Evidence for a Stable Association of Alkali Resistant RNA With DNase I Resistant DNA Within Mouse Genomic DNA

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Summary

Mouse L-1210 cells in log phase were radiolabeled with [^{14}C]uridine for 24 hours in medium containing all four deoxynucleosides (dN's). After removal of exogenous radiolabel the cells were subjected to serum arrest for 24 hours in medium containing all four dN's. The dN's were included to prevent incorporation of RNA radiolabel into DNA. Under conditions of serum arrest, DNA synthesis is inhibited, and most RNA transcription which is independent of DNA synthesis should continue, resulting in a dilution of radiolabel in RNA. The mouse cells or isolated nuclei were entrapped in agarose inserts, extracted in sodium dodecyl sulfate (SDS) detergent, and subjected to electroelution. When the DNA remaining in the insert was subjected to DNase I digestion, a soluble DNA fraction was recovered along with radiolabeled RNA. The ability to recover radiolabeled RNA under these conditions suggests that it may be associated with DNA at origins of replication. A substantial portion of this DNA associated RNA (DAR) appears to be sensitive to phospholipase C; an enzyme known to release DNA fragments from the nuclear matrix. DAR is composed of an RNase A sensitive fraction which appears to be flanking small segments of DNA and an RNase A/alkali resistant fraction (ARR) which is associated with

DNase I resistant DNA and represents less than 0.5% of the total RNA radiolabel. When DAR, heat-denatured DAR, and ARR are subjected to electrophoresis together with 123 base pair DNA ladder markers, they migrate as broad bands beginning at approximately 369 base pairs. More than one-half of each of the bands migrates faster than the 123 base pair marker. The migration rate and width of the bands are remarkably similar, indicating that heating and alkali treatments are incapable of reducing the size range of DNase I digested DAR. ARR from phenol extracted DNA is 10-14 times more resistant to alkaline hydrolysis than soluble RNA isolated from the same source. Phenol extraction removes two-thirds more ARR from DNA which has not been incubated in proteinase K than from DNA which was incubated in the enzyme prior to extraction, suggesting that ARR is tightly linked with native proteins. Gas chromatography and mass spectrometry (GCMS) reveal that ARR contains a uracil content which is an order of magnitude higher than that of whole cell DNA. ARR subjected to sequential digestion by nuclease P1 and alkaline phosphatase followed by high performance liquid chromatography (HPLC) analysis reveals that the [^{14}C]uridine is incorporated as uridine and not as thymidine or deoxyuridine. It also demonstrates that it is incorporated into ARR via 3' phosphodiester bonds. Because of the strength and intimacy of the association of DAR with high molecular weight DNA, its resistance to experimental conditions selective for the retention of RNA near origins of replication, its association with DNase I resistant DNA with a size range similar to origins of replication, the apparent sensitivity to phospholipase C, and it's resemblance to Okazaki RNA primers in uridine content and RNase A sensitivity, it is postulated that DAR's are stable, replication initiation primers situated at origins of replication within the nuclear matrix. The unusual chemical stability associated with a portion of its ribonucleoside structure suggests the presence of novel molecular bonds in this area.

Short Title: Alkali Resistant RNA

Key Words: DNA-associated RNA (DAR); alkali-resistant RNA (ARR); sodium dodecyl sulfate (SDS); deoxyribonucleosides (dN's); gas chromatography-mass spectrometry (GCMS); high performance liquid chromatography (HPLC).

Introduction

In both prokaryotes and eukaryotes, DNA is held together in independently supercoiled DNA domains. In prokaryotes, RNA appears to be involved in this process (Hecht et al, 1977; Kavenoff and Ryder, 1976; Pettijohn and Hecht, 1973; Stonington and Pettijohn, 1971; Worcel and Burgi, 1972; Worcel et al, 1973). The role of RNA in the establishment of independently supercoiled DNA domains in eukaryotes is less clear (Adolph et al, 1977; Cook, 1984; Cook and Brazell, 1976; Cook and Brazell, 1978). Proteins have been demonstrated to maintain supercoiling in DNA domains and Topoisomerase II DNA cleavage sites have been located near DNA attachment sites (Berrios et al, 1985; Cockerill and Garrad, 1986; Sperry et al, 1989). Additionally, supercoiling has been shown to be sensitive to the action of proteinase K (Cook, 1984). It does not necessarily follow, however, that the DNA domain anchorage sites are maintained by protein just because supercoiling is sensitive to proteases and these sites are near DNA domain attachment points. As has been shown in prokaryotic studies it is possible to relax the supercoiling in DNA domains without the concomitant destruction of the DNA domain attachment site (Pettijohn and Hecht, 1973; Worcel and Burgi, 1972). Other proteins which do not appear to be topoisomerases have also been found to be tightly linked to eukaryotic DNA and are resistant to extraction by phenol, SDS, proteases, and alkali (Krauth and Werner, 1979). Furthermore, they appear to reside within the nuclear matrix (Werner and Rest, 1987; Neuer and Werner, 1987; Chernokhvostov et al, 1989) associated with repetitive DNA sequences (Neuer-Nitsche et al, 1988) whose locations vary according to cell type (Werner and Neuer-Nitsche, 1989). There is also evidence that phospholipids may be involved with nuclear matrix DNA attachment sites (Cocco et al, 1980), and may be associated with DNA replication (Miscia et al, 1988).

Assuming that the establishment of eukaryotic DNA domains takes place in the nuclear matrix, then, the attachment sites must punctuate the DNA at periodic intervals. Perturbations in the

DNA which occur at periodic intervals most probably occur within such attachment sites. Three such perturbations are 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. The initiation of DNA synthesis appears be at the level of the nuclear matrix (McCready et al, 1980, Dijkwel et al, 1986, Jackson and Cook, 1986, Carriet al, 1986, Dijkwel and Hamlin, 1988) and DNA primase can be found associated with nuclear matrix (Tubo and Berezney, 1987, Hirose et al, 1988), suggesting that the origins of replication may be anchored there. 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). Together, these observations suggest that protein and phospholipid components may be involved in the establishment of DNA domain attachment sites at origins of replication--roughly paralleling the situation which exists in prokaryotes in which the chromosomal origin of replication is "permanently" attached to a mesosome or invagination of the cell membrane (for review see Firshein, 1989). These sites may be alkali-sensitive, A+T rich, and resistant to cloning. Alkali sensitive DNA sites usually indicate DNA damage (such as apurinic sites) or the presence of RNA, and resistance to cloning suggests the presence of unusual structures. One unusual structure associated with origins of replication are palindromes or "snap back" sequences (Vogt and Braun, 1977, Stow and McMonagle, 1983, Zannis-Hadjopoulos, M., 1984, Brykov and Kukhlevskii, 1988). Palindromes can be difficult to clone because of their unusual sensitivity to endogenous, recombinogenic nucleases (Leach & Stahl, 1983). Double stranded, cellular, heterogenous RNAs with unusual properties such as resistance to RNase A in high salt can be isolated from cellular nuclei. Some are tightly associated with the nuclear lamina (Herman et al, 1978) or associated with DNase I-resistant DNA at developmentally regulated nuclear matrix attachment sites (Patriotis et al, 1990). Others are known to have "snap back" qualities and hybridize with repetitive cellular DNA (Jelinek and Darnell, 1972). Other investigators have reported the discovery of actinomycin-D resistant double stranded RNA with snap back

regions which are present even after 60% of the RNA has been digested by RNase A (Stern et al, 1970). The snap back RNA regions are destroyed by alkaline hydrolysis (Jelinek and Darnell, 1972, Stern et al, 1970). Such RNA's could be associated with palindromic regions at DNA attachment sites within origins of replication. Although snap back regions within nucleic acids are generally considered to be palindromic, they may also be an indicator of cross links, either between daughter strands or non-daughter strands. If the snap back regions were the result of double stranded, palindromic RNA, then, this RNA should be destroyed by alkaline hydrolysis. However, if the snapback regions involved cross links, then, the most likely position for these cross links would be at the 2' hydroxyl positions of the ribose moiety and such bonds would be alkali resistant. Evidence for the presence of alkali resistant RNA at replication initiation sites has been demonstrated (Birkenmeyer, 1987). Therefore, the focus of this investigation was to test the possibility that an unusual 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 should be A+U rich. The initial results obtained supported the hypothesis that an RNA species containing an alkali resistant component was tightly associated with whole genomic DNA in an area that displayed an unusual resistance to DNase I digestion and was highly concentrated in the nuclear matrix (Abernathy, 1988). This data is being reported here together with additional data confirming that ARR is composed of alkali resistant RNA nucleotides partially removable by phenol extraction which are associated with a heterogenous size range of DNase I resistant DNA molecules, most of which are less than 123 base pairs in length.

