The KpnI sequences constitute the dominant, long, interspersed repetitive DNA families in primate genomes. These families contain related, but nonidentical sequence subsets, some of which border functional gene domains and are transcribed into RNA. To test whether these sequences perform an organizational function in the nucleus, their association with the nuclear matrix has been examined in African green monkey cells. DNase I treatment depleted the residual matrix of most of the KpnI 1.2- and 1.5-kilobase pair family sequences although significant amounts of each family remained in the loop attachment DNA fragments. Hybridization analysis of the KpnI and RsaI cleavage patterns of matrix loop attachment DNA indicate that some sequence subsets of these KpnI families are relatively less depleted than others. The nuclear matrix association of subpopulations of KpnI 1.2- and 1.5-kilobase pair families was also shown by metrizamide gradient centrifugation of nuclear matrix complexes cleaved by KpnI endonuclease. The gradients demonstrate that some KpnI segments are differentially associated with nuclear matrix proteins. Moreover, the procedures permit the preparative isolation and purification of the DNA-protein complexes containing these KpnI 1.2- and 1.5-kilobase pair sequence families. Speculations on the relationship between the matrix association of these KpnI family sequences and their possible roles in gene organization and expression are presented and discussed.

The nuclear matrix has been proposed as an attachment site for the anchoring of higher order chromatin structures referred to as DNA loops. These loops, with 60–100 kb of DNA, may contain functional domains of related genes whose replication, transcription, and/or regulation occur at the proteinaceous scaffold of the nuclear matrix (reviewed by Hancock, 1982a, 1982b). It remains unclear whether the repeating loop structures are anchored to the matrix scaffold via specific attachment sequences or whether the anchoring is random with respect to DNA sequences.

Likely candidates for DNA structural elements involved in the attachment of DNA loops are those which contain families of long interspersed repeats, now collectively referred to as LINEs (Singer, 1982). The LINE sequences in primate genomes are the KpnI families which are cleaved into discrete DNA fragments by KpnI endonuclease (Maio et al., 1981). The various families of KpnI sequences account for a substantial part (~4%) of the human and African green monkey (AGM) genomes and consist of related but nonidentical sequence subsets (Maio et al., 1981; Shafit-Zagardo et al., 1982a; Chimera, 1984). These primate KpnI families are related to the mouse LINE family sequences denoted BamHI or MIF-1. This relatedness includes shared sequence homologies although they are not identical (Singer et al., 1983). In addition, individual members of the primate KpnI and the mouse MIF-1 families have been found to be transcribed into RNA and to be present both at the boundaries and within β-globin gene domains (Shafit-Zagardo et al., 1982b, 1983; Heller et al., 1984). This relatedness of the different LINE sequences together with their sequence conservation indicates that they perform one or more important functions in genome organization. The observed microheterogeneity in the KpnI family subsets suggests that individual sequence subsets of these families may perform different nuclear functions. The observed transcription of some KpnI sequences into nuclear RNAs suggests their possible involvement in the control of transcription and/or RNA processing (Shafit-Zagardo et al., 1983). Their location at the boundaries of gene domains and their transcription suggest that these KpnI families, or specific sequence subsets, may participate in the nuclear matrix attachment and regulation of particular gene domains. Determining if there is a role for the KpnI sequences in the nuclear matrix structure will also increase our understanding of the functional importance of the evolutionarily conserved LINE sequence families.

The KpnI families have not been examined for a possible DNA loop anchoring function. For this reason, we have investigated the nuclear matrix association of the KpnI 1.2- and 1.5-kb families in AGM cells. While their relative content in residual matrix is reduced, the data indicate that some members (specific sequence subsets) of both families are present in the nuclear matrix and are differentially associated with matrix proteins.

(Received for publication, December 6, 1984)
Matrix Association of KpnI DNAs

EXPERIMENTAL PROCEDURES

RESULTS

KpnI 1.2- and 1.5-kb Sequence Families and Subpopulations—Cleavage of purified AGM nuclear DNA by KpnI released three distinct fragments of 1.2, 1.5, and 1.9 kb (Fig. 1, lane B). These discrete fragments are representatives of the KpnI 1.2-, 1.5-, and 1.9-kb interspersed, repetitive sequence families in the AGM genome. Analysis of this restriction pattern by blot hybridization using cloned human 1.2- and 1.5-kb sequence probes reveals sequence heterogeneity in these two KpnI families. The spacing and frequency of KpnI sites describe three populations of KpnI 1.2-kb family sequences. Over 60% of the family sequences were reduced to 1.5-kb fragments (KpnI-resistant population) of the sequences remained as longer fragments (KpnI-sensitive population), and less than 20% remained longer than 6.4 kb (KpnI-resistant population). As with the KpnI 1.2-kb family, Rsal reduced the KpnI 1.5-kb family to a simpler set of smaller fragments of 1.5, 0.927, 0.795, and 0.702 kb (Fig. 1, lane G). Less prominent, minor Rsal fragments which hybridize to the KpnI 1.5-kb probe are visible on longer autoradiographic exposures (data not shown).

