# Alkali-Resistant "RNA" Oligomers: I. Association with High Molecular Weight DNA in Mouse L-1210 Cells

Frank W. Abernathy* and Ralph E. Stephens

Institution: Department of Pathology, Room M 368, Starling-Loving Hall, Ohio State University Medical School, Columbus, Ohio 43210

Correspondence: Frank W. Abernathy, Dept. of Pathology, Room M 368, Starling-Loving Hall, Ohio State University Medical School, Columbus, Ohio 43210, (614) 293-8899

Condensed Title: Alkali-Resistant "RNA" Oligomers

## Abstract

DNA-associated "RNA" (DAR)[1] has been isolated from mouse L-1210 cells containing a sub-component of alkali-resistant "RNA" (ARR). The DAR is resistant to extraction by sodium dodecyl sulfate and electroelution, suggesting that it may be covalently bonded to the DNA as are Okazaki fragment RNA primers. However, unlike Okazaki RNA primers, the DAR demonstrates a resistance to treatment by RNAse A. It is postulated that the resistance of DAR to RNAse A is due to its ARR subcomponent which may be protected from alkali and RNAse A hydrolyses by a blocking group on the 2' position of the ribose moiety. A similar "ARR" phenomenon has been discovered within the origins of replication of the kinetoplast DNA of Trypansomes (Birkenmeyer et al, 1987). Origins of replication are putative sites for the permanent attachment of DNA domains (replicons?) within the nuclear matrix in eukaryotes (McCready et al, 1980; Vogelstein et al, 1980; Wanka,1982; Aelen et al, 1983; Smith,1984; Bekers et al, 1986; Carri et al, 1986; Dijkwel et al, 1986; Jackson and Cook,1986; Razin et al, 1986). Because of the intimate association of DAR with high molecular weight DNA (like Okazaki RNA primers), its unusual stability to alkali and RNAse A (unlike Okazaki RNA primers), and the possibility that it may reside within origins of replication; it is postulated that DAR may function in the establishment of DNA domain attachment sites within the nuclear matrix.

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[1]The abbreviations used are: DAR, DNA-associated "RNA", ARR alkali-resistant "RNA".

In both prokaryotes and eukaryotes, DNA is held together in the form of 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; Adolph et al, 1977; Cook and Brazell, 1978; Cook, 1984).

Proteins have been demonstrated to establish supercoiling in DNA domains (Cook, 1984). 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 the DNA domain anchorage sites are maintained by protein just because supercoiling is sensitive to proteases and these sites are near the DNA domain attachment points. As has been shown in prokaryotic studies it is possible to relax the supercoiling in DNA domains without the concommittent destruction of the DNA domain attachment site (Worcel and Burgi, 1972;Pettijohn and Hecht, 1973). There is also evidence that phospholipids may be involved with DNA attachment sites in the nuclear matrix (Maraldi et al, 1984). Therefore, the exact nature of DNA domain attachment sites in eukaryotes still remains a mystery.

Assuming that the establishment of eukaryotic DNA domains takes place in the nuclear matrix region, then, the DNA domain attachment sites maintaining this supercoiling within the nuclear matrix must punctuate the DNA at periodic intervals. Therefore, perturbations in the DNA which occur at periodic intervals most probably occur within the nuclear matrix and may be the result of DNA 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 (Djondjurovet 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; Carri et al, 1986; Dijkwel et al, 1986; Jackson and Cook, 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; Zyskindet al, 1981). 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, and A+T-rich. Discrepancies between calculated values for DNA domains and replicons suggest there may be a 4:1 ratio between replicons and DNA domains numbers (McCready et al, 1980). However, these values may reflect limitations in the ability of the assay procedures to detect very small DNA domains.

One explanation for the observations described above is that RNA is involved in the establishment of both prokaryotic and eukaryotic DNA domain attachment sites. The focus of this investigation was to test the possibility that an RNA species may be involved in the establishment of eukaryotic DNA domain attachment sites within the nuclear matrix. Radiolabeled uridine was considered as an excellent probe for these studies since any RNA associated with origins of replication may be A+U-rich.

The results of this work culminated in the isolation of DAR which was retained along with high molecular weight DNA following extraction in SDS, and electroelution in gel inserts. Its association with DNA was unaffected by RNase A treatment followed by electroelution. It was extracted after an extensive DNAse I digestion, ethanol precipitated, and then subjected to alkali hydrolysis. Ethanol precipitation was performed again leading to the discovery of a subcomponent of the DAR which was resistant to alkali hydrolysis (ARR). The ARR was probed with alkaline phosphatase to determine if the alkali resistance was due to phosphate end groups at the 2' portion of the ribose moiety. This was found not to be the case. Speculations into the structure and function of the DAR and its ARR subcomponent are discussed.

## EXPERIMENTAL PROCEDURES

### General Cell Maintenance

Mouse L-1210 cells were maintained in filter sterilized Eagles MEM medium (Earles) with 2X nonessential amino acids, 1.5 X essential amino acids, and 1.5 X vitamins (GIBCO). The medium was supplemented with 10% fetal bovine serum (K. C. Biologicals) and referred to as B-10 medium. The cells in suspension were kept in log phase at $37^0$C under a humidified atmosphere of 5% $CO_2$. Cells were counted using a hemocytometer, and cell viability was determined by trypan blue exclusion.

