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Nonrandom distribution of repeated DNA sequences with respect to supercoiled loops and the nuclear matrix

(satellite DNA/DNA organization/chromatin)

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ABSTRACT The DNA in a eukaryotic nucleus is arranged into a series of supercoiled loops that are anchored at their bases to the nuclear matrix. We have analyzed the DNA sequences that are closest to the matrix attachment points for their relative content of specific repeated sequences. Sequences were enriched (mouse satellite, human *Alu* family) or depleted (mouse *Eco*RI repeat, monkey α component), depending on the specific sequence and species examined. These results can be understood in terms of a nonrandom arrangement of DNA sequences with respect to nuclear DNA loops.

The DNA in a eukaryotic nucleus is structurally organized in a hierarchical fashion: the DNA is wound around nucleosomes (reviewed in ref. 1), the nucleosomes are arranged into supranucleosomal clusters (2-4), and these supranucleosomal clusters are packaged into loops, each containing ≈100,000 base pairs (bp) of DNA (5, 6). It is possible to visualize directly these loops in both metaphase chromosomes (7) and interphase nuclei (8) after extraction of histones. The loops appear as a halo of DNA anchored to a central scaffold or matrix (9). By nuclease digestion of these matrix-halo structures, one can progressively cleave DNA from the loops, thereby isolating DNA that is progressively closer to the nuclear matrix anchorage sites (10). In previous studies it has been shown that this DNA is enriched in the content of certain single-copy genes (10-12). However, much of the mammalian nuclear DNA is composed of sequences that are highly reiterated. To gain a fuller appreciation of the organization of nuclear DNA, we have assessed in this communication matrix-associated DNA for its content of different types of repeated sequences. The sequences were found to be distributed quite nonrandomly with respect to the nuclear matrix. The results can be understood in terms of a nonrandom arrangement of DNA sequences within nuclear DNA loops.

MATERIALS AND METHODS

Preparation of Matrix and Total Cellular DNA. Cells were labeled with [^{3}H]thymidine and grown as described (10). Cells at 4°C were suspended at 5×10^6 cells per ml in isotonic buffer (100 mM NaCl/50 mM KCl/20 mM Tris·HCl/0.1 mM EDTA, pH 7.4) containing 20 mM methylmethane thiosulfonate and 0.5 mM phenylmethylsulfonyl fluoride. Three volumes of high-salt buffer (2.67 M NaCl/17 mM KCl/20 mM Tris·HCl/4 mM $MgCl_2$, pH 7.4) containing 1.33% Brij-99 nonionic detergent (which is soluble in high-salt solutions) was then added. The suspension was incubated at 37°C after adding 50 units of DNase I per ml (EC 3.1.4.5; DN-CL, Sigma). Aliquots were removed at various times and matrices were pelleted by centrifugation for 20 min at $10,000 \times g$ at 4°C. Purification, restriction enzyme cleavage, and subsequent electrophoresis of DNA were carried out as described (10). Control experiments showed a linear relationship between the amount of restriction-cleaved DNA in a gel slot and the area under the appropriate ethidium bromide-stained fragment peak (assessed by densitometry) for each of the restriction fragments analyzed in this study.

Hybridization. DNA samples were heat denatured and spotted onto nitrocellulose filters (13). BLUR 8 plasmid DNA (14) containing an *Alu* family insert (provided by P. Deininger) was labeled by nick-translation. Hybridization and washing followed a protocol provided by K. Peden (personal communication). Each point was performed in triplicate, and the filters were autoradiographed on preflashed Kodak XAR-5 film. Control experiments demonstrated a nearly linear relationship between the amount of *Alu* sequence spotted on the filter and the intensity of the autoradiographic dot obtained after these procedures.