These results indicate that although 60% of the 1.2- and 1.5-kb family sequences in AGM DNA have KpnI sites at spacings of 1.2 and 1.5 kb, respectively, other sequence populations do exist. Moreover, in studying subfractions of AGM DNA or different preparations of nuclease-treated matrix DNA, it will be possible to analyze the three populations of each family either separately using KpnI restriction analysis or together by Rsal cleavage analysis. A fuller description of the short- and long-range organization of these KpnI sequence families will be presented elsewhere (Chimera, 1984).

Isolation and Characterization of Nuclear Matrices—Relatively mild digestion of DNA-rich nuclear matrix with low concentrations of DNase I (80 units/ml) resulted in the gradual cleavage and release of DNA loops from the matrix. Depending on the extent of digestion, from 67 to 2.8% of the total DNA remained attached to the residual nuclear matrix (Table I). This residual DNA represents portions of the loops that occur progressively nearer to the sites of loop attachment to the proteinaceous nuclear scaffold. Those DNA fragments remaining with the residual matrix will be referred to as attachment DNA (att-DNA) sequences.

Analysis of att-DNA fragment lengths indicated that undigested loops (>40 kb) were reduced to a mean length of 19 kb after mild digestion (Table I). It is possible that only a portion of the 10-kb att-DNA fragments is actually involved in anchoring a DNA loop to the proteinaceous scaffold. To examine this possibility, DNA-rich nuclear matrix was digested extensively with DNase I (160 units/ml). If a portion of the 10-kb att-DNA is indeed anchored to protein, then it should be partially protected from DNase I cleavage and, as a result, exhibit larger fragment sizes than unprotected control DNA. The data in Table II indicate that att-DNA remained consistently larger than similarly digested control DNA.

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2 Portions of this paper (including "Experimental Procedures" and Figs. 3 and 4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size fotocopies are available from the Journal of Biological Chemistry, 8650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-3694, cite the authors, and include a check or money order for $8.40 per set of fotocopies. Full size fotocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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3 J. A. Chimera and P. R. Musich, manuscript in preparation.
At the times indicated, aliquots were removed, the DNA was purified, and the fragment sizes were determined.

<table>
<thead>
<tr>
<th>DNase I incubation time (min)</th>
<th>DNA fragment size (kb)</th>
<th>DNA fragment purified</th>
<th>att-DNA</th>
<th>Control DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&gt;40</td>
<td>&gt;40</td>
<td></td>
<td></td>
</tr>
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<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>90</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

**RESIDUAL DNA**

<table>
<thead>
<tr>
<th>SIZE (kb)</th>
<th>1.2 kb FAMILY</th>
<th>1.5 kb FAMILY</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;40</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>9.7</td>
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<td>44</td>
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<tr>
<td>2.4</td>
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<td>24</td>
</tr>
<tr>
<td>0.4</td>
<td>17</td>
<td>25</td>
</tr>
</tbody>
</table>

**Fig. 2. Hybridization of KpnI probes to residual att-DNA.**

DNA-rich nuclear matrix was extensively digested with DNase I. At various times, residual matrix was isolated and the DNA was purified. Equal amounts of att-DNA samples were denatured and slot-blotted onto Zeta-Probe membrane for hybridization analysis using KpnI 1.2- and 1.5-kb family probes. The per cent hybridization (%) Hybrid) value equals the ratio of the amount of hybridization to the att-DNA divided by the hybridization to untreated matrix DNA.

The above analysis could not discriminate among the different sequence populations (KpnI-sensitive, intermediate, and KpnI-resistant) which constitute a KpnI family (see Fig. 1, lanes D and F). This ambiguity was resolved by mild DNase I digestion of DNA-rich matrix, followed by KpnI treatment of the purified att-DNA. Mild DNase I conditions were required, since KpnI segments are greater than 1 kb; they would be severely nicked under the extensive conditions used above and not resolvable as distinct restriction fragments.