Materials and Methods

General Cell Maintenance

Growing stocks of mouse L-1210 cells were maintained as suspension cultures in filter sterilized Eagles MEM (Earle's salts) with 2X nonessential amino acids, 1.5X essential amino acids, and 1.5X vitamins (GIBCO). The medium, supplemented with 10% fetal bovine serum (FBS, K.C. Biologicals),is referred to as B-10. Cells were maintained in log phase at 37°C under a humidified atmosphere of 5% CO_2. Stock cultures were maintained in the absence of antibiotics. Experimental cultures contained pen/strep (50U and 50ug/ml) Cells were counted by hemocytometer; cell viability was determined by trypan blue exclusion.

Selection of Experimental Culture Procedures

To enhance the possibility of identifying RNA, stably associated with DNA, two sources of misleading information were identified: 1) Okazaki RNA primers, though transiently associated, would contribute to the signal of recovered DNA associated RNA, and 2) labeled uridine could be incorporated into DNA as thymidine resulting in an erroneous signal. To minimize Okazaki effects, cells were serum arrested for 24 hours following radiolabeling to inhibit the initiation of new DNA synthesis while allowing completion of the round of synthesis already in progress, thereby allowing the release of any radiolabeled Okazaki primers. To minimize the misincorporation of uridine as thymidine, the ribonucleotide reductase pathway was inhibited by the addition of exogenous deoxyribonucleosides (dN's) (Klenow, 1962; Thelander and Reichard, 1979).

Initiation of DNA synthesis was inhibited by shifting the log phase cells from B-10 to B-0.5 medium. After 24 and 48 hours, the number of cells in B-0.5 compared to cells in B-10 was 70% and 30% respectively (data not shown). The viability of the B-0.5 cells was 96% and 80% respectively (data not shown). To further confirm the effectiveness of the serum arrest,

$3X10^6$ cells, aliquoted from the stock culture, were mixed with [^{3}H]thymidine (final concentration, 1.0 uCi /ml), incubated 30 minutes (37°C), and precipitated with an equal volume of 10% cold trichloroacetic acid. Triplicate control samples were taken at the initiation of serum arrest followed by samples at 24 and 48 hours. Precipitates were collected on Whatman GF/B glassfilters with cold 10% trichloroacetic acid followed by cold 95% ethanol washes. The filters were dried and counted in 10 mls of Aquasol (New England Nuclear). The addition of dNs (50ug/ml each of deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine) reduced the incorporation of [^{3}H]thymidine by 94% (resulting from dilution of the label). By 24 and 48 hours, after step down to B-0.5, incorporation was further reduced to 1% and 0.5% (data not shown). Both cell enumeration and [^{3}H]thymidine incorporation results indicate that these procedures may be effective in minimizing misleading signals. However, even though inhibition of radiolabeled thymidine uptake was more pronounced at 48 hours B-0.5, the reduction in cell viability was of such concern that we selected the 24 hour B-0.5 conditions as our general experimental protocol.

Radiolabeling Cells

Cells were aliquoted from growing stocks, centrifuged, and resuspended in fresh B-10 at $3X10^7$ cells/ml. To a 100 mm petri plate was added 4.4 mls of B-10 with or without dNs and 100 ul of the cell suspension. This culture was incubated for about 24 hours as described above to allow the cells time to recover from the subculturing procedure and dNs to equilibrate. At the end of this period, radiolabel was added to the cultures in 0.5 mls of B-10. For single label experiments,the radiolabel was 0.5 uCi of either [methyl-^{14}C]thymidine (0.25mCi/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). For double labeling experiments, 5 uCi of[methyl-^{3}H]thymidine (6.7 Ci/mmol, New England Nuclear) was added to the cultures along with 0.5 uCi of [^{14}C]uridine. The solutions were mixed and the cells allowed to incubate an additional 24 hours. At the end of this incubation, the cells were harvested, counted,

centrifuged, and resuspended at a concentration of $2X10^5$/ml in non-radiolabeled B-0.5. In those experiments in which ribonucleotide reductase was being inhibited, B-0.5 also contained dNs. The cells were incubated for 24 hours to allow for serum arrest. At the end of this time the cells were again counted and pelleted for nuclei preparations or for use directly in gel inserts.

Buffers

Buffers varied in terms of concentrations of Tris, EDTA, magnesium, and pH depending on the procedure. However they were of four basic types: 1) extraction buffer (10mM Tris, 10mM EDTA, 1% SDS, pH 8.0); 2) electroelution buffers (40mM Tris, 10 mM EDTA or {5 mM $MgCl_2$}, pH 8.0); 3) enzyme incubation buffers (10mM Tris, 5 mM 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}); and 4) precipitation buffers, (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 trichloroacetic acid precipitation. In some cases 250 ug/ml of carrier DNA was added to the precipitation buffers. Some of the buffer formulae were derived from 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 trichloroacetic acid 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.

Production and Extraction 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 ul in volume. The maximum concentration of cells or nuclei used in these gel inserts was $3X10^6$

cells or nuclei per 50 ul volume. Concentrations as low as $2X10^5$ cells per gel insert were used in early experiments but this was increased for isolation and characterization of ARR. Early gel inserts were formed in agarose wells made by a cork borer, but later gel inserts were made in agarose-embedded pipette sections. Gel inserts were incubated overnight in extraction buffer, rinsed, and used directly for electroelution or incubated two times in Tris-magnesium incubation buffer (at least 30 minutes per incubation) in preparation for enzyme incubations.

Gel Electroelution

The term electroelution is used to emphasize that what is being assayed is not emerging bands of DNA or RNA but rather the relative amounts of DNA and/or other associated molecules which are resisting electrophoresis because of their large size and/or complex structure. Gel inserts were electroeluted in either conventional slab gels or by horizontal tube gel electroelution. In horizontal tube gel electroelution, small 1 ml sections cut from a 10 ml plastic pipette are embedded in agarose and the inserts (50 ul) are formed in these sections. After gelling on ice the inserts are removed from their forms, extracted, processed as needed, and returned to clean 1 ml pipette sections. These small sections are connected by tubing to larger (4.5 ml), agarose-filled pipette sections, submersed in buffer in a horizontal position,and electroeluted. In later experiments, the small pipette section was not used and inserts were placed directly inside a buffer-filled depression within the larger tube. The majority of these experiments were performed with the horizontal tube gels because of the convenience of preparation, ease in handling, and improved reproducibility. After electroelution, the pipette sections were dismantled and the gel inserts removed. The inserts were either prepared for additional manipulations or placed into liquid scintillation vials along with buffer (0.5 ml), melted, mixed with 10 ml of aquasol, and counted. Tube gels (from the larger sections) were extruded from their sections directly into scintillation vials and, melted. Aliquots (0.5 ml) from the melted tube gels were transferred into fresh vials along with 10 ml of aquasol, mixed, and counted. Counts which entered the tube gel were tabulated and added together with the counts

remaining in the gel insert to obtain the "total" counts. The counts remaining in the insert were divided by the total counts to yield the relative retention of label within the insert. These numbers could then be used to obtain relative retention averages and for comparing controls to experimental groups.