RESULTS

When cells are incubated in buffers containing nonionic detergents and high concentrations of NaCl, the cellular and nuclear membranes are dissolved, and histones and other soluble proteins are removed from the nucleus (5, 6, 9). After being stained with ethidium bromide or other DNA binding agents, the nuclear DNA can be visualized (after relaxation of supercoiling) as a halo of DNA loops surrounding a residual nuclear skeleton or matrix (8). When DNase I is added, DNA is progressively cleaved from the matrix-halo structures. This is visualized as the progressive diminution in size of the ethidium bromide-stained halo. Depending on the extent of DNase treatment, from none to >99% of the total DNA can be cleaved from the matrix. It is important to note that the conditions of DNase I treatment were chosen to be extremely gentle; thus, DNA is gradually cleaved from the matrix (two double-stranded cuts in a loop will result in its detachment from the matrix), but DNA is not extensively degraded. The residual DNA that is recovered with the matrices after centrifugation represents those sequences that occur progressively nearer to the base of the DNA loops anchored to the matrix. This "matrix DNA" was isolated from a variety of cell types, and its relative sequence content of several repeated genes was assessed.

Some repeated sequences, such as satellite DNA, exist in long tandem arrays. One such satellite in mouse represents a substantial fraction (10%) of mouse nuclear DNA (15). When total nuclear DNA is cleaved with *Bst*NI, the 245-bp monomers (and multiples thereof) of the satellite DNA can be seen clearly upon gel electrophoresis (16). When matrix DNA from globin-producing mouse Friend erythroleukemia (FEL) cells [induced with dimethyl sulfoxide (17)] was cleaved with *Bst*NI, a far larger fraction of the DNA (relative to total nuclear DNA) consisted

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Abbreviations: FEL, Friend erythroleukemia; bp, base pairs.
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of the satellite sequence (Fig. 1*A*). It also can be seen from Fig. 1*A* that as more DNA was cleaved from the halo region, the matrix DNA was progressively enriched in the satellite sequence. This observation was quantitated by densitometry (Table 1, experiment A) and by measuring the [^{3}H]dT radioactivity in the appropriate gel bands of the various DNA preparations (data not shown). Both of these assays, densitometry and scintillation assay, showed that the matrix DNAs were enriched up to 2.6-fold in their content of satellite DNA sequences. Because 10% of the mouse genome consists of the satellite sequence (15), up to 26% of the DNA associated with the matrix consisted of the satellite sequence. This association of the mouse satellite sequence also was found in nuclei of other mouse cells. In Table 1, experiment B, it can be seen that the satellite sequence was substantially enriched in matrix DNA from the mouse fibroblast cell line SVB203. Furthermore, matrices from uninduced FEL cells also contained a high concentration of the satellite sequence (Fig. 2*A*; Table 1, experiment C).