### Serum Arrest Studies

Cells were aliquoted, centrifuged, and the pellet resuspended in fresh B-10 at a concentration of 3 X $10^7$ cells/ml. To each 100 mm petri plate was added 4.4 mls of B-10 containing 50 U/ml of penicillin and 50 ⫤ g/ml of streptomycin (Sigma) with or without 50 ⫤ g/ml of deoxyadenosine, deoxyguanosine, deoxycytosine, and deoxythymidine and 100 ⫤ l of the concentrated cell suspension. Each plate was incubated for about 24 hours as described above to allow the cells time to recover from the subculturing procedure prior to radiolabeling and also to allow them to be preincubated in any added deoxynucleosides which would inhibit the incorporation of uridine label into DNA as labeled thymidine via the ribonucleotide reductase pathway. At the end of this period, 0.5 mls of B-10 containing 0.5⫤ Ci of [methyl-$^{14}$C]thymidine (0.25 mCi/1.3 mg, New England Nuclear), [2-$^{14}$C] uridine(55.9 mCi/mM, New England Nuclear), or L-[1-$^{14}$C]leucine (55 mCi/mM, New England Nuclear) was added to the plates depending upon the experiment being performed. For double labeling experiments 5 ⫤ Ci of [methyl-$^3$H]thymidine (6.7 ci/mmol, New England Nuclear)was added along with 0.5 ⫤ Ci 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 the cells were counted, harvested, pelleted, and resuspended in non-radiolabeled medium containing only 0.5%

fetal calf serum (B-0.5) together with antibiotics. In those experiments in which ribonucleotide reductase was being inhibited the B-0.5 medium contained 50 ⫤ g/ml of each of the 4 deoxynucleosides. The cells at a concentration of 2 X 105/ml were incubated in an upright position for 24 hours to allow for serum arrest. At the end of this time the cells were counted and pelleted at $4^0$C for nuclei purification or for use directly in gel inserts.

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 ⫤ Ci /ml, incubated for 30 minutes($37^0$C) and then precipitated with cold TCA. The first sample (zero 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 ($4^0$C) and collected on Whatman GF/B glass filters with cold 10% TCA followed by a rinse in cold 95% ethanol. The filters were placed into glass scintillation vials along with 10 mls of Aquasol (New England Nuclear) and counted as triplicate samples.

Production, Extraction, and Electroelution of Gel Inserts

Cells or purified nuclei (modified from Basler et al, 1981) were added to low melting temperature sea plaque agarose (FMC Corp.) to produce gel inserts ranging from 50 to 250⫤ l in volume. The maximum concentration of cells or nuclei used in these gel inserts was 3 X$10^6$ cells or nuclei per 50 ⫤ l volume. Concentrations as low as 2 X $10^5$ cells per gel insert were used in some early experiments but were increased for isolation and characterization of ARR. Early gel inserts were formed in plugs made in an agarose bed but later gel inserts were made in agarose-embedded pipette sections. The latter arrangement greatly enhanced reproducibility.

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 interms of four 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) enzyme incubation buffers, (10mM Tris, 5mM EDTA, pH 7.8 {for proteinase K,}) or (10mM Tris, 5 or 10 mM $MgCl_2$, pH 7.4 {for DNAse I, RNAse A, and Phospholipase C}), and 4) ethanol and TCA precipitation buffers, (10 mM Tris, 10 mM EDTA or {5 or10 mM $MgCl_2$},0.1N NaCl, pH 7.4) for ethanol precipitation and (10 mM Tris, 1mM EDTA, 0.1N NaCl, pH 8.0) for trichloracetic acid precipitation. In some cases 250 ⫤ g/ml of carrier DNA was added to the precipitation buffers. Some of the buffer formulae were derived from other sources (Maniatis, et al, 1982). 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. Ethanol and TCA precipitation buffers were used to precipitate DNA and associated molecules (RNA and protein) extracted from gel inserts following gel insert extraction, electroelution, and enzymatic digestions.

An overnight incubation of gel inserts containing cells in extraction buffer resulted in the removal of as much as 37% of the DNA, 68% of the RNA,and 83% of the protein (data not shown). This overnight treatment was followed by 3 or more hours of electroelution in Tris buffer (5mM $MgCl_2$) to remove SDS, EDTA, residual protein, RNA, and other soluble contaminants. Losses of total cell

label following electroelution were 47% for DNA, 99.6% for RNA, and 99.8% for protein. Compared to electroelution in Tris buffer containing 10mM EDTA, electroelution in Tris-magnesium buffer not only retarded DNA loss but also provided an enzyme compatible buffer system for future treatments with EDTA-sensitive enzymes (DNase I). Therefore, SDS extraction in Tris-EDTA followed by electroelution in Tris-magnesium was adopted for subsequent studies.

Gel inserts were first electroeluted in agarose slab gels. In later experiments the gel inserts were electroeluted in horizontal tube gels under buffer and the counts which entered the tube gel were tabulated and added together with the counts remaining in the gel insert. This number was considered as "total" counts and used to normalize the results for comparisons between different gel insert/gel tube sets.

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. Carbon-14 uridine labeled nuclei in gel inserts were 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, St. Louis. 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 ($4^0$C) in a slab gel containing Tris-EDTA. All of the gel inserts were removed from the slab gel and incubated for 8 hours ($37^0$C) in Tris- EDTA (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 ($4^0$C). They were removed and incubated for an additional 8 hours at $37^0$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 ($4^0$C) in a second Tris-magnesium gel. They were removed and incubated for an additional 8 hours ($37^0$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 ($4^0$C). The inserts were removed, stained with 3 ≠ g/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 I.

Nuclear DNA was tested for the presence of RNA oligomers following SDS extraction, electroelution, RNase A digestion, and a second electroelution. Gel inserts containing cells or nuclei were extracted overnight. 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 ($37^0$C). One set of inserts was then incubated in RNase A (20 units/ml). They were electroeluted for 3 more hours at 40 volts and room temperature in fresh Tris-magnesium tube gels then incubated overnight in Tris-magnesium buffer ($37^0$C). All of the gel inserts except one set (which had received no RNase A treatment) were incubated in the presence of DNase I (20 units/ml). Macromolecules in the incubation buffers were either precipitated in ethanol or TCA as

described in the next paragraph. The results of the ethanol precipitation procedure are shown in Table 2.