To determine if mild DNase I treatment preferentially released particular sequence subsets from the nuclear matrix, the RsaI cleavage patterns of isolated att-DNA were analyzed. The results of this analysis are summarized in Table III. Hybridization with a KpnI 1.2-kb family probe revealed a reduction in all three RsaI fragments in the att-DNA. When greater than 97% of the matrix DNA was released, the att-DNA was depleted in 1.2-, 0.835-, and 0.405-kb fragments to 31, 45, and 23%, respectively. For the KpnI 1.5-kb family, all four major RsaI fragments were depleted: 1.5-, 0.927-, 0.795-, and 0.702-kb fragments were reduced to 49, 43, 23, and 23%, respectively. The differences in the calculated amounts of fragment depletion are not due to variations in hybridization efficiencies due to fragments of different size. The control DNA in each measurement consisted of fragments of the same size as the experimental fragments. Therefore, the different depletions for various RsaI fragment must reflect differential protection by the nuclear matrix proteins.

A significant difference was observed in the amount of hybridizable 1.2-kb fragments detected in the KpnI digest versus the RsaI digest of att-DNA (Tables I and III). This difference (18 versus 31%, respectively) reflects the contributions of fragments from the RsaI cleavage of the intermediate and KpnI-resistant populations in the att-DNA preparation (Fig. 1, lane E; Chimera, 1984). The hybridizable segments found in RsaI digests of att-DNA, therefore, represent the cumulative contributions from the three sequence populations in the 1.2-kb family. Likewise, the different amounts of hybridizable 1.5-kb (KpnI-sensitive) segments detected in the KpnI and RsaI digests of att-DNA have a similar explanation.

**Matrix Association of KpnI DNAs**

DNA-rich nuclear matrix and control (protein-free) DNA were digested extensively with DNase I (160 units/ml) at the times indicated, aliquots were removed, the DNA was purified, and the fragment sizes were determined.

**TABLE II**

*Comparison of DNA fragment sizes of att-DNA and control DNA after treatment with DNase I.*

DNA-rich nuclear matrix and control (protein-free) DNA were digested extensively with DNase I (160 units/ml). At the times indicated, aliquots were removed, the DNA was purified, and the fragment sizes were determined.

<table>
<thead>
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<th>DNA fragment purified</th>
<th>att-DNA</th>
<th>Control DNA</th>
</tr>
</thead>
<tbody>
<tr>
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<td>&gt;40</td>
<td>&gt;40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9.7</td>
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<td>2.7</td>
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</tr>
<tr>
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<td>2.4</td>
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</tr>
<tr>
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<td>0.4</td>
<td>0.4</td>
<td>&lt;0.1</td>
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</table>

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<tbody>
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<tr>
<td>9.7</td>
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<td>44</td>
</tr>
<tr>
<td>2.4</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>0.4</td>
<td>17</td>
<td>25</td>
</tr>
</tbody>
</table>

**Fig. 2. Hybridization of KpnI probes to residual att-DNA.**

DNA-rich nuclear matrix was extensively digested with DNase I. At various times, residual matrix was isolated and the DNA was purified. Equal amounts of att-DNA samples were denatured and slot-blotted onto Zeta-Probe membrane for hybridization analysis using KpnI 1.2- and 1.5-kb family probes. The per cent hybridization (%) Hybrid) value equals the ratio of the amount of hybridization to the att-DNA divided by the hybridization to untreated matrix DNA.

The most extensive DNase I treatment reduced att-DNA to a mean fragment length of 0.4 kb, compared to <0.1 kb for control DNA. Thus, some portions of the 10-kb att-DNA fragments appear to be more intimately associated with matrix proteins than do other portions.

**Presence of KpnI DNAs in the Nuclear Matrix**—To determine the relative amounts of KpnI sequences present in att-DNA, DNA-rich nuclear matrix was digested with DNase I to various degrees. The extensive digestion conditions were used to determine the content of KpnI sequences in the fragments most intimately associated with matrix proteins. Fig. 2 illustrates a gradual decrease in the total hybridizable 1.2- and 1.5-kb family sequences in att-DNA as the extent of degradation increases. The content of hybridizable 1.2-kb family sequences in att-DNA fragments of 0.4 kb was less than 20% of the untreated DNA value. Similarly, att-DNA fragments of 0.4 kb were depleted in hybridizable 1.5-kb family sequences to 25% of the initial value.

The above analysis could not discriminate among the different sequence populations (KpnI-sensitive, intermediate, and KpnI-resistant) which constitute a KpnI family (see Fig. 1, lanes D and F). This ambiguity was resolved by mild DNase I digestion of DNA-rich matrix, followed by KpnI treatment of the purified att-DNA. Mild DNase I conditions were required, since KpnI segments are greater than 1 kb; they would be severely nicked under the extensive conditions used above and not resolvable as distinct restriction fragments.

att-DNA fragments of 10 kb were reduced in their content of EBr-stained 1.2-kb segments to 64% of the amount present in undigested DNA (Table I). A depletion in EBr-stained 1.5-kb segments to 35% of the control amount was also observed. Blot hybridization of the same KpnI segments with 1.2- and 1.5-kb family probes indicated that hybridizable 1.2-kb segments were reduced to 18% and 1.5-kb segments to 20% of their respective contents in undigested DNA (Table I).