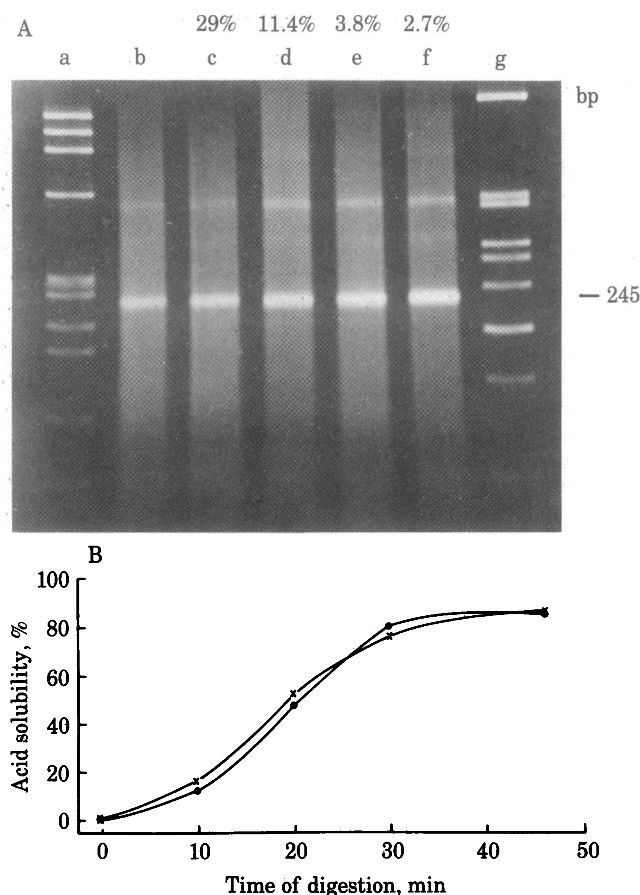

FIG. 1. (*A*) Enrichment of mouse satellite DNA sequences (245 bp and multiples thereof) in nuclear matrix DNA of induced FEL cells. Induced FEL cell total nuclear and matrix DNAs were digested with *Bst*NI and electrophoresed through a 4% polyacrylamide gel. Equal amounts of DNA were loaded in each gel slot. The various matrix preparations are identified by the percentage of total nuclear DNA remaining with the matrix after DNase I digestion. Lanes: a, marker ϕX174 *Hae* III fragments; b, total nuclear DNA; c, 29% matrix DNA; d, 11.4% matrix DNA; e, 3.8% matrix DNA; f, 2.7% matrix DNA; g, marker pBR322 *Hin*fI fragments. (*B*) Rates of digestion of mouse satellite DNA and total nuclear DNA by DNase I. ^{3}H-Labeled mouse satellite (×) and ^{14}C-labeled total nuclear DNA (●) of the same size were mixed together and digested with DNase I under the same salt and other ionic conditions used to digest the matrix–halo structures. Acid solubility was assessed at the indicated times of digestion.

Table 1. Quantitation of repeated sequences in nuclear matrix DNA preparations

Exp.	Source	DNA*	Relative sequence content
A	Induced FEL (mouse) satellite sequence†	Total	1.00
		Matrix 29%	1.42
		Matrix 11.4%	2.08
		Matrix 3.8%	2.21
		Matrix 2.7%	2.63
B	SVB203 (mouse) satellite sequence†	Total	1.00
		Matrix 4.7%	2.25
C	Uninduced FEL (mouse) satellite sequence†	Total	1.00
		Matrix 24%	1.42
		Matrix 7.5%	1.82
D	Uninduced FEL (mouse) *Eco*RI repeat†	Total	1.00
		Matrix 24%	0.60
		Matrix 7.5%	0.39
E	Vero (African green monkey) α component†	Total	1.00
		Matrix 22.5%	0.73
		Matrix 5.1%	0.58
F	A549 (human) *Alu* family sequence†	Total	1.00
		Matrix 31.9%	1.92
		Matrix 12.2%	2.42
		Matrix 7.1%	1.52
G	A549 (human) *Alu* family sequence‡	Total	1.00
		Matrix 31.9%	1.85
		Matrix 12.2%	1.98
		Matrix 7.1%	1.48
		Matrix 1.3%	1.62

* "Total" refers to DNA isolated from whole nuclei and "matrix" to DNA isolated from nuclear matrix that had been cleaved with DNase I so that the indicated percentages of nuclear DNA remained.
† As judged by densitometry of photographs of ethidium bromide-stained gels of restriction endonuclease-digested DNA.
‡ As judged by hybridization of ^{32}P-labeled BLUR 8 DNA to matrix DNAs spotted on nitrocellulose filters.

Although DNase I is often used as a "nonspecific" nuclease, it is known to show different rates of activity towards different sequences (18). Hence, it was important to show that the observed association of mouse satellite sequences with the nuclear matrix was not simply due to the relative resistance of mouse satellite DNA to DNase I digestion. To test this possibility, ^{3}H-labeled mouse satellite DNA and equivalent-size ^{14}C-labeled total mouse DNA were isolated by Ag^+/CsCl gradient centrifugation (19). They were then mixed and brought up to the same ionic conditions present during DNase I digestion of the matrix–halo structures. DNase I was added and the sample was incubated at 37°C. Aliquots were removed at various times and assessed for acid solubility. The rates of solubilization of ^{3}H and ^{14}C were very similar, thus showing that the mouse satellite is as sensitive to DNase I as is the average mouse DNA sequence (Fig. 1*B*).