Precipitation of RNA Oligomers

Precipitation methodologies involving ethanol and TCA were modified from other sources (Maniatis et al, 1982). In those experiments involving TCA or ethanol precipitation of DAR and ARR the gel inserts were incubated for about 24 hours at $37^0$C in 4-5 mls of Tris-magnesium containing 20 units/ml of DNase I. In later experiments 0.1N NaCl was added to the buffer to facilitate precipitation. In one experiment the gel inserts were melted and used to compare TCA 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 TCA precipitated in Tris, NaCl, and EDTA in the presence of 250 ≒ g/ml of carrier DNA. Later it was discovered that DAR ethanol precipitation in Tris, NaCl, and magnesium could be accomplished even in the absence of carrier DNA and the TCA precipitation procedure was discontinued since recovery of enzymatically appropriate substrate from the pellets was necessary for future experiments. The conditions used for TCA and ethanol precipitations were an overnight incubation at $4^0$C and $-50^0$C respectively. Samples were precipitated in approximately 2.5 volumes of ethanol to facilitate RNA precipitation followed by ultracentrifugation ($0^0$C) for 30 minutes at 106,600 X G (Maniatis et al, 1982).

The initial isolation of ARR oligomers involved ethanol precipitation in either Tris, NaCl and magnesium or in Tris, NaCl, and EDTA, and 250 ≒ g/ml DNA carrier. Buffers containing 10mM $MgCl_2$ are recommended for the isolation of small quantities of nucleic acids of small molecular weight (Maniatis et al, 1982).
As can be seen in Table III, higher recovery of DAR oligomers was obtained in the presence of carrier DNA. However, since carrier DNA could interfere with any further analyses, the Tris, NaCl, magnesium method of ethanol precipitation was adopted as the method of choice for most follow up experiments. The standard protocol involved the addition of 2.6 volumes of cold 95% ethanol to samples made up in buffer, overnight precipitation ($-50^0$C), and centrifugation at 106,600 X g for 30 minutes ($0^0$C). 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: 0.1N 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 10mls of aquasol and counted for 5 or 10 minutes. The results in Tables IV and V were from 50 minute counts.

Alkali Hydrolysis Procedures

Alkali hydrolysis was performed according to Bock, 1968. One-half ml of either pre-neutralized KOH with buffer (control) or 0.1N KOH was added to dried buffer-free ethanol precipitated pellets

which were vortexed and incubated (37$^0$C) for 5 minutes. They were vortexed again and boiled for 20 minutes. The tubes were chilled on ice and 0.25 mls of 0.2N HCl was added to neutralize KOH samples while 0.25 mls of 0.1N 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, 0.1N KCl, 40mM $MgCl_2$, pH 8.0, and ethanol precipitated.

Alkaline Phosphatase Incubation Procedures

DNase extracts were ethanol precipitated in Tris, NaCl, magnesium with 250 ≒ g/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 (0.4N Tris, 0.1N KCl, 0.04N $MgCl_2$, 4mM $ZnSO_4$, pH 8.0) followed by the addition of alkaline phosphatase (Bethesda Research Laboratories) added in a 10 ≒ l volume of alkaline phosphatase buffer (0.1N Tris, 0.1N KCl,0.01N $MgCl_2$, 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 that of Reid and Wilson, 1970. The alkaline phosphatase reaction solution was made by diluting 250 U/ ≒ l of alkaline phosphatase in the appropriate volume of alkaline phosphatase buffer to yield 25 U or 50 U of alkaline phosphatase per 10 ≒ l of alkaline phosphatase buffer. Ten ≒ l 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$^0$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 ofthe alkaline phosphatase treated pellets to alkali had been increased above the non enzyme treated controls.

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 most of the studies, exogenous deoxynucleosides were added to both high and low serum media 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 illustrated in Figure 1. Cell densities after 24 hours of exposure to low serum medium dropped to 68% of their high serum counterparts and thymidine uptake in low serum medium with deoxynucleosides was only 17% that observed at zero time. The presence of deoxynucleosides greatly enhanced the desired serum arrest effect. At 48 hours, values for both these parameters were even lower but cell viability had decreased by 20%.

The Effects of Enzymes on DNA Associated Carbon-14 Uridine Radiolabel

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). Purified nuclei were 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 $37^0$C for 8 hours with or without 40 units/ml of enzymes. The protocol used in this experiment is illustrated by the flow chart in Figure 2. The results, shown in Table I, suggested that treatment with proteinase K and RNase A had no discernable effect on the ability of the gel inserts to retain the radiolabel. 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 two and nine 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 somewhat 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 T1 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 activity.

DAR Oligomers

Attempts were made to isolate this uridine labeled material from the high molecular weight DNA for further characterization. Gel inserts containing cells radiolabeled with[$^{14}$C]thymidine, [$^{14}$C]uridine, or [$^{14}$C]leucine were prepared as before, extracted overnight in SDS, electroeluted for 3 hours, removed and subjected to incubations at $37^0$C for 16 hours in RNase A buffer with or without 20 units/ml of the enzyme, electroeluted for three more hours, and incubated at $37^0$C overnight in DNase I buffer with or without 20 units/ml of the enzyme (see Fig. 3 for protocol). The final incubation buffers were subjected to ethanol and/or TCA precipitation. The percent of total counts recoverable by ethanol precipitation are shown in Table II. 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}). 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}). 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 associated with the DNA as existing in an oligomeric form (TCA or ethanol precipitable) which is resistant to RNase A treatment.