To determine if mild DNase I treatment preferentially released particular sequence subsets from the nuclear matrix, the RsaI cleavage patterns of isolated att-DNA were analyzed. The results of this analysis are summarized in Table III. Hybridization with a KpnI 1.2-kb family probe revealed a reduction in all three RsaI fragments in the att-DNA. When greater than 97% of the matrix DNA was released, the att-DNA was depleted in 1.2-, 0.835-, and 0.405-kb fragments to 31, 45, and 23%, respectively. For the KpnI 1.5-kb family, all four major RsaI fragments were depleted: 1.5-, 0.927-, 0.795-, and 0.702-kb fragments were reduced to 49, 43, 23, and 23%, respectively. The differences in the calculated amounts of fragment depletion are not due to variations in hybridization efficiencies due to fragments of different size. The control DNA in each measurement consisted of fragments of the same size as the experimental fragments. Therefore, the different depletions for various RsaI fragment must reflect differential protection by the nuclear matrix proteins.

A significant difference was observed in the amount of hybridizable 1.2-kb fragments detected in the KpnI digest versus the RsaI digest of att-DNA (Tables I and III). This difference (18 versus 31%, respectively) reflects the contributions of fragments from the RsaI cleavage of the intermediate and KpnI-resistant populations in the att-DNA preparation (Fig. 1, lane E; Chimera, 1984). The hybridizable segments found in RsaI digests of att-DNA, therefore, represent the cumulative contributions from the three sequence populations in the 1.2-kb family. Likewise, the different amounts of hybridizable 1.5-kb (KpnI-sensitive) segments detected in the KpnI and RsaI digests of att-DNA have a similar explanation.

**Metrizamide Gradient Fractionation of KpnI-digested Matrix**—We have demonstrated that the DNA component of the

**TABLE III**

*Content of RsaI segments of KpnI families in att-DNA.*

DNase I-treated matrix, containing 2.8% of the input DNA, was isolated. Equal amounts of purified att-DNA were digested with RsaI, separated on a 1.0% agarose gel, and blotted to Zeta-Probe membrane for hybridization analysis with KpnI 1.2- and 1.5-kb family probes.

<table>
<thead>
<tr>
<th>Control DNA*</th>
<th>Att-DNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td></td>
</tr>
<tr>
<td>1.2-kb family RsaI fragment size (kb)*</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>0.835</td>
</tr>
<tr>
<td></td>
<td>0.408</td>
</tr>
<tr>
<td>1.5-kb family RsaI fragment size (kb)*</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>0.927</td>
</tr>
<tr>
<td></td>
<td>0.795</td>
</tr>
<tr>
<td></td>
<td>0.702</td>
</tr>
</tbody>
</table>

*The control DNA was prepared from undigested DNA-rich matrix.

*The content of the RsaI segments in the att-DNA was calculated as the per cent value relative to the content of respective fragment in the control DNA. The per cent values were calculated by densitometric quantitation of the bands in autoradiograms of the appropriate blot hybridizations.

*These RsaI fragments contain sequences from all three populations (KpnI-sensitive, intermediate, and KpnI-resistant).
residual matrix contains members from the KpnI 1.2- and 1.5-kb families. To gain an understanding of the possible role(s) of KpnI DNAs in the anchoring of DNA loops to the matrix, methods were developed to isolate KpnI DNA-protein complexes.

Nuclei were subjected to a series of high salt (2 M NaCl) buffer extractions and differential centrifugation steps to remove ionically bound histones and nonhistone proteins. Those proteins remaining are the nuclear matrix proteins. The DNA-rich nuclear matrix was digested with KpnI, and the digestion products were fractionated by isopycnic centrifugation in metrizamide gradients. The bulk of the DNA banded at a density of 1.14-1.16 g/cm³ and was contained in fractions 9, 10, and 11. Lesser amounts of DNA were found in fractions with higher densities (Fig. 3). This distribution represents a continuum of DNA complexed with increasing amounts of bound protein. In metrizamide gradients in low salt buffers, protein-free DNA bands at a density of approximately 1.12 g/cm³, while DNA-protein complexes were at densities ranging from 1.185 to 1.247 g/cm³ (Rickwood, 1976; Monahan and Hall, 1974). The densities reported here are 0.02-0.04 g/cm³ higher due to the 1 M NaCl in the gradients, which decreases the hydration of the macromolecules, resulting in an increased buoyant density (Rickwood, 1976). The two smaller peaks, observed in fractions 16-18 and 19-21, do not appear to be DNA and may represent ribonucleoprotein contaminants.

