DNAaseI-hypersensitive minichromosomes of SV40 possess an elastic torsional strain in DNA

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ABSTRACT
Previously, we have shown that DNA in a small fraction (2-5%) of SV40 minichromosomes was torsionally strained and could be relaxed by treating minichromosomes with topoisomerase I. This fraction was enriched with endogeneous RNA polymerase II (Luchnik et al., 1982, EMBO J., 1, 1353). Here we show that one and the same fraction of SV40 minichromosomes is hypersensitive to DNAase I and is relaxable by topoisomerase I. Moreover, this fraction completely loses its hypersensitivity to DNAase I upon relaxation. The possibility that this fraction of minichromosomes can be represented by naked DNA is ruled out by the results of studying the kinetics of minichromosome digestion by DNAase I in comparison to digestion of pure SV40 DNA and by measuring the buoyant density of SV40 chromatin in equilibrium CsCl gradient. Our data obtained with SV40 minichromosomes may be relevant to the mechanism responsible for DNAase I hypersensitivity in the loops or domains of cellular chromatin.

INTRODUCTION
The structure and conformation of a chromatin fiber is rearranged before the onset of transcription of a given gene in an eukaryotic cell; this rearrangement is thought to be a prerequisite for the initiation of transcription (for reviews, see (1-3)). The evidence for this statement is provided mainly by studies of chromatin digestion by different nucleases and, first of all, by DNAase I. The enzyme attacks preferentially the chromatin regions which are being transcribed or prepared for transcription as well as the neighbouring regions of chromatin. As a result, an elevated amount of DNA nicking - "DNAase I sensitivity" (4-11) and double-strand cutting at the early stages of hydrolysis - "hypersensitivity to DNAase I" (12-19, 7) is found in the regions of transcrip-
tionally active chromatin. The sites of double-strand breakage are often located upstream from the 5'-ends of transcribed genes. Although DNAAse I digestion turned out to be an important tool helpful in distinguishing transcriptionally active genes, the physical or chemical nature of (hyper)sensitivity to DNAAse I remains unclear.

As we found earlier, an important feature of transcriptionally active chromatin in eukaryotic virus SV40 was the elastic torsional strain of its DNA (20). In this report, we have shown that these were torsionally strained molecules of SV40 chromatin which were specifically hypersensitive to DNAAse I. Moreover, there is evidence that the DNA torsional strain itself may be the reason for DNAAse I hypersensitivity of transcriptionally active minichromosomes.

Circular minichromosomes of SV40 may represent a good model for a domain of cellular chromatin. For this reason, our results may indicate that transcriptionally active loops of cellular chromatin which are also (hyper)sensitive to DNAAse I possess a torsional strain in their DNA.

MATERIALS AND METHODS

Cells and virus

Monolayer cultures of African green monkey kidney cells (CV1) in roller bottles were infected with SV40, strain 776, and radioactively labeled as described (21).

Isolation of virus minichromosomes, preparation of topoisomerase I, electrophoretic analysis of topoisomers, and transcription in vitro were performed essentially as described (20).

Enzymatic treatment

Minichromosomes were treated with DNAAse I and/or topoisomerase I at 18°C after dilution to 0.2 M NaCl, 10 mM TRIS-HCl, 1 mM EDTA, pH 7.6. In cases not specified in legends to figures, DNAAse I (Worthington) was added in an amount of 0.005 μg per 1 μg of DNA. Then MgCl₂ was added to 6 mM. The reaction was stopped by addition of EDTA to 10 mM. Topoisomerase treatments took 1.5 h, and DNAAse treatments 5-10 min.

For electrophoretic analysis of DNA, SDS (0.1%) and pro-
teinase K (30 µg/ml) were added and the solution was incubated at 37°C for 1 h. Then DNA was precipitated by ethanol, dissolved in 20% sucrose, 1 mM EDTA, 0.01% bromphenol blue, 10 mM TEA-HCl, pH 7.6, and layered on agarose gels.

Ultracentrifugation in CsCl density gradient

The material was fixed by 1% formaldehyde for 24 h and then ultracentrifuged in a CsCl equilibrium density gradient as described previously (22).

RESULTS

Minichromosomes hypersensitive to DNAase I exhibit a higher sedimentation rate than the bulk of minichromosomes

First, we studied the distribution of minichromosomes hypersensitive to DNAase I along a sucrose gradient (Figure 1). The hypersensitivity was detected by the appearance of linear SV40 DNA molecules resulting from singular DNA double-strand cuts in SV40 circles at the early stages of digestion when a significant part of DNA remained superhelical.