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 $37^0$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 0.1N KOH without buffer and boiled for 20 minutes prior to neutralization and ethanol precipitation. The results of this procedure are shown in Table III. 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 to precipitate in Tris, NaCl, magnesium versus Tris, NaCl, EDTA buffers after alkali hydrolysis was found to be reduced to 78% and 56% of their respective untreated 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. If the ARR could be converted into an alkali-sensitive form after exposure to alkaline phosphatase, then, this would mean that the 2' positions of the RNA oligomers are being protected by phosphate groups which upon being removed by alkaline phosphatase treatment would render the RNA susceptible to alkali hydrolysis. The data for these experiments are presented in Table IV and analyzed in Table V. Gel inserts were exposed to DNase I for 22.5 hours at $37^0$C and ethanol precipitated in Tris, NaCl, magnesium in the presence of 250 ≠ g/ml carrier DNA. As can be seen from Table V the alkali-resistant label comprised about 80% of the total (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 $37^0$C for 3 hours followed by only 3 hours of electroelution (see Table IV, column 3 and Table V). 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 which are interconnected with other molecules and inaccessible to alkaline phosphatase treatment.

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 IV) 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 nine million nuclei were made from nuclei which had been stored in liquid nitrogen. The gel inserts were electroeluted for nine 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 nine hours of electroelution was 2.64% for DNA and 1.28% for the ARR form of DAR label (Table VI). 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 electroelution alone, and that the high molecular weight DNA and the ARR are covalently bonded together as are the Okazaki DNA fragments and RNA primers during DNA replication.

DISCUSSION

The results indicate that the ability of DAR to remain associated with high molecular weight DNA is resistant to SDS extraction, RNase A treatment, and electroelution. It is not resistant to DNase I followed by electrolelution. This tight association of the DAR with the DNA suggests that it may be covalently linked in a manner similiar to Okazaki RNA primers. However, its resistance to RNase A suggests that it is either not RNA or that the RNA exists in an unusal form. DAR can be ethanol precipitated after DNase I treatment from the DNase I extracts 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 the 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 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. At least three explanations for these results are possible: 1) the RNA was not "completely" hydrolyzed 2) the presence of 8 ≒ g of DNase I in each sample during boiling resulted in a denatured protein-nucleic acid precipitate which trapped otherwise soluble counts and 3) the "RNA" counts were incorporated into DNA counts by the conversion of the uridine label into thymidine via ribonucleotide reductase pathways. 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 0.1N 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 third possibility was remote for three reasons: 1) Such incorporation would be minor compared to the uptake of exogenous thymidine label by these same cells. Therefore, it would be hard to explain how incorporation of uridine as thymidine into DNA could account for ethanol precipitated pellets containing alkali-resistant [$^{14}$C]uridine label with six to nine fold more counts than similar pellets containing [$^{14}$C]thymidine label unless the specific activity of the incorporated [$^{14}$C]uridine were considerably higher than that for incorporated [$^{14}$C]thymidine. The specific activities of the two isotopes used were 46 ≠ Ci/mmol and 55.9 ≠ Ci/mmol for[$^{14}$C]thymidine and [$^{14}$C]uridine respectively. Even if the [$^{14}$C]uridine was incorporated as efficiently as [$^{14}$C]thymidine into DNA (which is extremely unlikely) the differences in specific activity could account for no more than a 1.2 fold difference in the observed counts.

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). 3) Mouse cell ARR was found to contain 100 times more uracil than whole cell DNA isolated from herring sperm when analyzed by mass spectrometry. In the latter case it could be argued that the high levels of uracil within the ARR sample resulted from the deamination of cytosine during alkali hydrolysis but no depletion of cytosine levels within the ARR sample was noted when it was compared to the whole cell DNA sample (Bergtold, personal communication). We concluded that the majority of these alkali-resistant uridine counts represented a form of RNA and did not reflect any significant incorporation of uridine into DNA.

Another explanation for the recovery of RNA label after alkali hydrolysis is that the RNA is chemically modified. 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. Molecules with properties similar to these ARR species have been discovered at the origins of replication of kinetoplast DNA in Trypansomes (Birkenmeyer et al, 1987).

The DAR appears to be composed of both alkali-sensitive and alkali-resistant components. Is all of this alkali-sensitive RNA merely a contaminant which has not yet been removed by electroelution or is some of it tightly bound to the DNA as is the ARR? In the earliest ARR study involving ethanol precipitation with Tris, NaCl, magnesium buffer the alkali-sensitive portion of the DAR was as much as 60% of the total label (Table III). This number was reduced to about 20% in later studies regardless of an increase in the electroelution time from 3 to 8 hours (Table V). Therefore, 20% is probably a more accurate estimate of the actual alkali-sensitive DAR content. However, DNA samples are capable of similar losses in label after alkali hydrolysis (Table III). Therefore, it is not

clear if the loss of RNA label is due to classical alkali hydrolysis of a susceptible RNA component or due to other causes related to the DNA label losses such as the presence of alkali-labile apurinic sites, etc. which would affect both DNA and RNA. The alkali resistance demonstrated by ARR indicates that at least this portion of the DAR could not represent Okazaki fragment RNA primers in the classical sense. The resistance of DAR to the inhibition of DNA synthesis suggests that it could be RNA primer that remains bound to the DNA at the end of replication in a form that renders some of it insensitive to alkali and RNase A. The DAR could represent a form of alkali-sensitive linker similar to those observed in green monkey kidney cell nuclei (Filippidis and Meneghini, 1977). However, a portion of the linker would be insensitive to both alkali and RNase A. 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.

It is suggested here that a signal sequence either within the DAR or near it may cause the retention of the DAR regions of two origins of replication following the simultaneous initiation of replication of two associated replicons which are base-paired together in a tetrameric fashion. This would be followed by diphosphate cross linkages between paired portions of the two non-daughter DAR regions resulting in ARR formation. Figure 4 is a hypothetical illustration depicting the tetrameric base paired region coupled by diphosphate bridges. Those regions of the RNA primer not involved in covalent cross-links would remain sensitive to alkali and RNAse A (Fig. 5). The Okazaki DNA fragment(s) within this tetrameric region would be flanked by the alkali and RNase A sensitive DAR and would be clipped out and lost following RNase A digestion and electroelution while the ARR portion would remain attached to the main DNA. Evidence for such a loss of DNA in response to RNase A treatment and electroelution was presented in Table II, data row 4.