The majority of the 1.2-kb family sequences were found in fractions 9, 10, and 11, which contained greater than 65% of the DNA loaded on the gradient (Fig. 4A). Some of these fragments represent protein-free sequences cleaved from the DNA loops of the matrix by KpnI endonuclease. Relative to the intermediate and KpnI-resistant populations there is an enrichment for the KpnI-sensitive population of segments (1.2 kb) in fractions with increasing density (see Table IV). For example, the DNA in fraction 13 exhibited an approximate 2-fold relative enrichment for 1.2-kb segments. The majority of 1.5-kb family sequences were also present in fractions 9, 10, and 11 (Fig. 4B), although there was an approximately 1.7-fold enrichment for the 1.5-kb segments in fraction 13 (Table IV). The higher buoyant densities in this portion of the metrizamide gradients (~1.19 g/cm³) indicate that these 1.2- and 1.5-kb fragments were associated with matrix proteins. Thus, certain members of both the 1.2- and 1.5-kb families appear to remain associated with greater amounts of matrix proteins and, by implication, must be differentially associated with these proteins. The amounts of protein in the higher density regions of the gradients were too low for their characterization by sodium dodecyl sulfate gel electrophoresis using the sensitive silver staining procedure (Wray, 1981).

Using the sensitive silver staining procedure and having only 1.2- and 1.5-kb families, the fractions were fractionated in the metrizamide gradient and then digested with KpnI. The DNA was then blotted to diazobenzyloxyethyl paper and hybridized with radioactive probes to either the KpnI 1.2- or the 1.5-kb family. The data presented here were quantitated by densitometric scanning. The ratios listed represent the relative amount of probe hybridization to the 1.2- and 1.5-kb segments relative to the total hybridization to the DNA in that fraction.

Table IV: Distribution of KpnI sequence families in metrizamide gradients

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>1.2-kb fragments*</th>
<th>1.5-kb fragments*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total DNA</td>
<td>0.42</td>
<td>0.45</td>
</tr>
<tr>
<td>9</td>
<td>0.95</td>
<td>0.30</td>
</tr>
<tr>
<td>10</td>
<td>0.48</td>
<td>0.36</td>
</tr>
<tr>
<td>11</td>
<td>0.70</td>
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</tr>
<tr>
<td>12</td>
<td>0.72</td>
<td>0.73</td>
</tr>
<tr>
<td>13</td>
<td>0.80</td>
<td>0.77</td>
</tr>
<tr>
<td>14-16</td>
<td>ND*</td>
<td>ND*</td>
</tr>
</tbody>
</table>

*The hybridization distribution patterns in the autoradiograms shown in Fig. 4 were quantitated by densitometric scanning. The ratios listed represent the relative amount of probe hybridization to the 1.2- or 1.5-kb segments relative to the total hybridization to the DNA in that fraction.

ND, not determined; the intensity of hybridization to the 1.2- and 1.5-kb fragments in fractions 14-16, although detectable, was too low for accurate quantitation.

DISCUSSION

The structural organization of the eukaryotic nucleus has been the subject of intense investigation. DNA is arranged in the nucleus as loops of 60-100 kb, which are anchored to a proteinaceous nuclear matrix (Cook and Brazil, 1975, 1976; Hancock, 1982b). The nature of the DNA attachment sequences which anchor the loops and their binding to the matrix proteins has not yet been established. The data presented here suggest that >18% of the AGM nuclear DNA, in DNA fragments of about 10 kb, is associated with nuclear matrix proteins. This value presumes that the nuclear matrix proteins afforded protection to att-DNA against mild DNase I digestion. More extensive DNase treatments indicate that within the 10-kb att-DNA fragments, one or more regions of about 0.4 kb appear to be more intimately associated with matrix proteins than other regions. Thus, when examining att-DNA for specific DNA sequence classes, it is important to consider how experimental conditions may influence the size and composition of the att-DNA fragments in the residual matrix. Mild nuclease digestion conditions may allow extraneous DNA in the loops to remain associated with the matrix, while extensive digestion conditions could release less protected but integral regions of the att-DNA fragments.

In control studies, bacteriophage T3 DNA, either intact or after cleavage with MboI (Sarkar et al., 1980), was incubated in the nuclear matrix preparation before loading in the metrizamide gradient. All the T3 DNA banded at a density of 1.14-1.15 g/cm³ (data not shown), indicating that there had been no redistribution of matrix proteins onto the bacteriophage DNA. In another gradient containing a KpnI digest of purified AGM DNA, all the KpnI fragments were found at a density of 1.14-1.15 g/cm³ (data not shown). Thus, the observed banding of KpnI 1.2- and 1.5-kb fragments at densities of ~1.19 g/cm³ does not appear to result from unusual density properties of these fragments, their size, or the formation of nonspecific DNA-protein complexes during gradient preparation.
ments of approximately 0.4 kb, and the resulting att-DNA must be operationally defined as those fragments that resist DNase I cleavage under these extreme digestion conditions.

