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The anatomy of supercoiled loops in the *Drosophila* 7F locus

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ABSTRACT

The genome in eucaryotes is organized into a series of supercoiled loops, topologically anchored at their bases by components of the nuclear matrix. Previous studies have shown that active genes are associated with the nuclear matrix. We wished to know whether loops in general were solely organized by active genes. We therefore examined a locus of the Drosophila X-chromosome comprising 163,000 bp of continuous DNA sequences and devoid of known active genes. Of the 52 EcoRI restriction fragments comprising this region, we found 5 anchored fragments which non-randomly organized this region into 4 DNA loops. Each of the 5 anchored fragments contained a transcribed sequence. These results strongly suggest that supercoiled loops are organized in a specific fashion with respect to DNA sequence, with the anchorage points exclusively demarcated by transcriptionally active genes.

INTRODUCTION

Numerous studies have indicated that the eucaryotic genome is organized into a series of supercoiled loops which are anchored to a nuclear skeleton or matrix (reviewed in 1). Although the composition of the nuclear matrix is a subject of controversy (2,3), several lines of evidence, including biophysical, morphological and biochemical experiments, support the idea that the genome itself is topologically constrained in the form of loops by a skeletal structure (4-9).

Several investigators have shown that transcribed genes are associated with the nuclear matrix (10-18). These studies have suggested a model for loop organization wherein sequences near active genes are responsible for anchoring loops. Because these observations on the association of active genes with the nuclear matrix have dealt with isolated examples of active genes, however, the exact relationship between active genes and loops has remained conjectural. We wished to test this loop organization model in an unbiased fashion by examining a relatively large region of DNA devoid of known active genes and determining if specific loop anchoring sequences spaced at appropriate intervals could be found. This would suggest that the genome was

Indeed organized non-randomly with respect to loops, with certain sequences forming the base of the loops and other sequences forming the loops themselves. We report here that the DNA in the Drosophila 7F locus is organized into a series of four loops of average size 39,000 bp. Each of the five anchorage points was near an actively transcribed gene. These results support a model for eucaryotic loop organization wherein supercoiled loops are exclusively demarcated by active genes, with the loops themselves largely comprised of sequences lying between transcriptionally active regions of the genome.

MATERIALS AND METHODS

Preparation of Matrix-Halo Structures

Drosophila Kc tissue culture cells (19) were grown in D-22 media at 23oC in T-flasks in the presence of 0.1 uCi/ml 3H-thymidine and harvested in late log phase. Cells were washed in Hanks' buffered saline and then resuspended at 1x107 cells/ml in IS Buffer: [100 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Tris pH 7.2, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.2% methyl-methanethiosulfonate (MMTS), (14)]. One-tenth volume of 10% NP-40 in IS buffer was then added while swirling the cells. Nuclei were pelleted by centrifugation at 400 x g for 5 min and resuspended in the same volume of IS buffer plus 1% NP-40. After ten strokes in a Dounce homogenizer with a tight fitting pestle, the nuclei were again pelleted. A fraction of the nuclei was removed for purification of total nuclear DNA. The remainder of the nuclei was resuspended in IS buffer at 2-3 x 106 cells/ml and an equal volume of 4 M NaCl, 20 mM Tris, 5 mM $MgCl_2$, 0.5 mM PMSF, 0.2% MMTS was then added to the nuclei. The resulting "matrix-halo" structures (12) were centrifuged through 4% glycerol in HS Buffer (2.1 M NaCl, 5 mM $MgCl_2$, 20 mM Tris pH 7.2) at 1500 x g for 15 min onto a cushion of 60% glycerol in HS Buffer. The structures were removed with a Pasteur pipet and diluted 20-40 fold with 100 mM Tris pH 7.2, 5 mM $MgCl_2$, 2 mM B-mercaptoethanol. Structures isolated by this method have virtually all the nuclear DNA present and the DNA is supercoiled (12,14).