It was possible that the satellite DNA became associated with matrices during their preparation. To test this possibility, ^{3}H-labeled mouse satellite DNA and ^{14}C-labeled mouse total nuclear DNA were added to unlabeled matrix–halo structures at the time of addition of high salt. The amounts of labeled exogenous DNA added resulted in similar concentrations of endogenous (unlabeled) and exogenous DNA in the mixture. After various extents of DNase I digestion, the matrices were analyzed for the binding of the exogenous sequences. Regardless of the extent of cleavage of the endogenous DNA from the matrix, very little (<0.2%) of the exogenously added DNA bound to the matrices, and satellite DNA binding was no greater than that of total mouse DNA.

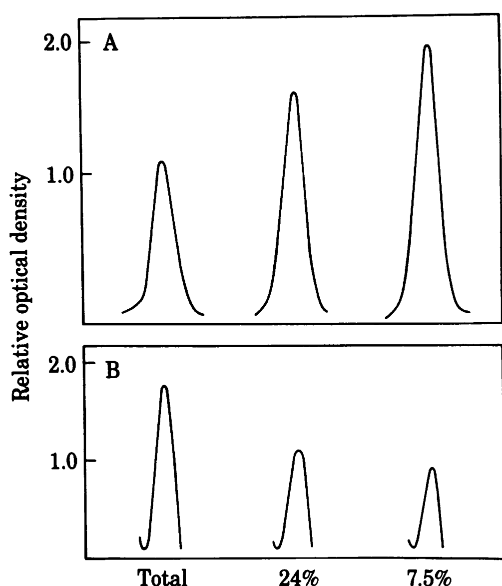

FIG. 2. Enrichment of mouse satellite sequences and impoverishment of *Eco*RI repeat sequences in nuclear matrix DNA of uninduced FEL cells. The various matrix preparations are identified by the percentage of total nuclear DNA remaining with the matrix after DNase I digestion. Peaks (left to right): total nuclear DNA, matrix 24%, matrix 7.5%. (*A*) Densitometric scan of the monomer bands from a 4% polyacrylamide gel of *Bst*NI-digested total nuclear and matrix DNA samples from uninduced FEL cells. Equal amounts of DNA were loaded in each gel slot. (*B*) Densitometric scan of the *Eco*RI band from a 1.5% agarose gel of the same total and matrix DNA samples as in *A* but this time digested with *Eco*RI.

Another repeated sequence found in mouse DNA is interspersed with other genes rather than tandemly repeated (20). Matrix DNA preparations from mouse cells were analyzed for their content of this interspersed repeat, which is cleaved twice by *Eco*RI, leaving a 1,300-bp fragment (20). The relative content of the *Eco*RI repeat sequences was decreased in the matrix DNA from uninduced FEL cells compared to total nuclear DNA (Fig. 2*B*; Table 1, experiment D). Similar results were found also for induced FEL cells and for the mouse fibroblasts (data not shown). The impoverishment was not due to preferential digestion of the *Eco*RI repeat by DNase I. Digestion of purified mouse total nuclear DNA by DNase I to sizes similar to or slightly smaller than that of the matrix DNA preparations shown in Fig. 2*B* was carried out under the same ionic conditions in which the matrix–halo structures were digested. Subsequent digestion with *Eco*RI and electrophoresis in agarose gels revealed contents of the *Eco*RI repeat in each sample of DNase I-digested total DNA that were identical (±5%) with that of purified DNA not previously digested with DNase I (data not shown).

The mouse satellite DNA is part of the nontranscribed heterochromatin preferentially located at the centromere (21, 22); hence, one of the interpretations for the enrichment of this sequence in nuclear matrix preparations is that the nuclear matrix is preferentially enriched in nontranscribed heterochromatin or centromeric DNA sequences. To assess this possibility, nuclear matrix DNA was prepared from African green monkey Vero cells. These cells contain a tandemly repeated, nontranscribed, centromerically located sequence called the α component, which makes up 20% of the total DNA (23, 24). The sequence can be detected by gel electrophoresis after cleavage of the nuclear DNA by the restriction endonuclease *Hin*dIII as a 172-bp monomer and multiples thereof (25). When DNA from whole nuclei or from nuclear matrices was cleaved with *Hin*dIII, a nonrandom distribution of the α-component sequence was observed (Fig. 3*A*). However, in contrast to the mouse satellite DNA sequence (Figs. 1*A* and 2*A*; Table 1, experiments A–C), the relative content of the α component was lower in the matrix DNAs than in the total nuclear DNA (Fig. 3*A*; Table 1, experiment E). As more DNA was cleaved from the matrix, the rel-