In a control experiment in which minichromosomes were incubated in the buffer used for digestion (but without DNAase I), we found some DNA double-breakage occurred in the fraction of minichromosomes sedimenting slower than their bulk, obviously due to some endogenous endonuclease activity (Figure 2a).

The addition of DNAase I to the incubation medium increases the amount of linear SV40 molecules mainly along faster sedimenting minichromosomes (Figure 2b). Figure 1b presents the quantitation of distribution of linear DNA molecules along the sucrose gradient as determined by radioactivity counting in bands excised from agarose gels. (Quantitations in Fig. 1 were made using other gels than those shown in Fig. 2.) One can see that the distribution of SV40 minichromosomes hypersensitive to DNAase I corresponds to the distribution of endogenous RNA polymerase activity depicted in Figure 1a. (See also (23)). This agrees with the notion that transcriptionally active chromatin is hypersensitive to DNAase I. It should be pointed out that the DNA of the peak itself was nicked more extensively by DNAase I than the DNA in the heavy shoulder. Possibly, in addition to transcriptionally active material, the shoulder...
The nuclei from \[^{3}H\text{-thymidine}\] labeled CV-1 cells infected with SV40 virus (strain 776) were extracted with 0.25% Triton X-100, 1 mM EDTA, 0.4 M NaCl, 10 mM TEA-HCl, pH 7.6, and the extracts were ultracentrifuged in an SW 25.2 rotor at 20,000 rpm and 4°C for 12 h through 10-30% sucrose gradients containing 1 mM EDTA, 10 mM TEA-HCl, pH 7.6, and 0.4 M NaCl. After analysis of \[^{3}H\text{-DNA}\] distribution, aliquots were taken from fractions of the gradient for measurement of the transcriptional activity according to Edenberg (50) (but 0.15 M (NH\(_4\))\(_2\)SO\(_4\) was added) and for treatment with DNase I or topoisomerase I. Fractions 14-16 were used for further analysis.

- Distribution of minichromosomes (closed line), and of their transcriptional activity (dashed line); b - distribution of linear form of viral DNA induced by either control incubation for 5 min in the buffer used for DNase I digestion (open circles) or incubation with DNase I (closed circles). Radioactivity was counted after excision of DNA-containing strips from agarose gels, dissolving them in 0.5 ml of 1 M HCl at 90°C, cooling to 60°C and addition of toluene-based Triton (30%) containing scintillator.
contained minichromosomes more protected against DNAase I nicking (for example, more compact particles).

Finally, the distribution of elastically strained SV40 minichromosomes, i.e. those relaxable by topoisomerase I action, was similar to that of the DNAase I hypersensitive material (Figure 2c). This suggested that the both properties could be characteristic of one and the same fraction of SV40 minichromosomes.

**DNAase I hypersensitive minichromosomes possess an elastic torsional strain in DNA**

To verify the above suggestion, we treated minichromosomes with DNAase I and topoisomerase I in different succession. We used the fractions of minichromosomes enriched with the transcriptionally active material. After enzyme treatments, DNA was isolated from the control and treated minichromosomes and run in agarose gel containing 0.01 µg/ml of ethidium bromide (Figure 3) to separate covalently closed but relaxed DNA molecules from those harbouring single-strand breaks (nicked circular molecules). The former ones move as a set of bands (topoisomers) just below (ahead) the band of nicked molecules. Covalently closed underreplicated double rings of SV40 DNA move, under these conditions, as a diffuse band just above (behind) nicked SV40 circles (Figure 3). Careful inspection reveals that this band is formed by a set of sub-bands (topoisomers). Under non-resolving electrophoretic conditions, all the three bands often comigrate.

Topoisomerase I treatment of minichromosomes relaxed a fraction of superhelical DNA which was identified as a set of topoisomers moving just below the band of nicked molecules (Figure 3, track 2). No molecules which possess double- or single DNA breaks appeared as compared to control minichromosomes. The limited digestion of minichromosomes by DNAase I yielded molecules harbouring single or double DNA cuts (track 3). However, when such minichromosomes slightly digested with DNAase I were treated with topoisomerase I, virtually no covalently closed but relaxed molecules were found notwithstanding the fact that approximately 80–90% of DNA molecules remained covalently closed and fully supercoiled (track 4). Hence, a
fraction of SV40 minichromosomes containing DNA relaxable by topoisomerase I was preferentially attacked by DNAase I. The treatment of minichromosomes with DNAase I after the pretreatment with topoisomerase I revealed that the former enzyme was no longer capable of producing DNA double-strand cuts (hypersensitivity) as was indicated by the absence of linear SV40 DNA molecules (track 5). The amount of DNA nicked by DNAase I was also somewhat lower in this case. The absence of linear DNA could not be attributed to the inhibition of DNAase I by topoisomerase I (as suggested by a lower content of nicked DNA in track 5) since the prolonged incubation of topoisomerase-treated minichromosomes with DNAase I led to a considerable increase in the amount of nicked DNA rather than linear DNA at the intermediate stages of hydrolysis (not shown).