Isolation of DNA Fractions

The DNA in the loops was digested with 20-40 units/ml of EcoRI (BRL, Bethesda, Maryland) at 37oC for 45 minutes at a density of 0.5-1 x 106 structures/ml. After digestion, DNA remaining anchored to the matrices (A-DNA) was separated from DNA severed from the matrix (S-DNA) by centrifugation at 10,000 x g for 20 min. DNA was purified from the

supernatant (S-DNA) and pellet (A-DNA) by proteinase K digestion followed by extraction with phenol and chloroform (34).

Electrophoresis, Southern Transfer and Hybridization

Purified A-DNA, S-DNA and various quantities of total nuclear DNA were cleaved with EcoRI, fractioned by electrophoresis through 1% agarose gels and transferred to nitrocellulose. Hybridization to 32P-labeled nick-translated phage DNA was performed as previously described (12). A portion of A-DNA (lanes marked A*) was not re-cleaved with EcoRI after DNA purification. By comparing the patterns of hybridization of the lanes marked A to those marked A*, one can verify that a limit EcoRI digest of the nuclear structures was achieved.

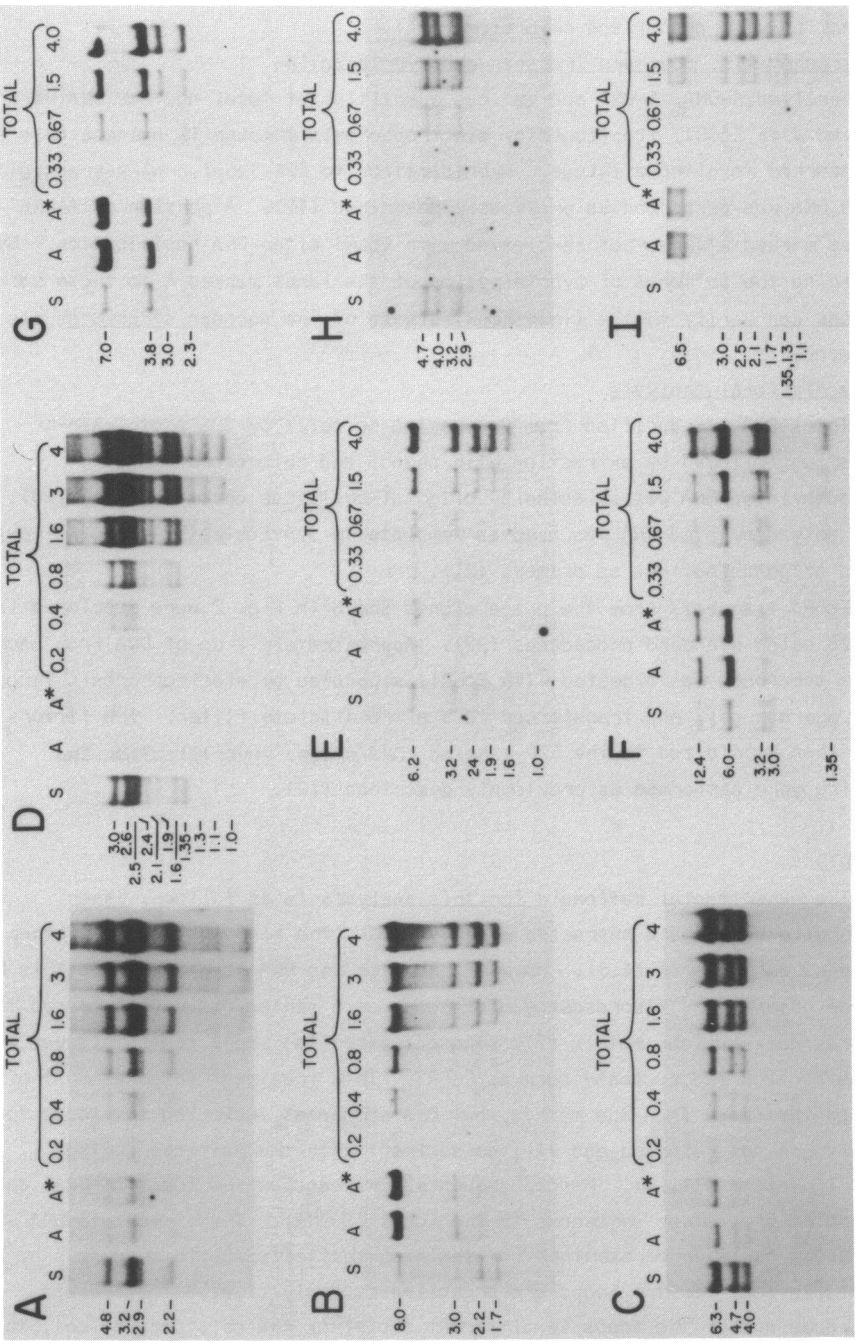
Transcriptional Analysis

Total RNA was purified from Drosophila Kc cells by SDS proteinase-K digestion followed by extraction with phenol and chloroform (34). Polyadenylated RNA was selected by oligo dT-cellulose chromatography (20). This polyadenylated RNA was used as template to synthesize 32P-labeled cDNA using oligonucleotides as primers (21).

EcoRI fragments from the phage clones shown in Fig. 2 were subcloned into pBR328 using standard procedures (22). Approximately 1 ug of DNA from each of these subclones was digested with EcoRI, separated by electrophoresis through a 1% agarose gel, and transferred to a nitrocellulose filter. The filters were then hybridized to the 32P-labeled cDNA probe. Hybridization and washing were performed as previously described (12).

RESULTS