In this paper emphasis has been placed on the possible role of RNA at DNA domain anchorage sites. Obviously other kinds of molecules must play an active role in the establishment of these sites as well. In the "Introduction" section of this paper a possible role for Topoisomerase II in the establishment of DNA supercoiling within individual DNA domains was discussed. A possible role for phospholipids in maintaining DNA attachment sites within the nuclear matrix was also mentioned in light of the fact that phospholipase C can cause the release of matrix DNA fragments from the matrix core (Maraldi et al, 1984). This latter observation is underscored by the observation from the present study that phospholipase C causes the partial removal of some of the DAR from high molecular weight DNA (Table I). Whether this loss is due to DNA damage, RNA damage, or damage to phospholipids remains to be seen. However, evidence from electron microscopy experiments suggests that the phospholipase C contains an endonuclease activity (data not shown). It is interesting to speculate that some of the DAR is bound to the inner membrane of the nuclear envelope and is released following hydrolysis of 2' RNA bonds to phospholipids. It would prove even more interesting if the removal of DNA from the nuclear matrix by phospholipase C is related to the removal of the DAR. Such an observation would lend credence to the idea that some of the DAR is involved with the establishment of DNA domain attachment sites within the contours of the nuclear envelope.

Table I. Total [$^{14}$C]Uridine Radiolabel Per Gel Insert After Successive Enzyme

Treatments Followed by Electroelution

| 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 I | 0 | (Complete loss) |

All gel inserts contained [$^{14}$C]uridine labeled mouse nuclei . They 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. 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. A flow chart depicting this protocol is illustrated in Fig. 2.

Table II. Percent of Total [$^{14}$C]Thymidine, [$^{14}$C]Uridine, and [$^{14}$C]Leucine Radiolabel Isolated From Single Labeled Gel Inserts and Their DNase I Incubation Buffers

| Radiolabel | Thymidine | | | Uridine | | Leucine |
|---|---|---|---|---|---|---|
| (D, R, S)* | S | D | R, D | D | R, D | R, D |
| Other† | 51.6 | 52.5 | 59.9 | 94.8 | 96.6 | 99.5 |
| Insert†† | 47.0 | 7.7 | 5.8 | 0.4 | 0.4 | 0.2 |
| Sup\|\| | 0.0 | 34.1 | 31.5 | 3.8 | 1.9 | 0.1 |
| Pellet\|\| | 1.4 | 5.7 | 2.8 | 1.0 | 1.1 | 0.2 |

Gel inserts contained mouse cells radiolabeled with either [$^{14}$C]thymidine, [$^{14}$C]uridine, or [$^{14}$C]leucine and extracted overnight in 1% SDS. They were electroeluted and then incubated in the presence or absence of RNase A. A flow chart depicting this protocol is illustrated in Fig. 3. Data columns 3, 5, and 6 are from gel inserts pre-incubated in RNase A. After the incubation period in RNase A 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 either RNase A or DNAse I. The oligomers in the DNase I incubation buffer were precipitated in either ethanol or TCA, centrifuged, and the pellets rinsed twice prior to solubilization and counting. The data is presented as percentages of total counts recovered. The TCA acid data is not shown but is equivalent to the ethanol data. *(D, R, S), (D) = DNase I treatment only, (R, D) = RNase A treatment followed by DNase I treatment, (S) = sham incubation. †The majority of these counts were in SDS extraction buffers and "pre-RNase A" tube gels. ††Percent of total CPM remaining per gel insert. ||Percent of total CPM within supernatants and the ethanol precipitated pellets from DNase I incubation buffers.

Table III. Ethanol Precipitated Radiolabel From DNase I Extracts of [$^{14}$C]Thymidine or [$^{14}$C]Uridine Single Labeled Gel Inserts With and Without Alkali Hydrolysis

| Radiolabel | Thymidine | Thymidine | Uridine | Uridine |
|---|---|---|---|---|
| Treatment | Buffer | KOH | Buffer | KOH |
| TNM* | 0.9 | 0.7 | 1.0 | 0.4 |
| TNE† | 1.8 | 1.0 | 0.5 | 0.3 |

Gel inserts contained mouse cells radiolabeled with either [$^{14}$C]thymidine or [$^{14}$C]uridine and were extracted overnight in 1% SDS buffer. They 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 $^{0}$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. *Percent of total cellular label isolated in Tris, NaCl, magnesium without carrier DNA †Percent of total cellular label isolated in Tris, NaCl, EDTA with 250 µg/ml carrier DNA.

Table IV. Effect on CPM of Alkaline Phosphatase on Ethanol Precipitated DNase I Extracts of [$^{14}$C]Uridine Labeled Gel Inserts With and Without Alkali Hydrolysis

| | | | |
|---|---|---|---|
| Hours of Electroelution | 8 | 8 | 3 |
| Units/ml of AP | 25 | 50 | 50 |
| Hours of Incubation | 1 | 3 | 3 |
| Pellets | CPM | CPM | CPM |
| Controls | 81 | 77 | 243 |
| KOH Twice | 60 | 59 | 200 |
| KOH + AP | 66 | 61 | 179 |
| KOH + AP + KOH | 65 | 66 | 185 |

Gel inserts contained mouse cells radiolabeled with [$^{14}$C]uridine which were 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 subjected to 100 $^{0}$C for 20 minutes in either 0.1N KCl or 0.1N KOH. The KOH samples were neutralized and all samples were buffered in a solution compatible with alkaline phosphatase. Samples were incubated in the presence or absence of alkaline phosphatase, ethanol precipitated, centrifuged, and rinsed once to remove buffer. They were subjected to 100 $^{0}$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. The actual counts recovered are shown in the table. 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 < 0.6% of total cellular CPM.

