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Localization of SV40 genes within supercoiled loop domains

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ABSTRACT

Recent studies indicate that eukaryotic DNA is organized into supercoiled loop domains. These loops appear to be anchored at their bases to an insoluble nuclear skeleton or matrix. Most of the DNA in the loops can be released from the matrix by nuclease digestion; the residual DNA remaining with the nuclear matrix represents sequences at the base of the loops, and possibly other sequences which are intimately associated with the nuclear matrix for other reasons. Using a quantitative application of the Southern blotting technique, we have found this residual DNA from SV40 infected 3T3 cells to be enriched in SV40 sequences, indicating that they reside near matrix-DNA attachment points. An enrichment of 3-7 fold relative to total cellular DNA, was found in each of three different lines of SV40 infected 3T3 cells. Control experiments with globin genes showed no such enrichment in this residual matrix DNA. This sequence specificity suggests that the spatial organization of DNA sequences within loops may be related to the functionality of these sequences within the cell.

INTRODUCTION

Numerous observations suggest that the structural organization of DNA within chromosomes may have a significant bearing on gene function[1-8]. In this regard, loops of DNA have been recognized as an important organizational component in several types of eukaryotic nuclei. In meiotic cells, the lampbrush appearance of chromosomes has been shown to result from the presence of loops, covered with transcriptional complexes emanating from a central scaffold[9, 10]. In addition, Paulson and Laemmli [11] have shown that metaphase chromosomes appear to consist of a series of DNA loops anchored to a central proteinaceous scaffold or matrix. It has also been shown that the DNA in interphase nuclei from Drosophila[12], humans[13], rodents[14-16] and yeast[17] cells is arranged in the form of negatively supercoiled loops. More recently, we have shown that the interphase loops can be visualized as a halo anchored to a central nuclear matrix[18].

In order to begin to determine the relationship of the looped organization

of DNA to nuclear function, we have asked whether specific DNA sequences can be localized on the loops with respect to their matrix attachment points. Most of the DNA in these loops can be released from the nuclear matrix by nuclease digestion[15, 17, 19]; the residual DNA remaining with the matrix represents sequences adjacent to the matrix-DNA attachment points. Using a quantitative application of the Southern blotting technique, we show here that the residual matrix DNA from SV40 infected 3T3 cells is significantly enriched in SV40 sequences. The significance of these findings in relation to the organization and function of nuclear DNA is discussed.

MATERIALS AND METHODS

Cells

SV40 transformed 3T3 cell clones SVB203, SVB209 and SVB213 were generously provided by Drs. G. Ketner and T. Kelly (The Johns Hopkins University School of Medicine, Baltimore, MD). These cells contain two or less copies of SV40 DNA sequences per cell, as discussed by Ketner and Kelly[21]. The cells were grown in roller bottles in Dulbecco's modification of Eagle's medium supplemented with 2% fetal calf serum (Microbiological Associates, Rockville, MD), 100 units/ml penicillin and 100 micrograms/ml streptomycin. Cells were labeled 48 hr with 0.1 μC/ml (methyl-^{3}H) thymidine (Moravek Biochemicals, City of Industry, CA, 50 Ci/mmol) before harvesting.

Preparation of Matrix and Total Cellular DNA

Cells were harvested by trypsinization and washed in Hanks's balanced salt solution. Detergent treated nuclei were prepared by treating these cells with isotonic buffer (100 mM NaCl, 50 mM KCl, 20 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) containing 1% NP-40 (Bethesda Research Labs., Rockville, MD). After low speed centrifugation, the nuclei were suspended in isotonic buffer, and then an equal volume of high salt buffer (4 M NaCl, 4 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4) was added slowly. All steps up to this point were performed at 4°C. These high salt treated nuclei were then incubated for 10-30 minutes with DNase I (DN-CL, Sigma, St. Louis, MO), 30 U/ml at 37°C. The reaction was stopped by placing the suspension on ice. The suspension was then layered on a cushion of 15% glycerol in 50% high salt buffer and pelleted by centrifuging for 20 minutes at 10,000 x g at 4°C. The amount of DNA remaining with the nuclear matrix was determined by measuring acid precipitable radioactivity. DNA was extracted from matrices or whole cells (total DNA) by the method of Gross-Bellard *et al.*[22]. DNA concentrations were determined by the method of Boer[23].