In order to examine the content of the individual KpnI sequence populations (KpnI-sensitive versus KpnI-resistant) in att-DNA, mild DNase I digestion conditions were chosen. Both general (EBr) and specific (blot hybridization) techniques revealed a reduction in the amount of KpnI 1.2-kb segments. However, a greater depletion was detected by hybridization analysis than by EBr staining. This suggests that some fragments in the 1.2-kb stained band represent a sub-population of the 1.2-kb sequence family that lacks homology to the cloned human probe used here. Furthermore, the difference between the staining and the hybridization results suggests that populations of the nonhomologous 1.2-kb segments may be enriched in the att-DNA relative to those studied here. To address this possibility, these non-homologous 1.2-kb KpnI fragments are being cloned from the att-DNA preparations for direct analysis. Analysis of 1.5-kb segments also revealed that they were present in att-DNA in reduced amounts, indicating that the bulk of the 1.5-kb sequences are not matrix-associated.

Individual KpnI family subsegments, released from att-DNA by Rsal, were also examined. All segments belonging to both the 1.2- and 1.5-kb families were depleted in mildly digested matrix. Some of the Rsal fragments in att-DNA were depleted more than other fragments, suggesting that Rsal cleavage analysis can resolve different sequence subsets in att-DNA preparations. The data in Table III indicate that in the KpnI 1.2-kb family the sequences in the 0.835-kb Rsal fragment are most protected and those in the 0.408-kb fragment are least protected against DNase I cleavage by the matrix proteins. The nature of these differences in matrix association with the various sequence subsets remains to be resolved. However, these and other observations suggest several interesting speculations about the possible function(s) of the KpnI sequences in the nuclear matrix. Nuclear transcription occurs at the matrix (Jackson et al., 1981) to which both active genes (Robinson et al., 1982; Hentzen et al., 1984) and heterogeneous nuclear RNA (hnRNA) (van Eekelen and van Venrooij, 1981) have been found associated. Shafit-Zagardo et al. (1982b, 1983) have observed an interspersion of KpnI sequences with gene domains and the transcription of KpnI families into hnRNA. Moreover, sequence analysis of a cloned, representative member of the KpnI 1.2-kb family of AGM DNA has revealed a copy of the hexanucleotide polyadenylation signal sequence (5'-AATAAA-3') within the 0.835-kb Rsal subfragment (Chimera, 1984). This signal sequence, characteristic of DNA transcribed by RNA polymerase II, implies that this cloned DNA may represent the KpnI 1.2-kb family sequences actually transcribed into hnRNA in the cell. This possibility is consistent with our observation that in the nuclear matrix the 0.835-kb Rsal sequence is relatively more resistant to DNase I than other 1.2-kb family sequence subsets. A similar relationship between the transcriptionally active and the relatively more DNase I-resistant Rsal subfragments in the KpnI 1.5-kb family is also possible but more speculative. Some support for these speculative proposals is in the finding of long open translational reading frames in the human KpnI families whose members are also represented in hnRNA (Potter, 1984; Shafit-Zagardo et al., 1983). Verification of these proposed functional-structural relationships will require further sequence characterization of genomic and cloned KpnI family members and their hnRNA transcripts, in addition to a more detailed analysis of the matrix att-DNA and matrix-associated RNAs in AGM cells.

The differential association of some KpnI segments with matrix proteins was also demonstrated by metrizamide gradient centrifugation. Although the bulk of the hybridizable sequences were in the protein-free DNA fractions, some KpnI fragments banded as protein-DNA complexes. Specifically, the KpnI-sensitive populations of the 1.2- and 1.5-kb families were enriched in fractions with densities characteristic of DNA-protein complexes. It should be noted that the continuum of the KpnI fragment distributions in the gradient has two possible interpretations. One possibility is that the DNAs of intermediate density contain fragments with decreasing amounts of bound protein per unit fragment length (i.e., the gradual shift of 1.2- and 1.5-kb fragments). Alternatively, a particular sequence may maintain a constant amount of bound protein per unit length, but may be linked in longer DNA fragments which, overall, have a reduced amount of bound protein. This possibility may apply to the intermediate and KpnI-resistant populations of the KpnI 1.2- and 1.5-kb families, which were found in att-DNA.