Table V. Pooled Data From Table IV

| 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 | -- |

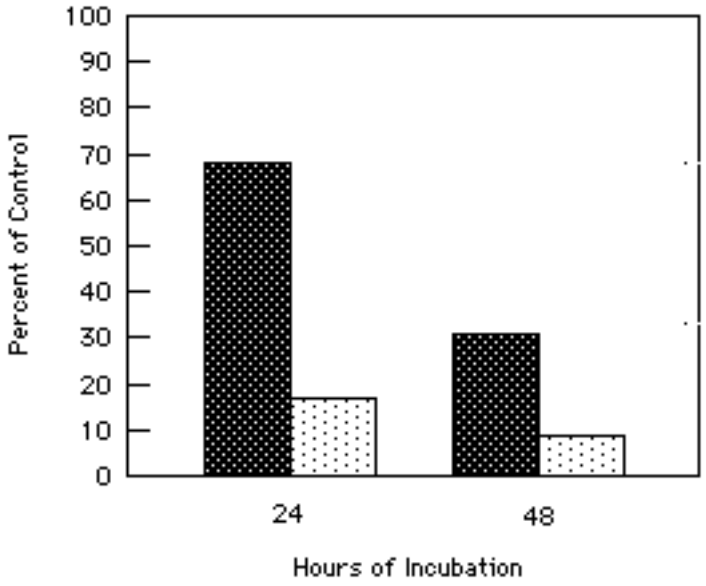
| AP effect assumed | Column 3 | n† |
|---|---|---|
| Control CPM, no alkali nor AP | 243 | -- |
| Alkali treated only CPM | 200 | -- |
| Average AP-treated CPM | 182 ± 2* | 2 |
| Total percent loss | 25 | -- |
| Possible percent loss due to AP | 7 | -- |

Data from Table IV was derivatized in two ways: assuming alkaline phosphatase exerted no effect on the system or assuming that it did had a partial effect on the system. Under the section of Table V 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 IV were averaged together. The data under column 3 was averaged together. Under the section of Table V 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 IV are shown in this section except that all alkaline phosphatase treated data were averaged together. AP is alkaline phosphatase, * is the standard error, and †n = number of data points.

Table VI. Comparative Loss of [$^{3}$H]Thymidine and [$^{14}$C]Uridine Double Radiolabel After SDS Extraction and Gel Electroelution

| | Nine Hours Electroelution | | | Eighteen Hours Electroelution | | |
|---|---|---|---|---|---|---|
| | Thymidine | | Uridine | Thymidine | | Uridine |
| A | 95399 | | 26898 | 101054 | | 29255 |
| B | 2964 | | 408 | 486 | | 69 |
| C | 3.1 | | 1.5 | 0.5 | | 0.2 |
| D | -- | 0.138 | -- | -- | 0.142 | -- |
| E | -- | | -- | 0.164 | | 0.169 |

Gel inserts contained nuclei which were double labeled with [$^{3}$H]thymidine and [$^{14}$C]uridine. The nuclei had been frozen in liquid nitrogen prior to use. Gel 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. Row A contains the total alkali-resistant counts collected from one set of gel inserts together with an average gel tube total count. Row B contains all the alkali-resistant counts recovered from the gel inserts alone. Row C is the percentage of total counts recovered from the gel inserts compared to the average total gel tube counts together with the alkali-resistant gel insert counts. Row D is the ratio of ARR counts divided by the alkali-treated DNA counts for 9 and 18 hours of electroelution respectively. Row E is the ratio of the 18 hour ARR or alkali-treated DNA counts divided by their 9 hour counterparts.




**Growth kinetics in cells incubated in low serum**

Figure 1. 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.