Protocol for Pilot Gel Retention Assay of Uridine Radiolabel

[^{14}C]uridine labeled nuclei in gel inserts were extracted overnight 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°C) in a Tris- EDTA slab gel. All of the gel inserts were removed from the slab gel and incubated for 8 hours (37°C) in 10mM Tris, 5mM EDTA, pH 7.8. In all cases but one the buffer contained 40 units/ml of proteinase K. Following this incubation the gel inserts were electroeluted in a second slab gel containing Tris-magnesium buffer overnight at 25 volts (4°C). They were removed and incubated for an additional 8 hours at 37°C in Tris-magnesium incubation buffer. The buffer contained no enzyme, or either 40 units/ml of RNase A, phospholipase C, or a combination of both. Following this incubation the gel inserts were electroeluted for 3 hours at 70 volts (4°C) in a second Tris-magnesium gel. They were removed and incubated for an additional 8 hours (37°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°C). The inserts were removed, stained with 3 ug/ml of ethidium bromide, and electroeluted for an additional 1.5 hours to remove excess ethidium bromide. The inserts and slab gels were photographed, melted, and counted in aquasol (see Figure 2 and Table I).

Protocol for Gel Retention Assay of DAR

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, with or without RNase A (20 U/ml), for 16 hours (37°C). 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°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 trichloroacetic acid as described in the next paragraph.

Precipitation of DAR

Precipitation methodologies involving ethanol and trichloroacetic acid were modified from established procedures (Maniatis et al, 1982). In those experiments involving trichloroacetic acid or ethanol precipitation of DAR and ARR the gel inserts were incubated for about 24 hours at 37°C in 4-5 mls of Tris-magnesium containing 20 U/ml of DNase I. In later experiments the DNase I concentration was increased to 40 units/ml and 0.1N NaCl or KCl 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 trichloroacetic acid precipitated in Tris, NaCl, and EDTA in the presence of 250ug/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 trichloroacetic acid precipitation procedure was discontinued since recovery of enzymatically appropriate substrate from the pellets was necessary for future experiments. The conditions used for trichloroacetic acid and ethanol precipitations were an

overnight incubation at 4°C and -50°C respectively. Samples were precipitated in 2.6 volumes of ethanol to facilitate RNA precipitation followed by ultracentrifugation (0°C) for 30 minutes at 106,600 X g (Maniatis et al, 1982). The supernatants were decanted and the tubes inverted and drained. 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 ethanol rinses to insure that the final pellet was relatively devoid of supernatant label. The pellets were drained as before and then placed into a vacuum desiccator and evacuated for 10 minutes at room temperature to remove the last traces of ethanol. 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. Samples were generally counted 5 or 10 minutes.

Alkaline Hydrolysis Procedures

Alkaline hydrolysis was performed according to established methods (Bock, 1968). One-half ml of either pre-neutralized KOH with buffer (control) or 0.1N KOH was added to dried, buffer-free ethanol precipitates, vortexed, and incubated (37°C) for 5 minutes. They were vortexed again and heated at 100°C 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 40 mM Tris, 0.1 N KCl, 40 mM $MgCl_2$, pH 8.0, and ethanol precipitated. From this point they were processed as described in the preceding paragraph in preparation for liquid scintillation.

Phenol Extraction and ARR Recovery

DNA from cells incubated in [^{14}C]uridine but no dN's were serum arrested and trapped in low melting temperature Sea Plaque gel inserts. The inserts were extracted overnight in TE buffer + 1% SDS and electroeluted in TM buffer for 3 hours at 70 volts. The inserts were either digested in DNase I as previously described or the inserts were melted at 65°C in TE buffer (10

mM Tris, 1mM EDTA, pH 7.8) containing 0.5% SDS, split into two samples and proteinase K added to one set of samples at a final concentration of 50 μg/ml. The samples in TE buffer were incubated overnight at 37°C. The DNased samples were adjusted to 10mM magnesium and all samples were phenol extracted, back extracted once, then, extracted in chloroform. The samples were ethanol precipitated and processed as described earlier for ARR preparation. All samples were counted for 10 minutes in Bray's scintillation cocktail.

Confirmation of Alkali Resistant Properties of ARR

Mouse cells were radiolabeled with either [^{3}H]uridine or [^{14}C]uridine with dN's and subjected to serum arrest as described earlier. The cells were harvested, incorporated into gel inserts, and extracted in TE buffer containing SDS. The extracts (which contained the majority of the uridine radiolabel incorporated by the cells) were saved and kept at 4°C. The gel inserts were electroeluted to remove non-bound RNA radiolabel. The gel inserts containing [^{3}H]uridine were added to the SDS extracts containing [^{14}C]uridine and vice-versa. They were heated to 65°C to melt the low melting point agarose so the gel insert DNA/DAR and SDS extract RNA radiolabels could mix together. The solutions were extracted with phenol and chloroform and ethanol precipitated. The pellets were rinsed with ethanol, subjected to alkaline hydrolysis, neutralized, ethanol precipitated, rinsed twice, dried, redissolved, and counted by liquid scintillation.

Molecular Sizing Assay

Ten tube gels were filled with 2% Sea Plaque agarose in 40 mM TE buffer, pH 8.0. Standard 123 base pair ladder DNA markers (BRL) were added to one tube. The markers were also mixed together with DAR, DAR heated to 100 °C for 20 minutes in 0.1 M KCl, or ARR and placed into three separate tubes. The remaining six tubes were filled with duplicate sets of either DAR, heated DAR, or ARR. All samples contained [^{14}C]uridine. The tubes were submerged and arranged horizontally in an electrophoretic chamber containing 40 mM TE buffer, pH 8.0 and

subjected to 3 hours of electrophoresis at 35 volts. All of the gels were removed from their tubes and six of the sample gels and the marker gel were stained with 3 μg/ml of ethidium bromide, aligned together along their long axes and photographed under UV light. All sample gels were sliced into 3 mm sections, melted and diluted in water in scintillation vials, mixed with 10 ml of Readysolve scintillation cocktail and counted.

GCMS Assay

Whole mouse cell DNA was purified by a modified conventional phenol-chloroform extraction procedure (Shih and Weinberg, 1982). It was ethanol precipitated and subjected to alkaline hydrolysis procedures as previously described for ARR isolation. The resulting pellet and an ARR pellet were sent to Dr. Dave Bergtold at the National Bureau of Standards (now the National Institute for Standards and Technology). They were subjected to acid hydrolysis to release the bases and processed for GCMS analysis. The resulting chromatographed peaks were compared by dividing the areas of all other peaks by the area established for thymine. The relative concentration of different bases for ARR was compared to that in whole mouse cell DNA by dividing ARR (base/thymine) ratios by whole mouse cell DNA (base/thymine) ratios.