The experimental rationale for this analysis is as follows. When Drosophila nuclei are extracted with 2 M NaCl, the histones and many other proteins are removed (4,6). However, none of the DNA is removed and this DNA can be observed by fluorescence microscopy as a series of loops anchored to the residual nuclear matrix (7). These supercoiled loops can be cleaved with a restriction endonuclease such as EcoRI. This treatment detaches most of the DNA in the loops from the matrix, but DNA sequences anchoring the loops to the matrix are not released and will co-sediment with the matrices during centrifugation (11,14). Hence, sequences that anchor the loops (A-DNA) can be separated from other sequences in the loops (S-DNA). These separate DNA fractions can then be examined for their content of specific sequences by DNA hybridization. If the loops are organized randomly (i.e., if different sequences anchor the loops in different nuclei of the cell population), then


A
B
C
D
E
F
G
H
I
S
A
A*
TOTAL
0.2
0.4
0.8
1.6
3
4
0.33
0.67
1.5
4.0
4.8
3.2
2.9
2.2
8.0
3.0
2.2
1.7
6.3
4.7
4.0
3.0
2.6
2.5
2.4
2.1
1.9
1.6
1.35
1.3
1.1
1.0
6.2
3.2
2.4
1.9
1.6
1.0
12.4
6.0
3.2
3.0
1.35
7.0
3.8
3.0
2.3
4.7
4.0
3.2
2.9
6.5
3.0
2.5
2.1
1.7
1.35,1.3
1.1

the content of any specific sequence in A-DNA will not be different from the content of that sequence in S-DNA or in total nuclear DNA. Conversely, if the loops are organized non-randomly, specific sequences will be enriched in A-DNA and concomitantly depleted in S-DNA.

We chose the 7F region of the Drosophila X-chromosome for this analysis for several reasons. First, Spradling and co-workers have successfully isolated 16 overlapping phage clones spanning 163,000 bp in this region (23). Since supercoiled loops in Drosophila have been measured at 36,000 bp by microscopic methods (24,25), and 85,000 by a biophysical method (6), this stretch of the genome should encompass 2-5 DNA loops. Moreover, the only known genes in this region (chorion genes) are not transcribed in Drosophila tissue culture cells (our unpublished data). Hence, this locus provided a large cloned domain perhaps representative of many areas of the genome of Drosophila cells.