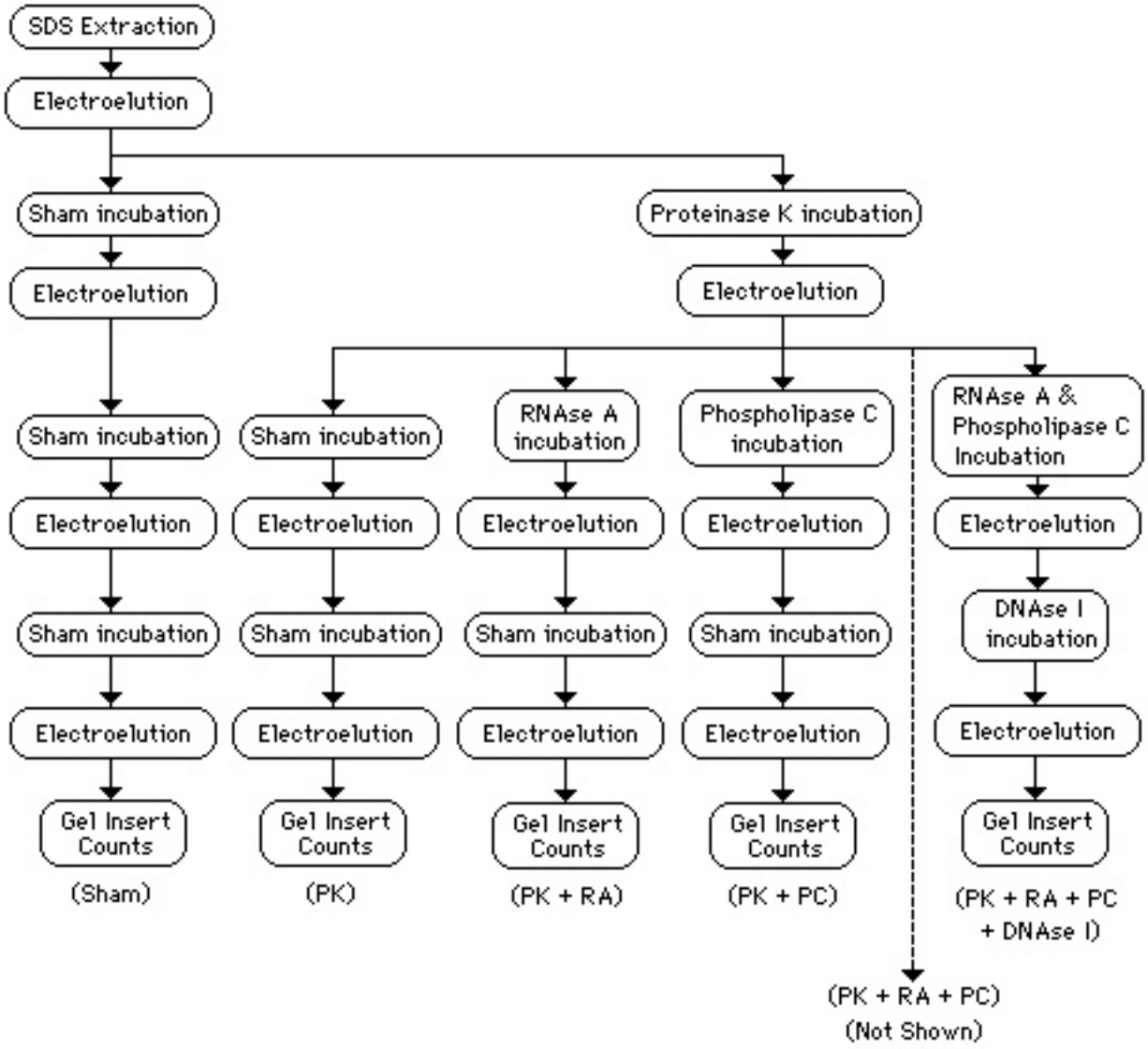



Figure 2. This flow chart illustrates the protocol used to generate the results displayed in Table I. The protocol is discussed in more detail in the legend for Table I and under "Materials and Methods". The cells used in this experiment were radiolabeled with [$^{14}$C]uridine and embedded in gel inserts. The inserts were subjected to an alternating series of incubations in extraction or enzyme solutions followed by electroelution. The purpose of the experiment was to determine the effect of varying combinations of enzyme treatments on the ability of the inserts to retain the [$^{14}$C]uridine radiolabel after electroelution. At the end of the experiment the inserts were removed from the gel wells and counted by liquid scintillation.

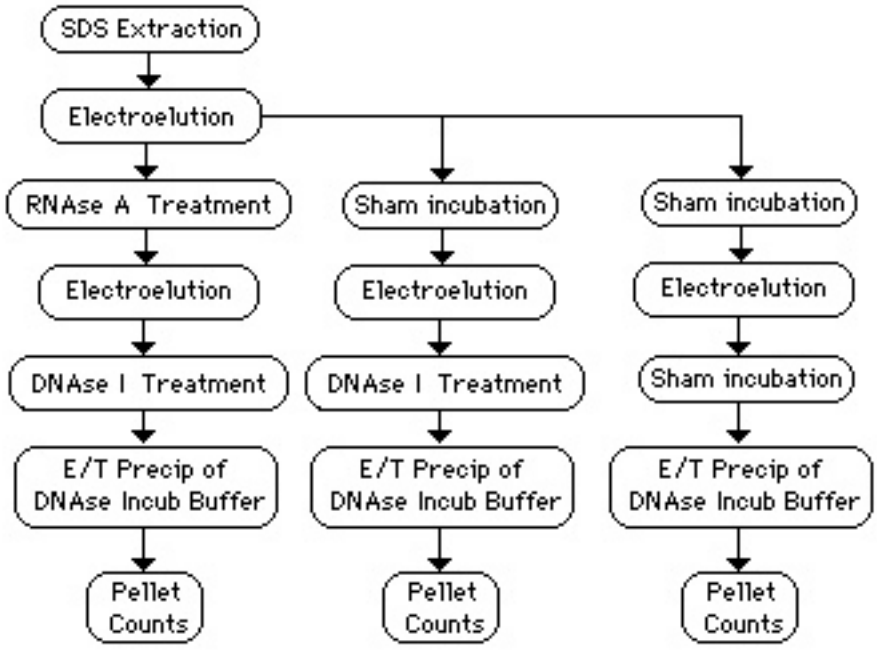



Figure 3. This flow chart illustrates the DAR isolation protocol used to generate the results displayed in Table II. The protocol is discussed in more detail in the legend for Table II and under "Materials and Methods". Cells were prepared in gel inserts, SDS extracted, and run in parallel through the above series of electroelutions, incubations, and precipitations in ethanol and TCA. All samples were counted by liquid scintillation.

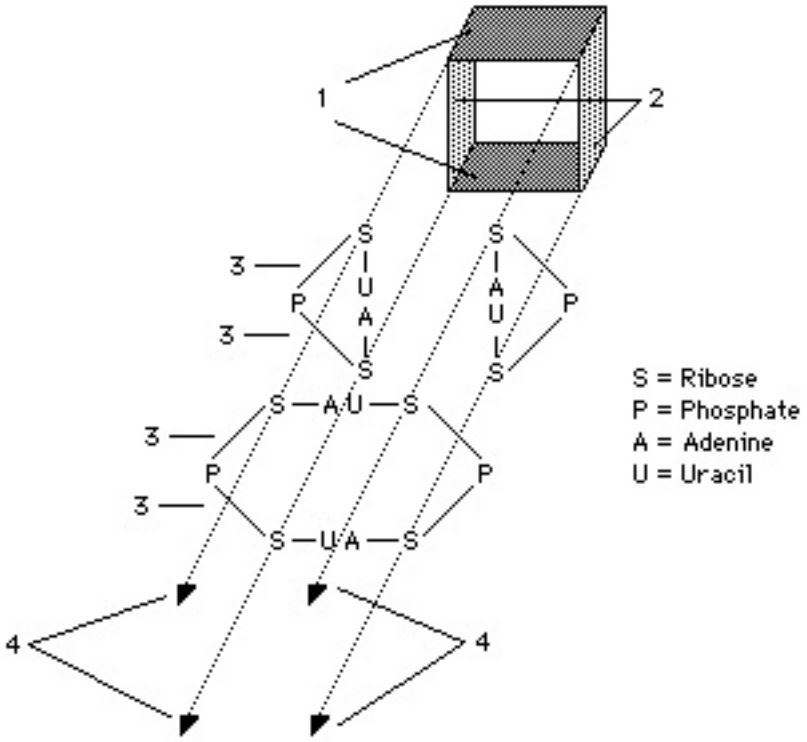



Figure 4. This is a three dimensional view of the 2' RNA crosslinks within the tetrameric strand region. The planes of base pairing between daughter strands or non-daughter strands are shown by (1) and (2) respectively. The 2' RNA phosphodiester linkages (3) between the phosphate bridge and the two non-daughter strands are illustrated. The axes (4) contain the 5', 3' phosphodiester backbones of covalently linked segments of Okazaki RNA primers and Okazaki DNA fragments.