HPLC Assay

Incubation parameters for nuclease P1 and alkaline phosphatase were derived from the literature (Linn and Roberts, 1982; Perbal, 1984). The ARR ethanol pellets were resuspended in 0.1 ml of nuclease P1 buffer (10mM Tris, 1mM $ZnSO_4$, pH 5.0) containing 40 units/ml nuclease P1 and incubated at 55°C overnight with shaking. They were then mixed with 0.9 ml of alkaline phosphatase buffer (0.1MTris, 1mM $ZnSO_4$, pH 8.1) containing 40 units/ml of bacterial alkaline phosphatase and incubated at 65°C at least two hours or overnight with shaking. HPLC parameters were derived from methods established in the laboratory of Dr. Ronald Trewyn at the Comprehensive Cancer Center at OSU. The samples were loaded onto an Altex, reversed-phase, C-18, HPLC column attached to a Beckman 171 UV spectrophotometric-

radiolabel flow detector and eluted at 1.0 or 1.5 ml/minute at 2000-2500 psi over a 20 to 40 minute period. UV peaks were detected at 260 nM. HPLC cocktail was 1% acetonitrile in 50mM ammonium formate buffer, pH 4.1.

Results

The Effects of Enzymes on DNA Associated [^{14}C]Uridine Radiolabel

Because a variety of enzymes have been shown to effect DNA structure, 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 dNs and quantitative tube gel techniques). The results, shown in Table I, suggest that treatment with proteinase K alone may increase the retention of uridine radiolabel relative to the control (1784 versus 1006 CPM). When inserts were incubated sequentially with proteinase K and then RNase A the counts increased still further from 1784 to 2095 CPM. The possibility that these numbers reflect real increases in retention have been under investigation in our laboratory but the evidence accumulated so far, although interesting, is still inconclusive. When gel inserts were treated with phospholipase C alone, retained counts dropped to 744 CPM and, in conjunction with RNase A, the counts from two separate inserts were reduced to 230 and 875 CPM. Together, these results represent a sizeable loss of counts when compared with the control insert. In the insert exposed to DNase I, the counts dropped to within background levels. These initial results suggested that some form of RNA ([^{14}C]uridine labeled) was resistant to SDS extraction 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 may be intimately associated with high molecular weight DNA which was 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 deoxyribonuclease activity.

Confirmation of DAR

The uridine labeled material was isolated from the high molecular weight DNA for further characterization as described in Experimental Procedures. The percent of total counts recoverable by ethanol precipitation are shown in Table II (pellet). Sham incubations 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 supernatant and 5.7% (119 CPM) in the pellet. Pre-treatment with RNase A followed by electroelution resulted in a slight further decrease in insert retention to 5.8% with less material being recovered in the supernatants (31.5%) and the pellet (2.8%, 40 CPM). 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 supernatant and pellet 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 one-half as much recovery in the supernatant (1.9%) and essentially the same amount of recovery in the pellet (1.1%, 310 CPM). 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 supernatant and pellet was only 0.1% and 0.2% (19 CPM) respectively. The results of this study partially characterized the RNA species associated with the DNA as existing in an oligomeric form (trichloroacetic acid or ethanol precipitable) which is partially resistant to RNase A treatment. The ability of RNase A to remove some of the DNA radiolabel can be explained in one of two ways: 1) The enzyme preparation contains a deoxyribonuclease resistant to conventional heat-inactivation

procedures whose activity may be enhanced by the use of buffers containing magnesium or 2) the DNA being clipped out is flanked by RNase A-sensitive RNA.

Characterization of DAR by Alkali Treatment (ARR)

The percentages of whole cell DNA recovered as DNase I resistant fragments after heating in buffer was 0.9% in Tris, magnesium, NaCl buffer and 1.8% in Tris, EDTA, NaCl buffer (Table III). The equivalent DAR counts were 1.0% and 0.5% respectively. When the DNA and DAR fragments were heated in alkali they were found to contain alkali resistant subcomponents which comprised 56-78% of the DNA and 40-60% of the DAR. The loss of DNA and DAR label during heating in alkali could be attributed to the loss of small fragments flanked by single strand nicks and/or fragments generated from staggered single stranded nicks on opposite strands, both of which could be produced by the DNase I treatment.

Effect of Phenol Extraction on ARR Recovery

The results shown in Table IV represent the percentage of total [^{14}C]uridine radiolabel released from gel inserts digested in DNase I or from melted gel inserts. About 25% of the counts are recovered as ARR from DNA which has been digested in DNase I and ethanol precipitated without phenol extraction (row 1). After phenol extraction about one-half of the counts retained in the ARR ethanol supernatant and one-third of the counts recovered as ARR are retained within the phenol and the phenol/aqueous phase (row 2). DNA which has not been digested in DNase I and phenol extracted loses 86% of its [^{14}C]uridine to the phenol/aqueous phase with only 2% and 3% retention in the phenol and ARR ethanol supernatants respectively. Ten percent of the total counts are recovered as ARR (row 3). If this DNA is digested in proteinase K and then phenol extracted the percentage of counts retained in the phenol/aqueous phase is reduced to 63%, the phenol counts are negligible, the ARR ethanol supernatant counts increase to 11%, and the ARR counts increase to 25% (row 4) which is the same level of recovery seen in DNased DNA which was not phenol extracted.

Confirmation of ARR

The double labeled counts obtained from the mixed ARR/SDS extract are shown in Table V. All counts in the tritium window attributable to carbon 14 have been subtracted out. The means for each set of control data (KCl treated) and their alkaline treated counterparts were compared by the student t test to determine if the losses of counts between the two sets of data were significant (data row 3). No significant loss of counts was detected in the ARR which was labeled with carbon 14 ($p >> 0.2$ for a one-tailed test). In contrast, the SDS extract which was labeled with carbon 14 showed a very significant loss in counts ($p < 0.0005$). A significant loss of counts was detected for the ARR which was labeled with tritium ($p < 0.0125$). However, the significance level for the counts lost in the tritiated SDS extract was much greater ($p << 0.00025$). The dramatic difference between label loss in ARR labeled with carbon 14 versus tritium might be attributable to proton exchange between water and the tritiated uridine under the harsh condition used. The means for the counts of the alkaline treated samples were divided by the control means (data row 5) and the resulting ARR ratios were divided by their respective SDS extract ratios (data row 6). These data indicate that the relative loss of counts due to alkaline hydrolysis were 10-14 times greater for SDS extracts than for their ARR counterparts. This provides very strong evidence that ARR is indeed far more alkali resistant than the majority of the cellular RNA.

Molecular Sizing of DAR, Heated DAR, and ARR

The graphs displayed in Figure 1 represent three sets of DAR, heated DAR, and ARR samples. The samples represented by the upper graph were mixed with molecular weight markers. The migration rate of four of the markers (123-492 base pairs) was determined from photographs of the tube gels which were stained with ethidium bromide and exposed to UV light. The remaining samples were not mixed with markers. The middle and bottom graphs represent samples stained or not stained with ethidium bromide respectively. The samples were not

detectable by ethidium bromide staining. The data points represent percentages of the total counts tabulated for each curve. These results clearly demonstrate that DAR, heated DAR, and ARR comigrate as a single broad band, most of which is less than 123 base pairs in length. Therefore, heating of DAR to 100 °C either in the presence or absence of alkali has little if any effect on the band migration rate, and hence does not contribute to any further size reduction of DNase I digested DAR.

The Base Composition of ARR

Analysis of acid-hydrolysed ARR pellets by GCMS indicates that uracil, thymine, cytosine, adenine, and 5'methylcytosine are present (data not shown). When the relative concentrations of bases are compared to whole cell DNA, as described in Experimental Procedures, the ARR/DNA base ratios for guanine, cytosine, and 5'methylcytosine were 0.8, 1.1, and 0.9 respectively (adenine was not determined). These ratios suggest that there is no significant difference in the relative amounts of these bases in ARR as compared to whole cell DNA. However, the ARR/DNA ratio for uracil was 11.9, indicating a ten-fold higher concentration of uracil in ARR as compared to whole cell DNA.