Restriction, Electrophoresis and Hybridization of DNA

DNA was digested to completion with HindIII (Bethesda Research Labs., Rockville, MD) and electrophoresed at 1 V/cm on a 1.5% agarose gel. Fragments were transferred to nitrocellulose sheets (Schleicher and Schuell BA 85, Keene, NH) by the method of Southern[21]. Filters were preincubated for 6 hours at 65°C in 10 x Denhardt's[24] buffer (Denhardt's buffer = 0.02% each of bovine serum albumin, polyvinylpyrrolidone and Ficoll) in 6 x SSC (SSC = 0.15 M NaCl, 0.015 M Na citrate).

SV40 DNA was a gift of Dr. David Shortle (The Johns Hopkins University School of Medicine, Baltimore, MD). Inserts from mouse alpha and beta globin cDNA clones were a gift of Dr. H. Kazazian (The Johns Hopkins University School of Medicine, Baltimore, MD). Hybridization probes were prepared by nick translation of DNA[25] using ^{32}P-dATP and ^{32}P-dCTP (Amersham, Arlington Heights, IL, 400 Ci/mmol) to a specific activity of 5-10 x 10^7 dpm/µg. Filters were hybridized with 1 microgram of labeled probe in 10 ml of 1 M NaCl, 10 x Denhardt's buffer, 50 mM Na phosphate, 2 mM EDTA, pH 7.0. After hybridization for 66 hours at 65°C, filters were washed extensively at 65°C in buffers of decreasing ionic strength to 0.5 x SSC, 0.3% SDS (K. Peden and D. Nathans, manuscript in preparation). Filters were subjected to autoradiography at -70°C with Kodak XR5 film. Grain density was quantitated by densitometric tracing using a Clifford Densicomp model 445 densitometer. Areas under peaks were quantitated using a Hewlett-Packard 9874A digitizer.

RESULTS

When nuclei are subjected to treatment with 2 M NaCl, histones and other proteins are removed[26, 27]. Upon mild nicking of the DNA strands to release supercoiling, loops of DNA unwind and, upon staining with ethidium, can be seen as a halo of DNA surrounding a central nuclear matrix[18]. With progressive nuclease action, the loops are gradually cleaved from their nuclear matrix attachment points; this can be observed directly with the fluorescent microscope as the gradual shortening of the halos until they disappear[18]. After sedimentation through a glycerol cushion, the DNA that is recovered in the nuclear matrix pellet represents sequences that are intimately associated with it. The amount of DNA recovered with nuclear matrix is a function of how much of the DNA in each loop was removed with the nuclease treatment; a longer treatment will of course remove more of the DNA.

The DNA from the nuclear matrix preparations can then be isolated and its relative content of a given DNA sequence determined by hybridization.

Because of the small amounts of DNA recovered with nuclear matrix, it is convenient to use a hybridization assay which requires only small quantities of DNA. The Southern method of hybridization[20] provides such an assay. In this method, DNA restriction fragments are separated by electrophoresis in agarose gels, transferred to nitrocellulose filters and hybridized to an excess of a ^{32}P-labeled DNA probe. The filter is then washed and autoradiographed.

To use the Southern method in a quantitative manner, however, it is necessary to show that there is a direct relationship between the amount of a specific DNA sequence present in the sample and the grain density of the autoradiogram obtained. To determine this relationship, we loaded various amounts of SV40 DNA, or DNA from SV40 infected 3T3 cells, onto gels after restriction, and, after the Southern procedure, measured the grain density of the resultant bands by autoradiography. It was found that over an 8-fold range, the concentration of DNA in a given restriction band is linearly related to the grain density for the DNA fragments between 500 and 1700 base pairs (bp) in size ($r = 0.946$; $p < .001$). In these experiments, we were comparing the density of the same restriction fragment in different slots of the same agarose gel. The Southern technique has also been used in a quantitative manner by other investigators[28-30].