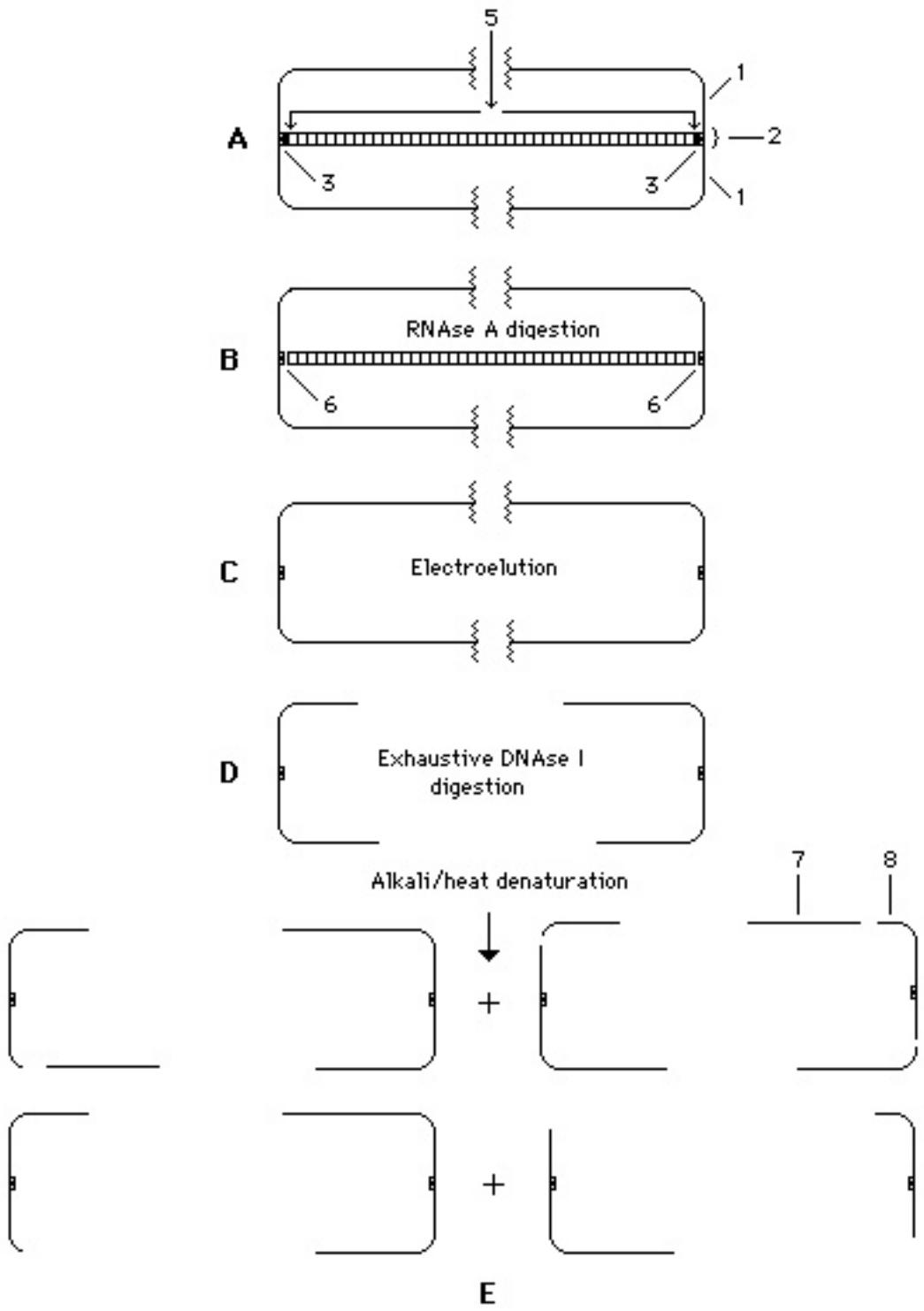



Figure 5. Fig. 5a is an illustration of upper and lower replicons (1) bound together into the tetrameric region (2) by ARR crosslinks located within a partially alkali resistant Okazaki RNA primer region (3). The tetrameric region is composed mainly of a single Okazaki DNA fragment (5) which is flanked by alkali and RNase A sensitive portions of the DAR. RNase A digestion (Fig. 5b) destroys the sensitive portion of the DAR which clips out the Okazaki DNA fragment at the junction of the DAR and DNA (6). During gel electroelution of "intact" DNA (Fig. 5c) the Okazaki

DNA fragment is removed but the fused replicon DNA remains behind together with the ARR subcomponent of the DAR. The gel inserts are then digested in DNAse I (Fig. 5d) which destroys the replicons leaving only assymetric, highly nicked fragments of DNA connected to the ARR hairpins. 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 II). In Fig. 5e the DNA/ARR fragments have been subjected to alkali hydrolysis and heating which releases small DNA fragments (7) flanked by DNase I nick sites and denatures all fragments into assymetric single strands. The presence of pieces of DNA in association with ARR (8) is responsible for the ability of ARR to precipitate in the presence of ethanol or TCA.

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