The Nucleoside Composition of ARR

Even with the exogenous addition of dNs to inhibit ribonucleotide reductase, there is still need of direct proof that the labeled uridine is present in ARR as labeled uridine and not some other nucleoside. The nucleoside composition of ARR from serum arrested cells labeled with [^{14}C]uridine was analyzed by HPLC (Table VI). Two samples, containing equal numbers of cells, were labeled and processed for ARR concurrently. Cells in Sample 1 were not incubated in dNs; cells in Sample 2 were incubated in dNs as described in Experimental Procedures. Sample 2 cells incorporated 2.1 times as much uridine label as Sample 1 cells (data not shown). Sixty percent of the label incorporated in Sample 1 cells was removed by SDS extraction compared to 92% of the label taken up by Sample 2 cells (data not shown). Therefore, Sample 1 cells

retained about 2.4 times more non extractable label than did Sample 2 cells. The small losses of label resulting from electroelution indicate that most of the non-extracted label is DAR (data not shown).

Retention times for nucleoside peaks were determined by simultaneous UV and radiolabel detection of carbon 14. Radiolabel coeluted only with uridine even though thymidine was present in both samples as determined by UV absorption (Table VI). Deoxyuridine was prominent in Sample 1 (-dNs) but virtually absent in Sample 2 (+dNs). No radiolabel coeluted with deoxyuridine. Thymidine and uridine peaks were larger in Sample 2 than in Sample 1. The amount of radiolabel coeluting with uridine was about 1.6 times greater in Sample 2 than in Sample 1 (data not shown).

Discussion

When cells are radiolabeled with [^{14}C]uridine followed by a 24 hour incubation in serum arrest medium without radiolabel a portion of the [^{14}C]uridine can be recovered from the cells in the form of DAR, i.e., RNA which is tightly linked to high molecular weight DNA as demonstrated by its ability to resist removal from agarose-bound DNA during extraction in SDS followed by electroelution. Since only the initiation of DNA synthesis and not DNA synthesis itself is inhibited by conditions favorable for serum arrest, any radiolabeled RNA associated with DNA following a pulse chase should be found near the origins of replication (assuming that RNA transcription which is independent of DNA replication is not being interrupted as well). This tight association of the DAR with the DNA suggests that it may be covalently linked to the DNA in a manner similiar to Okazaki RNA primers. However, the ability of DAR to remain associated with the DNA even when replication is being inhibited suggests that it is not an Okazaki primer in the traditional sense.

Exhaustive incubation in SDS followed by exhaustive electroelutions are incapable of removing all of the DAR. Remarkably, exhaustive incubations in proteinase K and/or RNase A seem to increase the retention of the RNase A resistant label. However, some RNase A sensitive label is released (together with a small amount of DNA) following exposure to RNase A in magnesium-containing buffers and electroelution. Whether this indicates the presence of a contaminating heat-resistant deoxyribonuclease or the clipping out of DNA flanked by RNase A sensitive RNA remains to be seen. The inability to recover the RNase A sensitive portion of the DAR in the pellet after DNase I digestion suggests that this portion of the DAR is associated with DNase I sensitive DNA and/or contaminating RNases within the DNase I preparation are removing it during the incubation period.

Much of the DAR is removed after exposure to phospholipase C in buffers containing magnesium followed by electroelution. Whether this effect is due strictly to the enzyme or a contaminating deoxyribonuclease must be confirmed. Preliminary data obtained from DNA spreads examined by electron microscopy suggest that the enzyme preparation may contain a contaminating nuclease activity. However, data from electrophoretic gel migration patterns of PM2 plasmids incubated with the enzyme show no evidence of nuclease activity (not shown). The DAR is completely released (presumably with the DNA) after exposure to DNase I followed by electroelution.

DAR can be ethanol precipitated from the DNase I buffer in which the gel inserts are incubated. The ability to recover the DAR from the buffer suggests it has been converted into a freely diffusable form. And the capacity for ethanol precipitation suggests this form must be oligomeric, although it is uncertain how much of this capacity is due to DNA, RNA or protein. It has been reported that DAR is associated with protein at the level of the nuclear matrix (Patriotis et al, 1990). DNased I digested DNA and undigested DNA contain at least two major

ARR components, one is removable by phenol extraction and the other is not. The ARR removed by phenol from DNased DNA distributes itself approximately 4:3 between the phenol/aqueous interface and the phenol phase respectively with 32% of the ARR been lost to either one or both of these fractions. In undigested, and hence higher molecular weight DNA, 63% of the ARR is removed by phenol extraction and is sequestered almost entirely within the phenol/aqueous interface. Incubation of undigested DNA in proteinase K releases this ARR so the total amount recovered equals that recovered from DNased DNA which was not phenol extracted. The ability of the phenol/aqueous interface to retain more than twice as many [^{14}C]uridine counts from undigested DNA than from DNased DNA suggests that a positive relationship exists between the size of the DNA molecules and the ability of the interface to retain ARR and that the DNA and its ARR constituent are being partitioned between the phenol and aqueous phases as a result of an association with protein. The observation that smaller pieces of DNA/ARR are less retained by the phenol/aqueous interface than larger pieces (17% versus 10% respectively) suggests that the protein component is physically separated from the ARR by a segment of DNA that can be clipped by DNase I. Clipping this DNA segment could separate a portion of the ARR from the protein component, preventing the ARR from being removed by phenol extraction.

The observation that the size range of DNase I digested DAR is unaffected by heating or alkali suggests that DNase I digested DAR and ARR are identical. This is supported by the data shown in Table V which indicates that no [^{14}C]uridine radiolabel is lost due to heating in alkali. The effect of heating and alkali on DAR secondary structure is less clear. It seems unlikely that the DAR/DNA complex is single stranded in native form, but denatured daughter strands might comigrate during electrophoresis. Another possibility is that the DAR is crosslinked either by proteins or through other novel bonds and hence cannot be separated by heating or alkali treatments. There is evidence to suggest that at least 5% of the ARR retains double stranded character after heat denaturation and hydroxylapatite elution and is, therefore, either crosslinked, contains inverted repeats, or both (data not shown). Less stringent conditions for

reannealing may be required to demonstrate that most or all of the ARR contain crosslinks and/or inverted repeats. The fact that the majority of the DNase I resistant DNA/RNA fragments are less than 123 base pairs in length is compatible with the hypothesis that ARR is associated with Okazaki DNA fragments, possibly as primers at origins of replication (100-300 base pairs).

When DNase I digested DAR isolated from cells incubated in dN's together with either [^{14}C]uridine or [^{14}C]thymidine is heated in alkali, a portion of the DAR and DNA can be lost (Table III). Since alkali treatment is unable to remove [^{14}C]uridine from undigested DAR (Table V) the loss of DNase I digested DAR and DNA during heating in alkali suggests that alkali labile bonds may be present which are important for DAR retention during heating of DNase I digested fragments but are not critical when the DAR is associated with undigested DNA. Since RNase A is incapable of removing additional material from DNase I digested DAR (Table II) these cryptic alkali sensitive bonds may be analagous to those associated with RNase A insensitive DAR described elsewhere (Patriotis, et al 1990). When freeze-damaged DNA is electroeluted from thawed inserts, the rates of release of ARR and DNA are coupled (data not shown). Also, thymidine is isolated alongside uridine from ARR after nuclease P1 and alkaline phosphatase digestions. Therefore, it appears that at least the alkali-resistant portion of the DAR consists of RNA linked to DNase I-resistant DNA. DNase I resistant DNA fragments up to 20 bp in length are known to exist in regions upstream from promoters (Lobanenkov, 1988).