Using this assay, we examined the nuclear matrix DNA for enrichment of SV40 sequences in SVB203 cells. In these cells, there is one integration site per cell, containing a partial tandem duplication of the SV40 genome[21]. Matrix preparations from SVB203 cells were obtained which contained either 3.5% or 9.0% of the total cellular DNA. The DNA extracted from matrix and the total cellular DNA reference were digested with HindIII, and equal amounts of these DNA samples were electrophoresed, transferred, hybridized and autoradiographed as above. The results are shown in Figure 1 and Table 1. The enrichment is similar for all the fragment sizes (1768, 1169/1101, 526 bp) measured. The matrix associated DNA is 4-7 fold enriched in SV40 sequences, relative to total cellular DNA. It is also notable that the DNA from the matrix preparation that contained only 3.5% of the total cellular DNA is enriched in SV40 sequences relative to the DNA from the matrix that contained 9.0% of the total cellular DNA. The fact that greater enrichment is found as more DNA is removed from the matrix implies that the SV40 sequences lie very close to DNA-matrix attachment points.

Control experiments showed that this result was not an artifact of the experimental method. Test transfers showed similar transfer efficiencies of

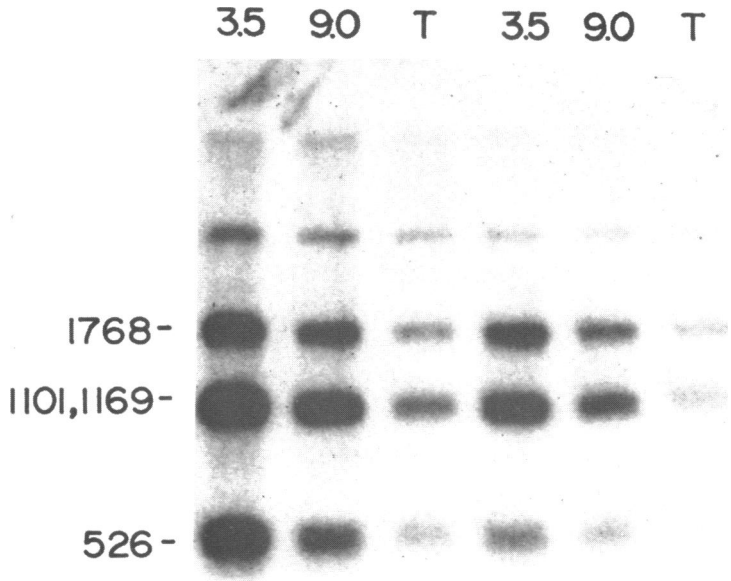

Figure 1. Enrichment of SV40 DNA Sequences in Matrix DNA.
Equal amounts of total cellular DNA from SV40 transformed 3T3 cell line SVB203 and DNA from matrix preparations containing 3.5% or 9.0% of the total cellular DNA restricted with HindIII were electrophoresed on a 1.5% agarose gel, transferred to nitrocellulose sheets and hybridized with ^{32}P SV40 DNA. The three left lanes contain 5 µg of DNA and the three right lanes contain 2 µg of DNA. Slots with total cellular DNA are labeled T; slots with DNA from matrix preparations containing 9.0% of total cellular DNA are labeled 9.0; slots with DNA from matrix preparations containing 3.5% of total cellular DNA are labeled 3.5 The fragments of length greater than 1768 bp represent "joint fragments" which contain both SV40 and cellular DNA sequences. They were not quantitated because they did not fall within the fragment size range wherein we had linearly correlated autoradiographic density and SV40 sequence content.

total cellular and matrix DNA restriction fragments in the size classes of interest (data not shown). Moreover, using ^{32}P-labeled mouse beta globin cloned cDNA as a hybridization probe in the Southern hybridization assay,

TABLE 1

Enrichment of SV40 Sequences in Matrix DNA

Autoradiograms of hybridization bands of size 1768, 1135 bp (an unresolved doublet of 1169 and 1101 bp) and 526 bp from matrix preparations from SV40 transformed 3T3 cells were compared, by densitometric tracing, to the homologous bands from total cellular DNA to compute enrichment.