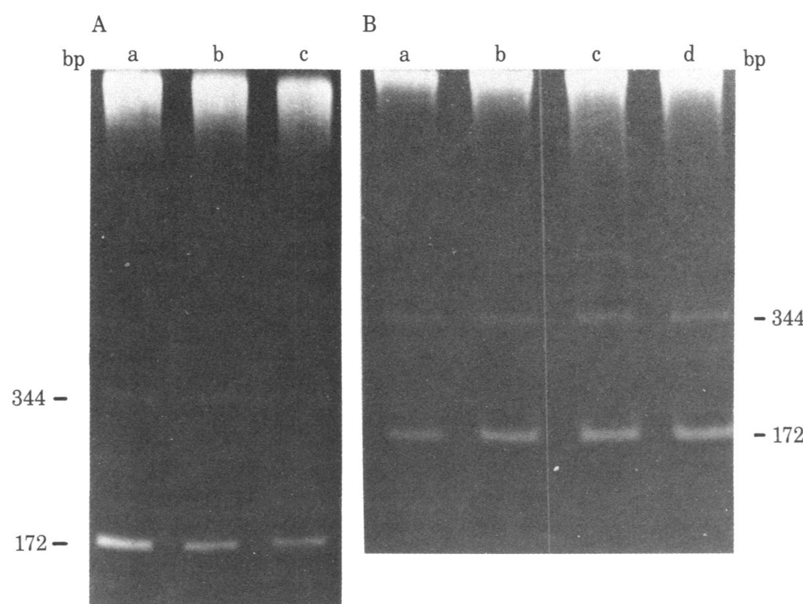

FIG. 3. (*A*) Impoverishment of α-component sequences (172 bp and multiples thereof) in Vero nuclear matrix DNA. Total nuclear (lane a), 22.5% matrix (lane b), and 5.1% matrix (lane c) DNAs from Vero cells were digested with *Hin*dIII and electrophoresed in a 4% polyacrylamide gel. Equal amounts of DNA were loaded in each gel slot. (*B*) Equal content of α-component sequences in undigested and DNase I-digested Vero total nuclear DNA. "DNase control I and II" DNAs were digested with DNase I to fragment lengths slightly less than the Vero 22.5% and 5.1% matrix DNA samples in *A*, respectively. Total nuclear DNA (lane b) and DNase control I (lane c) and II (lane d) DNAs were digested with *Hin*dIII as in *A*. Half the amount of *Hin*dIII-digested total DNA (lane a) was also electrophoresed to show the level of impoverishment that would be expected if DNase I preferentially cleaved the sequence enough to result in an α-component content 50% that of the total DNA.

ative content of the α component decreased. As in the case of the mouse *Eco*RI repeat, this decrease was not due to preferential degradation of the α component by DNase I digestion. Purified monkey DNA was digested (under the same conditions used to digest the matrix–halo structures) to sizes similar to or slightly smaller than the matrix DNA preparations shown in Fig. 3*A*. Subsequent digestion with *Hin*dIII of these DNA samples resulted in contents of α component virtually identical with that found in DNA not previously digested with DNase I (Fig. 3*B*).