Preliminary results indicate that when cells are grown in the presence of deoxynucleosides to inhibit the uptake of labeled uridine into DNA by way of conversion to deoxyuridine or thymidine there is an increase in DNA replication as demonstrated by a higher level of thymidine incorporation into DNA (indicative of a greater number of 4N cells), a drastic decrease in deoxyuridine levels, an increase of label uptake into soluble RNA and ARR, but a decrease in its overall incorporation into DAR (data not shown). The decrease in DAR label

coupled with an increase in ARR label suggests that uptake of uridine label into DAR is being inhibited in one pathway and stimulated in another one involved in ARR formation. The simplest explanation for the decrease in label uptake into DAR is that the uridine to thymidine pathway is blocked by the presence of exogenous thymidine. The ability of exogeous deoxynucleosides to dampen uridine to thymidine conversion while amplifying ARR production suggests that ARR is not simply an artifact of the isolation procedures.

The presence of a portion of the DAR, part of which may be linked to protein, that is 10-13 times more resistant to alkaline hydrolysis than is the majority of the cellular RNA containing a 10 fold higher level of uracil than whole cell DNA in the form of deoxyuridine and uridine is an important discovery which must be carefully scrutinized in light of the more conventional and accepted procedures utilized for isolating and purifying RNA's. Variations in phenol and chloroform extraction procedures, including the ionic strength of the buffers used for extraction are known to result in a wide variation in the ability to recover A+T rich satellite DNA's (Skinner and Triplett, 1967; Rae et al, 1976). The recovery of DNA's with high A+T content (matrix attachment sites?) may be diminished by such procedures along with any ARR associated with them. Furthermore, small pieces of DNA associated with proteins can be lost in the phenol phase (Bodnar et al, 1983). If ARR is associated with DNA-protein complexes at attachment sites which have been highly fragmented during preparation, then, ARR could be extracted out along with these complexes.

A considerable amount of ARR was recovered in the soluble RNA fractions, which indicates that ARR can be extracted out of the gel inserts during the SDS incubation. Although it is tempting to dismiss the recovery of soluble ARR counts as artifacts of the isolation procedure, the large percentage of counts recovered (almost 10% of the carbon 14 radiolabel) suggests that incomplete hydrolysis or inefficient removal of hydrolysed counts cannot fully account for such a large recovery. A more plausible explanation is that some component of the ARR is being

extracted out of the insert and recovered. Whether this soluble ARR is associated with simple linear pieces of DNA fragments and/or more complex structures remains to be clarified. Recent investigations in our laboratory provide evidence that supercoiled DNA structures are being released during both SDS extraction and gel electroelution (data not shown).

Exactly how unmodified uridine radiolabel can be recovered from ARR following nuclease P1 and alkaline phosphatase digestions remains to be determined. One plausible explanation for the recovery of uridine from ARR is that during alkaline hydrolysis, uridine or whatever ribonucleoside is immediately adjacent to DNase I-resistant DNA cannot be removed. It is known that RNA dimers composed of one 2'-o-methylated nucleoside and one unmethylated nucleoside are resistant to alkali (Bock, 1968; Maden and Salim, 1974; Rottman, 1978; Nishimura, 1979). A deoxynucleoside adjacent to an unmethylated ribonucleoside should provide similar protection against alkaline hydrolysis. A second explanation for the alkali resistance of ARR is chemical modifications at the 2' hydroxyl position of the ribose moiety which confers alkali/RNase A resistance. The two most likely protecting groups are methyl and phosphate groups. Methyl groups have been found to be associated with ribosomal RNA (Maden and Salim, 1974), transfer RNA (Nishimura, 1979) and capped messenger RNA's (Rottman, 1978). The largest alkali-resistant RNA fragments isolated to date belong to ribosomal RNA's which are 2'-o-methylated ribonucleotides 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 an ethanol precipitation procedure following such extensive treatments as SDS extraction and electroelution. Finally, the ability of alkaline phosphatase to isolate uridine from ARR suggests that any protecting group at the 2' hydroxyl position of ribose would probably be phosphate and not a methyl group. Phosphate groups at the 2' hydroxyl position of the ribose moiety have been found to be associated with nuclear RNA's (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 tri-phosphorylated ribonucleosides has been demonstrated (Wallace and Edmonds, 1983; Ruskin et al, 1984; Kiberstis et al, 1985). In the absence of nuclease P1 treatment and under more gentle heat treatments ARR is at best only slightly sensitive to alkaline phosphatase (not shown). After digestion with nuclease P1 and under more stringent conditions of heating, alkaline phosphatase does release uridine from ARR. The RNA tetranucleotide model described in Figure 2A can be cleaved by nuclease P1 at all 3' positions except the one adjacent to the phosphorylated 2' position (Wallace and Edmonds, 1983). If this is digested by alkaline phosphatase, the end product generated will be a trinucleotide. Therefore, such a model cannot explain how nuclease P1 and alkaline phosphatase can release uridine from ARR. On the other hand, if the ARR were complementary to the RNA splice site as shown in Figure 2B, then, the 2' phosphorylated position would be adjacent to a 5' instead of a 3' position. The 3' positions would be available for hydrolysis by nuclease P1 so that the product in Figure 2C would be generated, and after alkaline phosphatase treatment, free uridine could be released (Figure 2D). It is of interest to note that 79% of the triphosphorylated nucleosides associated with RNA splice sites are adenosine (Wallace and Edmonds, 1983) which would mean that 79% of the complementary ARR splice sites should be uridine. Of course, the releasing of free uridine from ARR by sequential treatments with nuclease P1 and alkaline phosphatase could also be achieved from uridine not modified at the 2' position if it was contiguous with linear DNA nucleotides. The final explanation for the uridine release will require additional study into the nature of the nucleotides generated from ARR by nuclease P1 digestion.

The resistance of DAR to the inhibition of DNA synthesis, its tight and stable association with DNase I resistant DNA fragments comparable in size to Okazaki fragments, its apparent association with protein, its uridine composition, and its high uracil content compared to whole cell DNA, all suggest that DAR could be Okazaki RNA primers that remain bound near to or at the origins of DNA replication in a form that renders them partially insensitive to alkali and RNase

A. Similar observations have been described elsewhere (Birkenmeyer, 1987). As such, it is a good candidate for alkali sensitive linkers and A+T rich regions, and because it is a mosiac of DNA, protein, and ARR, it is also a good candidate for unclonable areas. Because of the potential for cross links between ARR and other matrix elements it is tempting to suggest that ARR may be involved in attaching origin DNA to other elements within the nuclear matrix. The apparent sensitivity of DAR to phospholipase C (albeit preliminary) hints of linkages between DAR and phospholipids, thus implying structural roles for DAR within the nuclear matrix. Results reported by others suggest that DAR is tightly associated with DNA at matrix attachment sites which are developmentally regulated (Patriotis, 1990). The dual observation that RNase A digestion can generate a small loss of DNA and RNA and at the same time increase overall retention of the DNA and remaining RNase A resistant DAR in gel inserts during electroelution is remarkable and suggests that DAR may play an important role in the integrity of DNA superstructure.

We recognize that the stability of the DNA/DAR association during the 24 hour pulse-chase/serum arrest incubation could represent nothing more than uptake of uridine into primer which is not being removed because of a replication block. And, the ability of RNase A to clip out pieces of DNA along with a portion of the DAR may represent the loss of Okazaki DNA fragments flanked by DAR. However, the intimate relationship of DAR with DNase I resistant DNA, its unusual behavior during phenol extraction, and the unusual chemical stability of its ARR subcomponent provide adequate justification for continued investigations of these unusual structures and into possible novel functions DAR may be performing at the level of the nuclear matrix.