There are 52 EcoRI restriction fragments within the cloned region examined. Each fragment was tested for its concentration in A-DNA, S-DNA and total nuclear DNA using a Southern hybridization technique. Representative autoradiographs are shown in Fig. 1 and the results are depicted graphically in Fig. 2A. Immediately apparent from these figures is the fact that the organization of DNA in this region was non-random. Some sequences were enriched considerably in A-DNA while others were significantly depleted. Since A-DNA contained 25% of total nuclear DNA, the greatest possible enrichment would be four fold. Of the 52 EcoRI fragments covered by the 16 overlapping phage clones, 5 fragments were enriched more than 2.5 fold. These fragments were apparently anchored to the matrix in a large fraction of the nuclei (at least 2.5 x 25% = 62% of the nuclei). Fragments adjacent to these anchorage points were sometimes slightly enriched while most of the other fragments were depleted. The results with S-DNA were always consistent with the A-DNA findings: if a sequence was enriched in A-DNA, it was depleted in S-DNA. Because the S-DNA represented 75% of the total nuclear DNA, only small

Figure 1: Analysis of Anchored Sequences at the 7F Locus.
A-DNA (1.0 ug), A*-DNA (1.0 ug), S-DNA (1.0 ug), and various quantities of total nuclear DNA (0.2-4.0 ug) were fractionated by electrophoresis, transferred to nitrocellulose, and hybridized with ^{32}P-labeled DNA from phage clones of the 7F locus (7). The relative position of the phage clones is indicated in Fig. 2. One preparation of A-DNA and S-DNA was used for 1A-1D, while another preparation was used for Fig. 1E-1I. Cross-reactive high molecular weight fragments not contained within the 7F locus can be seen in blots A, D, and I. See Materials and Methods for an explanation of the preparation of A*-DNA.
A:15; B:3601; C:A05; D:2203; E:2807; F:2304; G:2912; H:A06; I:1904