Human nuclear matrix DNA was then examined for its content of the *Alu* family sequences, a class of repeated sequences that is interspersed throughout much of the genome (26). Nuclear matrices containing various amounts of DNA were isolated from human A549 cells. The DNA was purified and cleaved with the restriction endonuclease *Mbo* I, an enzyme which cleaves the consensus *Alu* family sequence in two places, leaving a 180-bp fragment (14). Electrophoresis revealed a 1.5- to 2.4-fold enrichment of *Alu* family sequences in the matrix preparations (Table 1, experiment F). However, a relatively high background of fragments similar in size to the 180-bp *Alu* family fragments was present in these gels. Hence, to check the gel electrophoresis results, a hybridization method was used. Equal quantities of RNase-treated DNA from the various nuclear matrix preparations were spotted onto a nitrocellulose filter and hybridized to a ^{32}P-labeled *Alu* family probe (13). There was up to a 2-fold enrichment for the *Alu* family sequences in the matrix DNAs, thus confirming the electrophoresis result (Fig. 4; quantitated in Table 1, experiment G). From Table 1, experiments F and G, it can be seen that although matrix preparations containing 1.3% to 31.9% of the total DNA were all enriched in the *Alu* family sequences, the enrichment did not, in general, get significantly higher as more DNA was cleaved from the matrix. This was in contrast to the situation with the other repeated sequences of mouse and monkey cells shown in Table 1, experiments A–E; this may indicate more heterogeneity of the *Alu* family sequence members in terms of their relative positions within DNA loops.

It was necessary to demonstrate that the enrichment of *Alu* family repeats in human matrix DNA was not due to resistance to DNase I digestion. Total ^{3}H-labeled nuclear DNA from A549 cells was digested with DNase I to an average fragment size of $\approx$7,000 bp under the same conditions used to digest matrix–halo structures. The DNA was then purified, electrophoresed (without restriction endonuclease cleavage) through an agarose gel, transferred to nitrocellulose (27), and hybridized to ^{32}P-labeled *Alu* clone BLUR 8. After being washed, the filter strip was cut into slices, and the radioactivity was assayed by scintillation counting. It was found that the distribution of ^{32}P was almost superimposable on the distribution of ^{3}H. Specifically, the ratio of ^{32}P to ^{3}H was not increased in the larger-sized DNA molecules, as would be expected if the *Alu* sequences (or sequences surrounding them) were relatively more resistant to DNase I than was the bulk of the DNA.

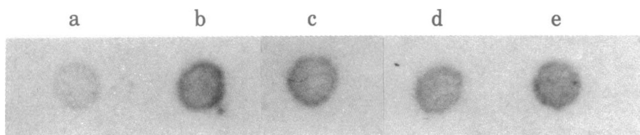

FIG. 4. Enrichment of human *Alu* family sequences in nuclear matrix DNA; autoradiograph of equal amounts of A549 total nuclear (spot a) and matrix DNAs (spots b–e) spotted on nitrocellulose filters and hybridized with nick-translated BLUR 8 DNA. Matrix DNAs: 31.9% (spot b), 12.2% (spot c), 7.1% (spot d), and 1.3% (spot e).

DISCUSSION

The results clearly showed that there was a nonrandom distribution of repeated sequences with respect to the nuclear matrix. Sequences were enriched (mouse satellite, human *Alu* family) or depleted (mouse *Eco*RI repeat, monkey α component) depending on the specific sequence and species examined. This nonrandom distribution seemed to be species specific, rather than cell-type specific because nuclear matrix DNAs from both erythroleukemic and fibroblastic mouse cells were enriched for satellite sequences but had proportionately less of the interspersed *Eco*RI repeat.

It has been suggested that any DNA sequence will be associated with the matrix at the point in the S phase during which it is replicated (8). However, an individual replicon is only "active" for $\approx$0.5 hr out of a cell cycle time of 12–24 hr (for mammalian cells), so the percentage of cells that are in the process of replicating a replicon containing any given DNA sequence is always <5%. Hence, association of newly replicated DNA with the nuclear matrix should not result in the preferential association of any specific sequence in a population of logarithmically growing or stationary cells.

The nonrandom arrangement of DNA sequences shown here is consistent with previous reports demonstrating the association of specific single-copy (10–12) and ribosomal RNA genes (28) with the nuclear matrix. The results reported here are also consistent with reports showing association of satellite DNA with the nuclear matrix of bovine kidney cells (29) and enrichment of rapidly renaturing DNA with chromosome scaffold DNA from Chinese hamster cells (30).