Cell Line	%DNA*	Restriction Fragment(s)+	Enrichment‡
SVB203	3.5	1768	5.5
		1101/1169	6.3
		526	7.1
	9.0	1768	4.0
		1101/1169	4.5
		526	3.1
SVB209	2.4	1768	4.6
		1101/1169	4.7
	5.0	1768	2.9
		1101/1169	3.6
SVB213	4.6	1768	3.8
		1101/1169	5.0
		526	5.2
	8.8	1768	3.1
		1101/1169	4.1
		526	4.1

*percent of total cellular DNA remaining with the nuclear matrix.
+bp
‡relative to total cellular DNA

we have shown that mouse beta globin sequences are not preferentially associated with the matrix of SV40 transformed 3T3 cells (Fig. 2). Similarly, we have found that mouse alpha globin sequences are not preferentially associated with the nuclear matrix (data not shown). These results are discussed below.

SV40 does not integrate at a unique site in the host genome[31, 32]. It was therefore possible that the association of SV40 DNA sequences with the nuclear matrix was limited to cloned cell line SVB203, and unrelated to SV40 DNA *per se*. Experiments similar to that shown in Figure 2 have shown that matrix DNA from independent clones of SV40 transformed 3T3 lines SVB209 and SVB213 are similarly enriched in SV40 DNA sequences (Table 1). Thus, SV40 DNA sequences appear to be specifically associated with the nuclear matrix

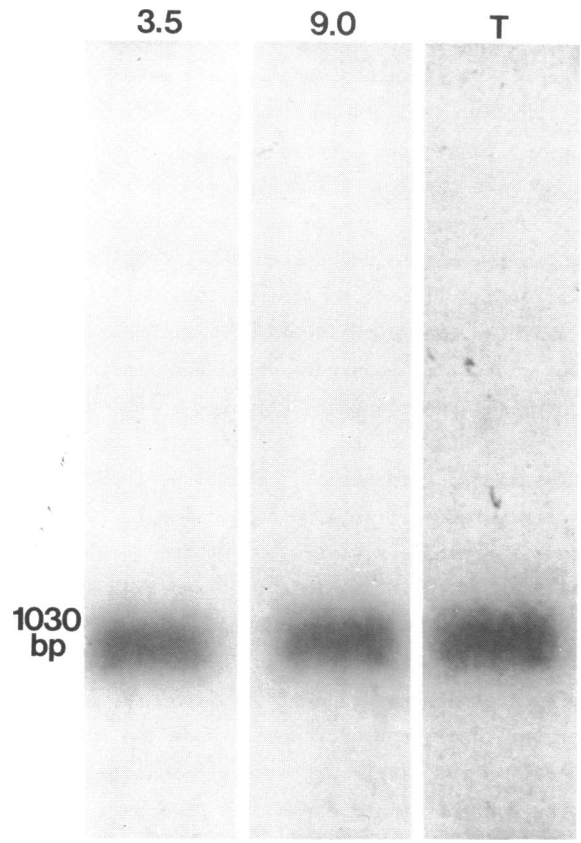

Figure 2. Determination of Globin Sequences in Matrix DNA.
Total cellular DNA from SV40 transformed cell line SVB203 and DNA from matrix preparations of the same cell line containing 3.5% or 9.0% of the total cellular DNA were restricted with HindIII, electrophoresed, transferred to nitrocellulose sheets and hybridized with ^{32}P-labeled nick translated mouse beta globin cDNA clones. Each slot contained 10 µg of DNA. T, 9.0 and 3.5 refer to the same DNA preparations as in Figure 1.

of SV40 transformed 3T3 cells.

DISCUSSION

We have shown specific enrichment of SV40 DNA sequences in matrix associated DNA. Although the transcribed sequences within the SV40 genome would be expected to be preferentially digested upon DNase I treatment of nuclei[3-7], [33-35], the preparation of nuclear matrix removes those components of chromatin

thought to be responsible for conferring resistance (histones; ref. 36-38) or sensitivity (high mobility group proteins 14 and 17; refs. 5-7) to DNase I. Instead, after the high salt incubation, DNase I makes random cuts in the DNA, severing much of the DNA in the loops from the matrix. Thus, DNA associated with the matrix after this DNase I treatment will be enriched in those sequences which occur at the base of the loops.