This method of digesting DNA-rich matrix with restriction enzymes and fractionating the DNA-protein complexes from the protein-free DNA has numerous applications to the study of regulatory and structural DNA-protein interactions. In repetitive sequence families, the members exhibiting differing degrees of (matrix) protein association can be separated on metrizamide gradients according to the amount of associated protein. Thus, particular sequence fragments with their associated proteins can be isolated for further detailed study. Relative to the present study, scaling up of the procedure will permit the isolation of the KpnI 1.2- and 1.5-kb family members actually present in DNA-protein complexes. The sequence subset composition of the DNA and the protein composition of the complexes can then be determined. Such experimental approaches are necessary to further our understanding of the DNA sequence and polypeptide specificities of the DNA-protein interactions for gene regulation and the dynamic structural organization of the nucleus.

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Matrix Association of KpnI DNAs

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MINIPRINT SUPPLEMENT

The Association of the Interspersed Repetitive KpnI Sequences with the Nuclear Matrix by
Joseph A. Chimera and Phillip R. Musich

EXPERIMENTAL PROCEDURES

DNA Preparations--4M total nuclear DNA was prepared from strain Cfu-7 cells as described by Kornblum and Maio (1975). Control matrix DNA was prepared similarly from undigested DNA-rich nuclear matrix lysates (described below). DNA was isolated from nucleosome digested matrix samples by treatment with Protease K in an 200-EDTA buffer at 37°C for 1 hour, then phenol extracted and ethanol precipitated. To prepare DNA from metrizamide gradients fractions the metrizamide was first removed by precipitation of the DNA with ethanol. The precipitated DNA-protein complex was then treated as described above for the nucleosome digested matrix samples.

Bacteriophage T3 DNA was a gift from Umadas Maitra. The plasmid DNA used in this study were carried in E. coli strain C600 cells and were obtained from Joseph J. Maio. For preparation of their DNA the plasmids were amplified using chloramphenicol and isolated using the alkaline lysis procedure (Sambrook and Hol7, 1979).

Isolation of DNA-rich Nuclear Matrix--Randomly cycling CV-1 cells were collected by centrifugation at 5000 X g for 10 minutes and resuspended in L1 buffer. To lyse the nuclei and release the DNA-rich nuclear matrix from the chromatin proteins an equal volume of 4 M NaCl, 2 M NaCl, 10 M Tria-HCl (pH 7.4) was slowly added with gentle stirring with a Teflon rod. The sample at this stage, referred to as the DNA-rich nuclear matrix lysate, was the starting material for the experiments outlined below.

DNA I Treatment of DNA-rich Nuclear Matrix--The DNA-rich nuclear matrix lysate was adjusted to a DNA concentration of 0.5 mg/ml with a high salt (HS) buffer containing 2 M NaCl, 1 M MgCl2, 10 M Tria-HCl (pH 7.4). The lysate was then centrifuged at 4°C for 5 minutes before addition of DNAse I. For mild digestion studies DNAse I was added to 80 units/ml while the enzyme was added to 160 units/ml for strong digestion. The various time intervals equal aliquots of the digestion solution were removed and added to 4 volumes of ice cold HS buffer to stop the DNase I reaction. The residual nuclear matrix was collected by centrifugation at 5000 X g in a swinging bucket rotor (Beckman J2-21) for 25 minutes at 4°C. The DNA was purified from the residual matrix as described above.

DNA II Digestion of Purified DNA--In control experiments purified high molecular weight DNA was adjusted to 80 units buffer conditions and at a final DNA concentration of 0.5 mg/ml. The gradients were run from 0.05 to 0.80 (w/w) (400 units/ml) in 500 ml of 1.0 M NaCl-10% sucrose in 1 M NaCl-10% sucrose (v/v) and dialyzed (The Research Laboratories, Northfield, N.J.) in 1 M NaCl-1 M KCl-10% sucrose (v/v) in a dialysis bag for 3 days.

DNase I was digested into the lysate by the high salt from the DNA-rich nuclear matrix, a series of HS buffer washes and differential centrifugation steps were used. The nuclear lysate was diluted to 250 ml with HS buffer and centrifuged at 5000 X g for 30,000 rpm and 30°C for 12 hours in a Beckman Ti70 rotor. The wash step was repeated twice to ensure complete removal of all nuclear proteins dissociated in the 2 M NaCl conditions. The resulting DNA-rich nuclear matrix was dialyzed against 1000 volumes of KpnI restriction buffer (6 M Tris-HCl (pH 7.5), 6 M NaCl, 6 M MgCl2, 6 M 2-mercaptoethanol) for 16 hours at 4°C. After dialysis the sample was treated with 1% (w/v) restriction enzyme (Bethesda Research Labs., Gaithersburg, MD) added to 1 unit/mg DNA and the sample incubated for 10 hours at 37°C. A second aliquot of enzyme (0.5unit/mg DNA) was added and the incubation continued for three additional hours. The digest was then chilled to 4°C and made 1 M in NaCl-L-N buffer and 5% (w/v) in centrifuge tubes (Accurate Chemical and Scientific Corp., Nevisbury, N.Y.).