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Table I. *Retention of [^{14}C]Uridine Radiolabel in Gel Inserts Following Successive Enzyme Treatments and Electroelutions*

Experimental Procedures	CPM/Gel Insert(s)
EE -> SI -> EE -> SI --------> EE -> SI -> EE	1006
EE -> **PK** -> EE -> SI --------> EE -> SI -> EE	1784
EE -> PK -> EE -> **RA** -------> EE -> SI -> EE	2095
EE -> PK -> EE -> **PC** -------> EE -> SI -> EE	744
EE -> PK -> EE -> (PC + **RA**) -> EE -> SI -> EE	875, 230
EE -> PK -> EE -> (PC + RA) -> EE -> **DI** -> EE	0

Seven gel inserts containing [^{14}C]uridine labeled mouse nuclei were subjected to overnight extraction in 1% SDS buffer followed by a series of electroelutions and incubations in appropriate buffers with, or without, enzymes. The sham incubated gel insert (top row) received no enzyme treatments. All other inserts were subjected to various parallel incubation treatments as depicted under Experimental Procedures. Successive treatments are indicated by arrows and changes in the protocol, as one proceeds down the table, are shown in bold face type. SI = sham incubation (enzyme buffer, no enzyme), PK = proteinase K, RA = RNase A, PC = phospholipase C, DI = DNase I, and EE = electroelution. After all treatments were completed, the inserts were melted and counted by liquid scintillation (CPM/Gel Insert).

Table II. *Percent of Total [^{14}C]Thymidine, [^{14}C]Uridine , and [^{14}C]Leucine Radiolabel Isolated From Single Labeled Gel Inserts Subjected to Electroelution With or Without Treatments in RNase A and DNase I*

	PERCENT RADIOLABEL					
Label	Thymidine			Uridine		Leucine
Treatment	SI	DI	RA->DI	DI	RA->DI	RA->DI
Sample						
Insert	47.0	7.7	5.8	0.4	0.4	0.2
Supernatant	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
SDS Extraction	51.6	52.5	59.9	94.8	96.6	99.5

Gel inserts contained mouse cells radiolabeled with either [^{14}C]thymidine, [^{14}C]uridine, or [^{14}C]leucine and extracted overnight in 1% SDS buffer. They were electroeluted and then sham incubated in buffer (SI) or in the presence of RNase A (RA). After the incubation period the gel inserts were electroeluted a second time and then sham incubated in buffer or in the presence of DNase I (DI). Inserts incubated in RNase A, electroeluted, and incubated in DNase I are denoted as follows: (RA->DI). The DNase I or final sham incubation buffers were precipitated overnight in ethanol or trichloroacetic acid, centrifuged, and the pellets rinsed twice prior to solubilization and counting. The trichloroacetic acid data is not shown but is equivalent to the ethanol. The data is presented as percentages of total counts recovered (Pellets). Other data presented include the percentage of counts recovered from the ethanol supernatants (Supernatants), the inserts, (Inserts), and the SDS extraction buffers and tube gels (SDS Extraction). Most of the radiolabels were in SDS extraction buffers and pre-RNase A tube gels.

Table III. *Percentage of Whole Cell [^{14}C]Thymidine or [^{14}C]Uridine Radiolabels Isolated From Gel Inserts Subjected to Electroelution, DNase I Digestion, Heat, and Alkali*

	PERCENT RADIOLABEL			
Ethanol Buffer	Thymidine (Buffer)	Thymidine (KOH)	Uridine (Buffer)	Uridine (KOH)
TNM	0.9	0.7 (78)	1.0	0.4 (40)
TNE	1.8	1.0 (56)	0.5	0.3 (60)

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 (TNM) or Tris, NaCl, EDTA (TNE) buffers, centrifuged, rinsed once to remove buffer, and subjected to 100°C for 20 minutes in the presence of either Tris buffered solution, pH 8.0 or in 0.1N KOH. After hydrolysis the tubes containing KOH were neutralized and buffered. All solutions were ethanol precipitated, centrifuged, and the pellets rinsed twice, resolubilized, and counted. The data is presented as percentages of total counts recovered. TNE also contained 250 ug/ml carrier DNA. Data in parentheses represent label recovered under alkaline conditions as a percentage of label recovered in buffer.

Table IV. *Effect of Phenol Extraction on ARR Recovery*

Samples	p/a	phenol	chlor	sup 1	sup 2	ARR
A	--	--	--	74.4	0.5	25.1
B	27.5	21.0	0.1	34.2	0.1	17.1
C	85.9	1.7	0.1	2.6	0.1	9.6
D	62.7	0.6	0.1	10.9	0.0	25.7

The "total" [^{14}C]uridine counts described in this table are those associated with inserts after SDS extraction and gel electroelution. In column 1 the samples A and B represent DNased digested DNA and C and D denotes DNA released from melted inserts. Only samples B, C, and D were subjected to phenol extraction and these were vortexed during the extraction procedures. Fifty µg/ml of proteinase K was added to sample D and both samples C and D were incubated overnight at 37 °C. In column 2 " p/a" represents the percentage of total counts released from the inserts remaining in the phenol/aqueous interface after removing the aqueous phase and back extracting once. Columns 3 and 4 indicate the percentage of total counts retained in the phenol and chloroform respectively. Columns 5 and 6 contain the percentage of total counts recovered from ethanol supernatants before and after alkaline hydrolysis respectively. Column 7 contains the percentage of the total counts which can be precipitated by ethanol as ARR.

Table V. *Comparison of Relative Alkali Resistance of ARR to non-ARR*

	Set 1		Set 2	
	[^{14}C] uridine	[^{3}H] uridine	[^{3}H] uridine	[^{14}C] uridine
	Gel insert	SDS extract	Gel insert	SDS extract
0.1 M KCl	1461 ± 44	210898 ± 1652	10585 ± 1009	24092 ± 1093
0.1 M KOH	1469 ± 99	6793 ± 3199	4566 ± 729	2365 ± 1476
t statistic	-0.05	37.79	3.23	7.89
signif level	>> 0.2	<< 0.001	< 0.013	< 0.001
KOH/KCl	1.01	0.03	0.43	0.10
Index	10.10	-----	14.33	-----