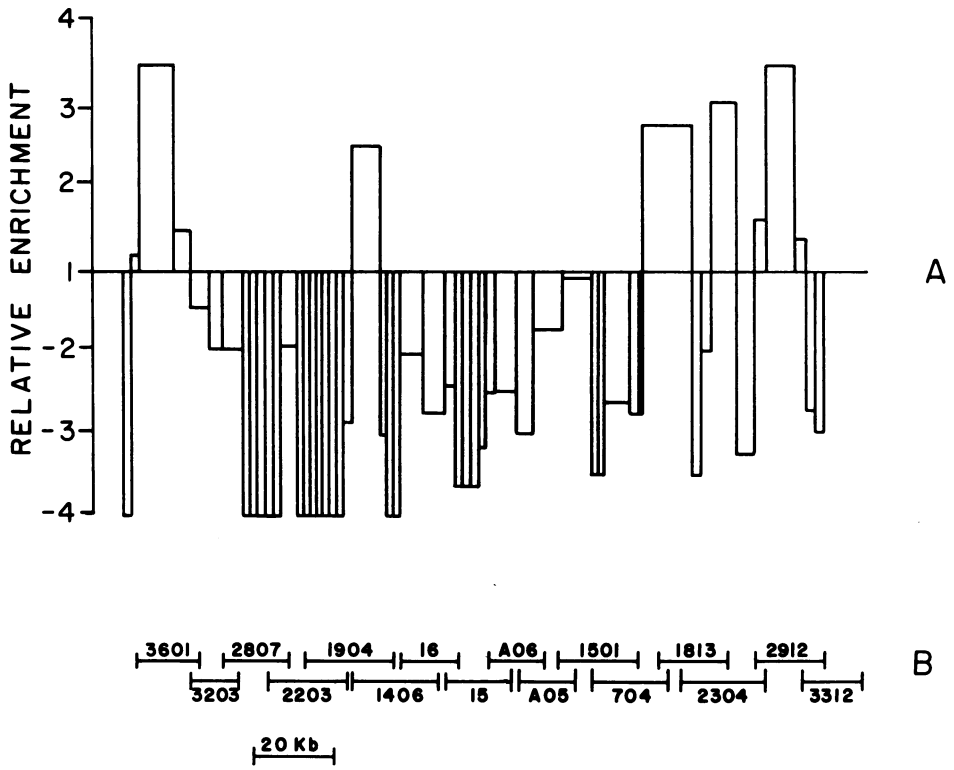


Figure 2: Anchorage of EcoRI Fragments in the 7F Locus.

A. Enrichments and depletions of the 52 EcoRI fragments in the 7F locus of the X-chromosome of Drosophila Kc cells in A-DNA relative to total nuclear DNA were determined by hybridization analyses such as those shown in Fig. 1. The numbers shown represent the average of 5 independent experiments and were obtained by densitometry. In none of the five experiments did any individual value differ by more then 30% from the values shown in the graph.

B. The location of the 16 phage clones used as hybridization probes. The chorion protein coding genes are located within phages 15 and A05 (23).

enrichments were possible (100%/75%=1.33 fold). When sequences were depleted in A-DNA, they were present in S-DNA in at least equal representation to total nuclear DNA.

These results were reproducible in five independent preparations of A-DNA and S-DNA. Limit EcoRI digests of the structures were achieved, as can be seen by comparing lanes A* with lanes A. It should also be noted that the fragments enriched in A-DNA were among the larger EcoRI fragments in the domain tested. However, the observed enrichment was not due simply to the

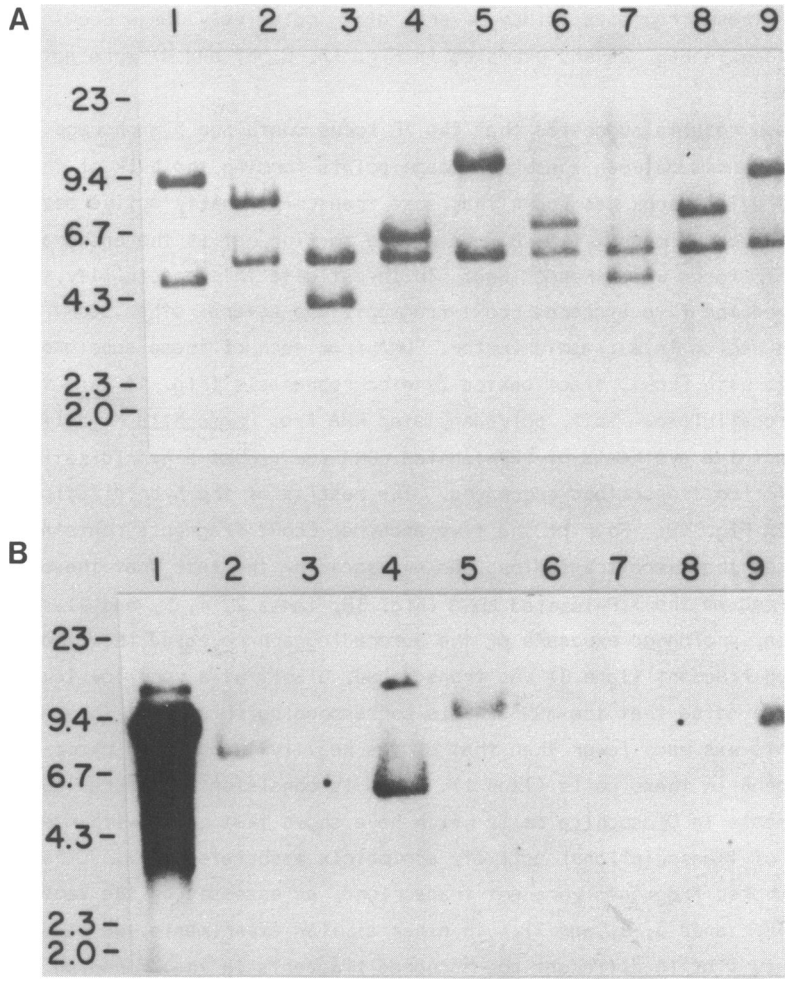


Figure 3: Transcriptional Analysis of the Anchored DNA Fragments from the 7F Locus.