Metrizamide Gradient Centrifugation--The DNA digested nuclear matrix were subjected to metrizamide gradient centrifugation. The gradients were prepared in the centrifugation tubes by dissolving metrizamide in 20 ml of 1 M NaCl-L-N buffer to a final concentration of 40% (w/w). This solution was underlayered with 5.0 ml of KpnI digest prepared as described above. The gradients were centrifuged at 5000 X g for 12 hours in a Beckman Ti70 rotor. The digest were collected from the top of the fractions by horizontal-agarose gel electrophoresis and photography the DNA

DNase I Digestion of DNA-rich Nuclear Matrix--To remove the nuclear proteins released into the lysate by the high salt from the DNA-rich nuclear matrix, a series of HS buffer washes and differential centrifugation steps were used. The nuclear lysate was diluted to 250 ml with HS buffer and centrifuged at 5000 X g for 30,000 rpm and 30°C for 12 hours in a Beckman Ti70 rotor. The wash step was repeated twice to ensure complete removal of all nuclear proteins dissociated in the 2 M NaCl conditions. The resulting DNA-rich nuclear matrix was dialyzed against 1000 volumes of KpnI restriction buffer (6 M Tris-HCl (pH 7.5), 6 M NaCl, 6 M MgCl2, 6 M 2-mercaptoethanol) for 16 hours at 4°C. After dialysis the sample was treated with 1% (w/v) restriction enzyme (Bethesda Research Labs., Gaithersburg, MD) added to 1 unit/mg DNA and the sample incubated for 10 hours at 37°C. A second aliquot of enzyme (0.5unit/mg DNA) was added and the incubation continued for three additional hours. The digest was then chilled to 4°C and made 1 M in NaCl-L-N buffer and 5% (w/v) in centrifuge tubes (Accurate Chemical and Scientific Corp., Nevisbury, N.Y.).
The hybridization probes used in this study were the recombinant plasmids pBK(1.2)12 and pBK(1.5)54. These plasmids contain human DNA inserts representing the KpnI 1.2 kb and the KpnI 1.5 kb families, respectively, of the primate interpersed repetitive DNAs (Shafit-Zagardo et al., 1982a). The plasmids were radiocatively labelled in the following manner. Plasmid DNA (100 µg/ml) was partially cleaved with 0.23 units/ml of micrococcal nuclease (Worthington, Freehold, N.J.) in 5 mM Tris-HCl (pH 7.4), 1.5 mM CaCl₂ buffer at 25°C for 1 minute and the DNA purified by phenol extraction and ethanol precipitation. This treatment reduced the plasmid DNA to a heterogeneous collection of fragments between 100 and 1000 bp in length with 5'OH termini. These termini were labelled with ATP gamma 32P (crude) (ICN, Irvine, CA) using T4 polynucleotide kinase (Bethesda Research Labs, Gaithersburg, MD) as described by Hanisic et al. (1982).

The minimum specificities obtained were 2 x 10⁷ cpm/µg DNA.

The hybridization of the probe to the membranes containing DNA were performed in a 3X SSC-10X Denhardt's buffer and autoradiographed as described previously (Musich et al., 1980). The probe was stripped off the Zeta Probe membranes or the DBM papers using 0.1 N NaOH at 42°C for 30 minutes. The filters could then be rehybridized with another probe.

Figure 3. Isopycnic centrifugation of nuclear matrix complexes in a Metrizamide gradient. DNA-rich matrix was digested with KpnI and subjected to density gradient centrifugation in metrizamide. Fractions were collected from the top and their refractive index measured to determine the density (ρ). Equal aliquots were removed, the DNA was purified and its concentration determined by the relative EBFluorescence assay.

Figure 4. Distribution of KpnI 1.2 kb and 1.5 kb family sequences in metrizamide gradient fractions. The DNA in equal volume aliquots of the gradient fractions (see Fig. 3) was purified, redigested with KpnI to ensure complete restriction cleavage, and electrophoresed in a 0.7% agarose gel. The gel included a KpnI digest of total ACM nuclear DNA as an internal standard. The DNA was transferred from the gel to DBM paper for hybridization with either a KpnI 1.2 kb (Panel A) or a KpnI 1.5 kb (Panel B) family probe.