However, several other studies seemingly conflict with the findings reported here; some of these reports (28, 31, 32) of experiments with various rodent cells show similar distributions of repeated DNA sequences in matrix and total nuclear DNA. However, careful examination of the data reveals significant differences in the procedures used to isolate matrix DNA in these studies. (*i*) In two of these reports (28, 32), nuclease treatment was done before removal of histones and other chromosomal proteins. At this stage, the nuclear DNA is in a conformation that renders some sequences less or more susceptible to nuclease action (33, 34). Any enrichment (or absence of enrichment) for specific sequences in the matrix DNA might well be due to such sensitivity. (*ii*) A study (31) showing a random distribution of mouse satellite DNA in nuclear matrix preparations digested after histone removal used micrococcal nuclease, which has a high degree of specificity towards various DNA sequences (35). Unless the enzyme used to digest the matrix–halo preparation is known not to display a differential rate of activity towards the sequence examined, results showing the association (or lack of association) of that sequence with the nuclear matrix must be interpreted with caution. (*iii*) A similar criticism can be made with reference to analyses of DNA prepared by digestion of matrices or chromosome scaffolds with restriction endonucleases. Studies have shown the enrichment of mouse (36) or human (37) satellite sequences in matrices prepared by *Eco*RI or *Hae* III digestion, respectively. However, the restriction enzymes used did not cleave the satellite sequences examined; therefore, as pointed out previously (36), these results are most easily explained by the decreased activity of the restriction enzyme for loops containing these satellite sequences.

Based on the data presented here and the reports summarized above, it appears that at least some sequences are arranged nonrandomly with respect to supercoiled loops and the nuclear matrix. These data can be explained by the model shown in Fig. 5. In this model, all DNA in the nucleus is arranged into a series

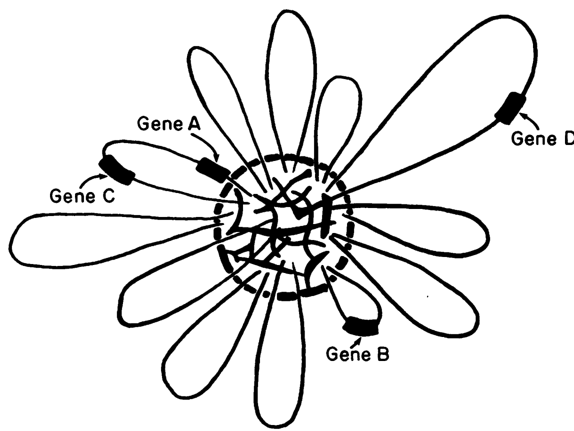

FIG. 5. A histone-depleted nucleus is depicted with the DNA loops extended and anchored to the central nuclear matrix. Gene A is associated with the nuclear matrix because it resides at the base of a loop. Gene B is associated with the nuclear matrix because it resides in a loop smaller than the average. Gene C is impoverished in matrix DNA because it lies at the end of a loop. Gene D is impoverished because it lies within a loop larger than the average.

of supercoiled loops that are anchored at their bases to the nuclear matrix; the size and orientation of each loop is determined by the sequences comprising it. According to this scheme, there are two ways in which a gene could be preferentially associated with the nuclear matrix. Gene A in Fig. 5 would be associated with the nuclear matrix after the bulk of the loops have been removed because it lies close to the nuclear matrix anchorage points of the loop in which it resides. Transcribed sequences are bound to the matrix (10–12, 38) and, thus, operationally would form the bases of the DNA loops in which they reside. Accordingly, *Alu* family sequences may be enriched in nuclear matrix DNA by virtue of the fact that they are transcribed (39, 40). Another way for a gene to appear to be associated with the nuclear matrix is that it lies in a loop shorter than the average (Fig. 5, gene B). Small loops would have less DNase I cleavages per loop and, therefore, would be more difficult to separate from the matrix. According to this hypothesis, the mouse satellite sequence would be enriched in nuclear matrix preparations because the sequences are arranged in loops shorter than the average. It is worth noting, in this regard, that the size of loops attached to an individual metaphase chromosome scaffold, measured by electron microscopy, varies widely (7).

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