Approximately 1 ug of DNA from each of 8 subclones was digested with EcoRI and fractionated by electrophoresis through an agarose gel. A photograph of the ethidum bromide stained gel is shown in A. This DNA was transferred to nitrocellulose and hybridized with a ^{32}P-labeled cDNA probe representing total cellular polyadenylated RNA. The resulting autoradiograph is shown in B. Lane 1: Genomic clone coding for the cytoplasmic actin gene, used as a positive control. Lane 2: anchored 7.0 kb EcoRI fragment from phage clone 2912. Lane 3: non-anchored 3.0 kb EcoRI fragment from phage clone 2912. Lane 4: anchored 6.0 kb EcoRI fragment from phage clone 2304. Lane 5: anchored 9.5 kb fragment (a subfragment of the 12.4 kb EcoRI fragment) from phage clone 1813. Lane 6: non-anchored 6.3 kb EcoRI fragment from phage clone A05. Lane 7: non-anchored 4.7 kb EcoRI fragment from phage clone A05. Lane 8: anchored 6.5 kb EcoRI fragment from phage clone 1406. Lane 9: anchored 8.0 kb EcoRI fragment from phage clone 3601.

size of these fragments, since several other relatively large EcoRI fragments (see graph in Fig. 2A and examples in Fig. 1A, C, E, and H) were not enriched in A-DNA.

These results suggested that the 7F locus contained 5 anchorage points, with sequences between these anchorage points forming the bulk of the loops. Since previous work has shown that some transcriptionally active sequences are anchored to the matrix (10-18), we wished to find out if the anchorage points in the 7F locus were transcribed. To investigate this possibility, we first subcloned the five anchored EcoRI fragments and several other EcoRI fragments in this region in a plasmid vector. DNA from each of these subclones was digested with EcoRI, fractionated by electrophoresis (Fig. 3A) and transferred to nitrocellulose. Next, polyadenylated RNA from Drosophila Kc cells was used to direct the synthesis of 32P-labeled cDNA for use as a hybridization probe specific for transcribed sequences. The results of the hybridization are shown in Fig. 3B. Four of the five anchored EcoRI fragments contained sequences that were transcribed, as evidenced by the fact that these fragments hybridized to the 32P-labeled cDNA (Fig. 3B, lanes 2, 4, 5, and 9). In addition, prolonged exposure of the autoradiograph revealed that the fifth anchored fragment (lane 8) was transcribed, albeit at a very low level. It should be noted that the mRNA levels corresponding to each of these anchored fragments was much lower than that of the heavily transcribed cytoplasmic actin gene in these cells (lane 1). This is consistent with previous experiments in Drosophila cells which have shown that genes with even very low levels of transcriptional activity are matrix associated (17). Other non-anchored fragments were not transcribed, as assessed by the same assay (Fig. 3B, lanes 3, 6, and 7). In other similar experiments, we have found that only 1 of 16 different non-anchored fragments in the 7F region showed transcriptional activity in this assay. Hence, although all transcribed sequences may not be anchored, it appears that all anchored fragments contain transcribed sequences.

