryonic development. As evolution proceeded, more and more of the individual progenotic chromosomes lost their centromeric regions as they began fusing together to form the chromonema of subchromatids. This resulted in the need to strengthen the superstructure of the remaining centromeres to prevent chromosomal detachment from them during mitosis. This could have been accomplished by an amplification of small, structural DNA replicons resulting in the large amounts of repetitive DNA observed in centromeres today. Eventually the diffuse centromere was reduced to the large complex moncentric forms observed today in most modern eukaryotes (Figures 59f-j). The only remaining attachment sites to the nuclear envelope were centromeres, telomeres, and secondary constrictions (subchromatid junctions?). Of these, only the centromeres contained the proper constituents for attaching to the mitotis spindle.

The development of telocentric eukaryotic chromosomes probably proceeded that of metacentric and submetacentric chromosomes. These latter chromosomes may be fusion chromosomes of two telocentric chromosomes joined together at their centromeres. Subchromatids may be the end products of metacentric or submetacentric chromosomes which fused with other chromosomes and eventually lost their centromeric attachment region. Telomeres may represent the original attachment point of progenotic telocentric chromosomes. Their inability to fuse with other chromosomal elements may be the result of an absence of "free" origins of replication, i.e., areas of "sticky" DNA which allow tetrameric base pairing to occur between two origins of replication.

Figure 59

Eukaryotic Nuclear Development

Figures 59a-e illustrate cell division during embryogenesis in organisms like the nematode worm Ascaris and in Dipteran insects like Sciara (Swanson, 1981). The giant fusion chromosome (1A) shown in 59a was derived from three telocentric chromosomes which are still attached to the inner membrane of the nuclear envelope (2) by their original telocentric ends (3a, b, and c). The centriole is shown by (4). In 59b the centriole has divided and is moving to opposite poles. In 59c during cell division non-chromosomal fibers (5) attach to the cell membrane at the site of cytokinesis. Each telocentric chromosomal subunit of the giant fusion chromosome is attached to a spindle fiber (6a, b, and c) and in 59d the giant chromosome is pulled apart in a lateral movement. Note that the nuclear envelope has disintegrated. The attachment of spindle fibers along the entire length of the fusion chromosome is called a diffuse or holocentric centromere. Figures 59f-j illustrate cell division in a modern eukaryote. In 59f and g the three telocentric chromosomes forming the fusion chromosome (1B) are still connected by their original telocentric ends (3a, b, and c) to the inner membrane of the nuclear envelope. At metaphase (59h) the spindle fibers (5) attach to the cell membrane at the site of cytokinesis but only one set of chromosomal fibers (6b) attaches to the chromosome (1B). The point of attachment is at the original location of the middle telocentric chromosome described in 59c resulting in a metacentric chromosome. When the daughter chromosomes separate, the daughter chromatids are pulled behind the centromeres instead of moving parallel with them (59i).

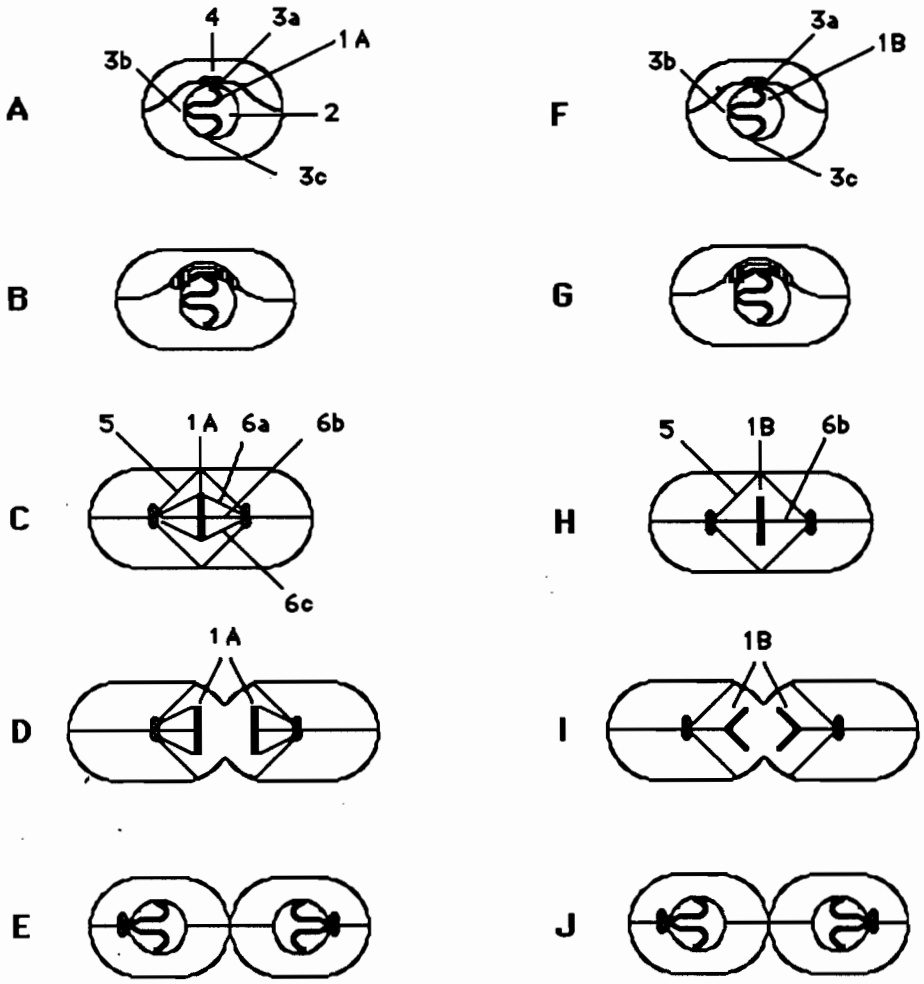


Figure 59

Summary

The theme of the theoretical discussions in Appendix D has been simply that during the course of prokaryotic, mesokaryotic, and eukaryotic cellular evolution, a series of genetic and cytoplasmic vestiges have been retained in living organisms which may be serving as evolutionary "sign posts". Whether these sign posts are being accurately

interpreted or not is simply a matter of debate. More subtle sign posts have no doubt been discovered since the original inception of these theories but the same caveats apply to them as well.

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