This random cutting might explain why the SV40 sequences are not quantitatively retained in the matrix associated DNA. Complete protection of sequences would afford an 11-fold enrichment in a matrix DNA sample containing 9% of the total cellular DNA; such levels of enrichment are never seen (Table 1). Random cutting of sequences would preferentially, but not quantitatively, leave proximal DNA sequences attached to the matrix. In addition, some of the DNA strands attached to the matrix might be too small to form the appropriate restriction fragments[39]; the size of DNA strands left anchored to the matrix ranges from 1500 base pairs to 10,000 base pairs in matrix preparations containing 2.4% to 9.0% of the total cellular DNA (unpublished data). It is also possible that the SV40 sequences are not preferentially associated with the nuclear matrix in a certain fraction of the cells. This would also result in less than quantitative enrichment.

Although some experiments showed a mild loss of the globin genes in the nuclear matrix preparations, the differences were too small to be reliably quantitated with our assay. There are several possible ways to explain how the globin sequence content of nuclear matrix DNA could be similar to total nuclear DNA. It is possible that the globin genes are situated near, but not at, the base of the loops (at the nuclear matrix attachment point). Because of the randomness of DNase action, this could result in their retention in only a fraction of the nuclear matrices. Alternatively, the globin genes, unlike the SV40 DNA sequences, may be randomly distributed with respect to the nuclear matrix, so that in only some cells are they associated with the nuclear matrix.

There are several possible explanations for the association of SV40 DNA sequences with the nuclear matrix. As mentioned in the Introduction, the matrix is the site of anchorage for the DNA loops. Hence, if SV40 integrated at sites near the bases of the loops, they would be found in association with the matrix. Razin *et al.*[16] and Cook and Brazell[30] also proposed that if a sequence is enriched in the DNA associated with the nuclear matrix, then it must be at the base of the loops. However, such interpretations must be made with caution. We have previously found that the genes for ribosomal DNA are enriched in nuclear matrix preparations[19, 40] and since these genes are

tandemly repeated[41], the interpretation that these genes occur only at the base of loops is unlikely.

There are several other interpretations that must be considered. For example, loops which are involved in transcription may exist in a different conformation, relative to the nuclear matrix, than the bulk of the loops. In fact, it has previously been proposed that the nuclear matrix is involved in transcription, processing and/or transport of DNA[42]. RNA transcription and/or processing could take place on a stationary support within the nuclear matrix, such as has been proposed for DNA replication[43]. Evidence supporting these proposals includes the association of hnRNA and snRNA with the nuclear matrix[44-46], specific binding of steroid hormones on the nuclear matrix in target tissues[47, 48], and the preferential association of ribosomal DNA with the nuclear matrix[19, 40]. This communication is also consistent with a transcriptional role for the nuclear matrix, since transcribed SV40 sequences are enriched on the matrix, whereas matrix DNA was not found to be enriched in nontranscribed globin DNA sequences. Concentration of transcribed sequences in a pellet fraction of high salt extracted chromatin has been demonstrated by Bekhor and colleagues[49, 50]; however, the relationship between this chromatin fraction and the nuclear matrix is unclear at present.

Alternative explanations for the association of loop domains containing SV40 DNA sequences with the nuclear matrix should be considered. For example, SV40 maintains 3T3 cells in a transformed state, and it is possible that its association with nuclear matrix is related in some way to transformation. Moreover, sequences near the SV40 origin of replication bind specifically to T antigen[51]. If T antigen is found in the nuclear matrix of transformed cells, as is the case for polyoma[52] and SV40 productively infected cells[53], then the association of SV40 DNA with the nuclear matrix may be due to its binding to T antigen. It is also possible that the SV40 origin of replication may be associated with the matrix because of the matrix's function in DNA replication[18, 43, 54, 55]. Studies to further elucidate the nature and function of DNA sequences associated with the nuclear matrix are in progress.

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