DISCUSSION

We have found that DNA in the 7F locus of the X-chromosome of Drosophila is organized non-randomly with respect to DNA loops. There were five anchorage regions which organized the DNA into four loops of 75, 52, 15, and 14 kb (with loop sizes measured as the distance between the centers of anchoring fragments). Interestingly, the average size of the loops mapped in this fashion was 39,000 bp, which is in agreement with the average size of

36,000 bp determined by morphological methods (24,25). The large variation in the size of loops in this region is consistent with measurements made by electron microscopy showing a large variation in the size of individual loops (26).

The precise sequences within the five anchored EcoRI fragments that mediate their attachment to the matrix are not known. However, previous experiments have shown that other anchored restriction fragments are bound through 2-3 kb regions which include the 5' end of active genes (17). Cooperative interactions appear to be important for this anchorage, as internal digestion of the anchored fragments to produce fragments of less than 1 kb resulted in their release (17). Hence, the size of the fragment produced by an individual restriction endonuclease is to some degree important to the anchorage of that fragment: the restriction fragment must be large enough (2-3 kb) to contain the sequences at the 5' end of an active gene which cooperate to form a binding site. Since over 80% of the 7F locus was comprised of restriction fragments greater than 2.5 kb, our analysis should have detected most of the anchored sequences within this locus. However, it is possible that the enzyme used to prepare these structures cleaved an additional anchoring fragment(s) within this locus in such a way as to release it, thereby precluding its detection by our methods.

The fact that each anchored fragment at the 7F locus contained expressed sequences strongly suggests that expression is crucial for anchorage. This anchorage is probably not due to interaction with RNA polymerase or other components of transcriptional complexes, for the following reasons. Nuclear transcription of the five anchored fragments occurs at a very low rate, with less than 1 in 10 cells containing an RNA polymerase molecule engaged in active transcription of any of these five fragments (Small and Vogelstein, unpublished data). In addition, conditions which remove more than 99.7% of nascent RNA do not result in relase of anchored fragments from the 7F locus (data not shown) or from other regions of the genome (17). Finally, fine structure mapping has shown that the anchoring fragments are located near the 5' end of expressed genes and these regions do <u>not</u> include the bulk of the transcriptional unit (17).

It is important to put our work in the proper context. The results that we obtained are dependent on the conditions we used to prepare the loop structures analyzed. For example, using different preparative procedures, Mirkovitch <u>et al</u>. (18), have shown that somewhat different sequences are bound to the nuclear matrix than those found with a standard high salt procedure

(17). In particular, Mirkovitch et al., found relatively small (several hundred base pair) fragments near the 5' end of active genes that mediated attachment (18), while with high salt preparations the 5' ends of genes were attached to the nuclear matrix through multiple interactions within larger (2-3 kb) fragments (17). There is no evidence that any of these looped structures (whether isolated in low or high salt) are biologically significant. Moreover, there are no conclusive experiments demonstrating that the existence of the loops themselves is not a result of aggregation or other artifacts induced by the experimental procedures. With these cautions in mind, we have analyzed the organization of specific DNA sequences within the structures used to demonstrate the existence of DNA loops by a large number of investigators over the last ten years (6,7,26,27,30). Within these structures, we have shown that the loops appear to be organized quite specifically with respect to DNA sequence. The anchored regions of the loops are provided by transcriptionally active genes (probably the 5' end of active genes) and the loops themselves are largely comprised of the DNA sequences lying between active genes.

It is of interest to relate the above findings to other observations about loops. In particular, it has been shown that loop size in various organisms shows a strong correlation with replicon size (24,25) and that DNA replication itself takes place in association with the nuclear matrix (7,27-30). Since, as shown above, loop sizes appear to be determined by distances between transcriptional units, this suggests a connection between DNA replication and transcription. One might speculate that of the many potentially active origins of replication, the ones that are actually functional in a given cell are those that are near transcriptionally active genes in the cell. This subset of replication origins would be near nuclear matrix anchorage points allowing preferential access to components of the replication machinery which are bound to the matrix (31,32). This speculation may explain the experimental observations that transcriptionally active genes are replicated during the early portion of S-phase (reviewed in ref. 33).

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