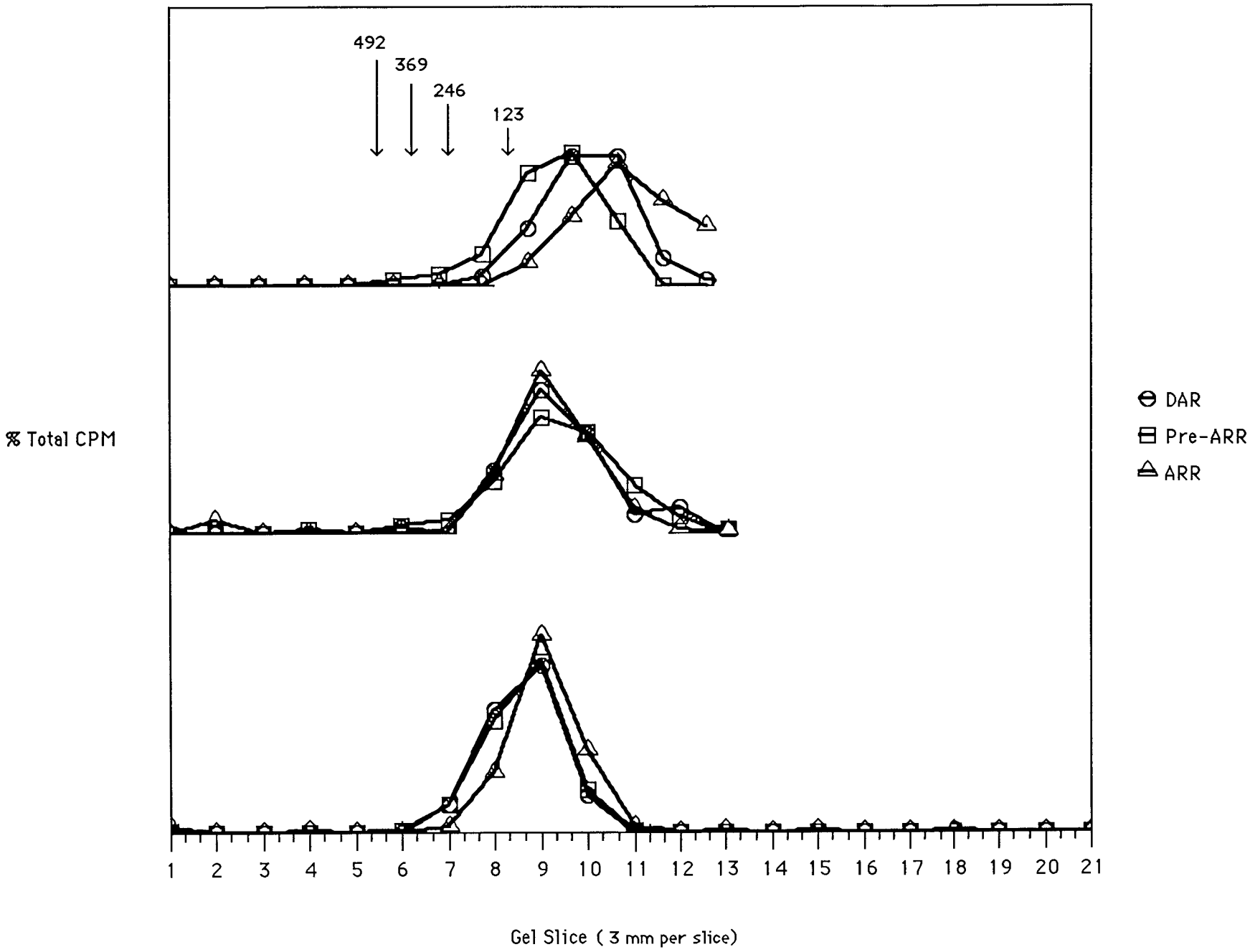
Mouse L-1210 cells were radiolabeled with either [^{3}H]uridine or [^{14}C]uridine, encapsulated into gel inserts and extracted with SDS. The inserts were electroeluted to remove non-bound RNA label. The inserts containing [^{3}H]uridine were added to the SDS extracts containing [^{14}C]uridine and vice-versa. The two combinations were heated to melt the inserts and the two labels were mixed. The final solutions were subjected to phenol/chloroform extraction and ethanol precipitation. The pellets were redissolved, split, ethanol precipitated, rinsed free of buffer, heated in either KCl or KOH, neutralized (where applicable), and again ethanol precipitated. These pellets were rinsed twice, dissolved, and counted by liquid scintillation. Samples were assayed in triplicate and the means and standard errors of the counts are displayed in the first two data rows. Differences in the two means (KCl versus KOH treated) were measured by the student t test (data row 3). The levels of significance are for one-tailed tests at 4 degrees of freedom (data row 4). The ratios of the two means (KOH/KCl) are shown in data row 5. The index on the left in data row 6 was calculated by dividing the KOH/KCL ratio of the inserts containing [^{14}C]uridine by the KOH/KCL ratio of the SDS extracts containing [^{14}C]uridine. The corresponding [^{3}H]uridine index is shown on the right.

Table VI. *HPLC Retention Times of ARR Nucleosides From Cells Labeled with [^{14}C]Uridine*

	RETENTION TIMES					
	Uridine		Deoxyuridine		Thymidine	
	UV	^{14}C	UV	^{14}C	UV	^{14}C
Standards	4.03	--	6.34	--	18.23	--
Sample 1 (-dNs)	4.10	4.10	6.65	ND	18.80	ND
Sample 2 (+dNs)	3.30	3.30	(see legend)		18.10	ND

ARR pellets single labeled with [^{14}C]uridine were digested sequentially in nuclease P1 and alkaline phosphatase to generate nucleosides as described in Experimental Procedures. Retention times (RT, recorded in minutes) of uridine, deoxyuridine, and thymidine standards were recorded following UV detection at 260 nm. Immediately following, ARR samples were injected sequentially and analyzed by both UV and radiolabel. In both samples all of the radiolabel was confined to a single peak coeluting with the uridine. Sample 1 is ARR from cells not exposed to exogenous deoxynucleosides (dNs). Sample 2 is ARR from cells exposed to 50 ug/ml of the four dNs as described in Experimental Procedures. Compared to the non-radiolabeled standards, the RTs for uridine, deoxyuridine, and thymidine of Sample 1 were slightly increased; the RTs for uridine and thymidine of Sample 2 were slightly decreased. Deoxyuridine was virtually absent in Sample 2. ND = none detected.

Fig. 1. DAR, DAR heated to 100 °C, and ARR were loaded into separate submersible tube gels containing 2% Sea plaque a garose and electrophoresed for 3 hours at 35 volts. The first set of gels (DAR, heated DAR, and ARR) each contained 123 base pair DNA ladder markers (top curve). The second and third sets of gels contained no markers (middle and bottom curves respectively). All the gels were removed from their tubes and sets 1 and 2 were stained with ethidium bromide, aligned along their long axes, and photographed under UV light. No samples were detectable with ethidium bromide staining alone. All the gels were sliced into 3 mm sections which were diluted in water, melted, mixed with scintillation cocktail, and counted. All data was tabulated as percentages of total counts. Four markers (123-492 base pairs) were measured in the photographs and their migration distances calculated and superimposed on the graph.


492
369
246
123
% Total CPM
DAR
Pre-ARR
ARR
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Gel Slice (3 mm per slice)

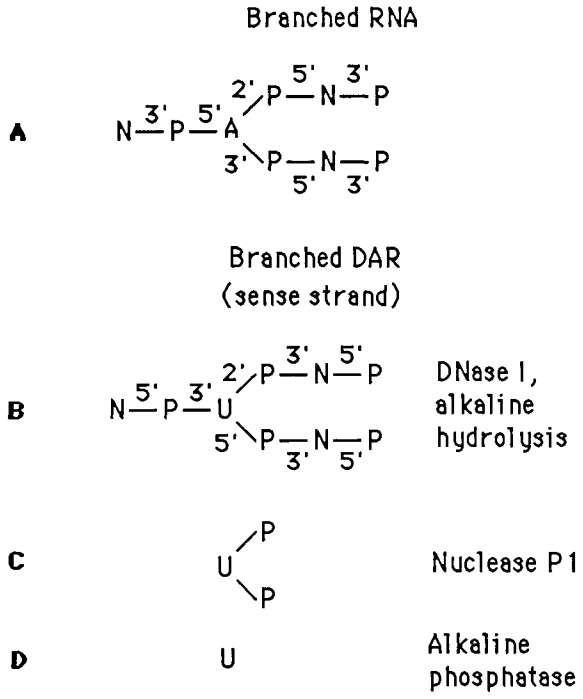


Fig. 2. A diagram of a tetranucleotide RNA splice site is shown in 1A, the splice site is primarily composed of adenosine (after Wallace and Edmonds, 1983). A, U, N, and P represent adenosine, uridine, any nucleoside, and phosphate respectively. The model in 1B represents a portion of the DNA sense strand after DNase I digestion and alkaline hydrolysis. It is composed of ARR and DNA and is responsible for the transcription of the RNA splice site. When it is digested by nuclease P1 the 3' bonds are digested, converting it into uridine 2', 5' diphosphate (1C). Free uridine is released following digestion in alkaline phosphatase (1D).