The experiment was repeated with a lower concentration of DNAase I added to minichromosomes before (track 6) or after (track 7) the treatment with topoisomerase. The result was the same in qualitative terms, but the marginal amount of DNA relaxable by topoisomerase I remained after the pretreatment with DNAase I (track 6).

Our conclusion is that minichromosomes hypersensitive to DNAase I possess an elastic torsional strain in their DNA.

When naked superhelical SV40 DNA is digested under these particular conditions (but not some others—see Discussion), the hypersensitivity is not revealed (absence of linear form).

**Figure 2. Electrophoresis of DNA from minichromosomes taken from different fractions of sucrose gradient.**

- a - After control incubation in the buffer used for DNAase I digestion; b - after addition of DNAase I; c - after incubation with topoisomerase I. The lane numbers correspond to fraction numbers. Arrows indicate the positions of the main peak of minichromosomes. (Quantitation of linear DNA in Figs. 1a, b, however, was made using other gels than given in Fig. 2a, b.) Different amounts of superhelical dimers (SD) on panels a and b result from variation between different minichromosome isolation. The rightmost lanes contain pure SV40 DNA treated with either EcoRI endonuclease (a and b) or topoisomerase I (c). The electrophoretic buffer contained 30 mM NaH₂PO₄, 40 mM Tris, 1 mM EDTA (pH 8.0). The slab gel presented on panel (c) was subjected to electrophoresis in the presence of 0.01 μg/ml of ethidium bromide for 36 h. Abbreviations are deciphered in the legend to Figure 3.
Figure 3. Electrophoretic analysis of SV40 DNA from mini-chromosomes treated with DNAase I (5x10^{-3} μg in lanes 3-5 or 2.5x10^{-3} μg in lanes 6, 7 per 1 μg of DNA) and/or topoisomerase I in different succession. Minichromosomes were taken from fractions 14-16 of the gradient depicted in Figure 1a. Electrophoresis was run for 36 h at 1800 and 2-3 V/cm in 1.6% agarose gel made on 20 mM NaH2PO4, 40 mM Tris, 1 mM EDTA (pH 8.0), and 0.01 μg/ml of ethidium bromide.

1 - control minichromosomes; 2 - minichromosomes treated with topoisomerase I for 2 h; 3 - minichromosomes treated with DNAase I for 5 min; 4, 6 - minichromosomes treated with DNAase I and then, after addition of EDTA, with topoisomerase I; 5, 7 - minichromosomes treated with topoisomerase I and then with DNAase I; 8 - naked SV40 DNA treated with DNAase I (2.5x10^{-3} μg per 1 μg of DNA).

Abbreviations: S - superhelical SV40 DNA; L - linear SV40 DNA; R - relaxed SV40 DNA; N - nicked SV40 DNA; SD - superhelical dimers of SV40 DNA; RI - other replicative intermediates of SV40 DNA.

and only single-strand breaks are accumulated (track 8, see also the next section).

DNAase I hypersensitive fraction of minichromosomes is not a naked DNA.

First, we analysed dose dependence of DNAase I digestion
Figure 4. Dose dependence for digestion with DNAse I. Minichromosomes were taken from fractions 14-16 of the sucrose gradient depicted in Figure 1a.

a - a photograph of agarose gel after electrophoresis of DNA from minichromosomes (lanes 1-6) or naked DNA (lanes 7-12) digested with DNAse I.

DNAse doses were (in µg per 1 µg of DNA): 0 (lanes 1, 7); 0.00025 (lanes 2, 8); 0.001 (lanes 3, 9); 0.0025 (lanes 4, 10); 0.005 (lanes 5, 11); 0.025 (lanes 6, 12).

b - quantitation of the digestion dose-response determined by counting DNA radioactivity in agarose strips excised from the gel presented in panel (a). The radioactivity was counted as described in the legend to Figure 2. The kinetics was corrected by taking into account the supercoiled dimers (SD). Solid lines - percentage of DNA harbouring both single and double-strand breaks. Dashed lines - percentage of DNA harbouring double-strand scissions. Closed circles - naked DNA; open circles - minichromosomal DNA.
Figure 5. Ultracentrifugation in a CsCl density gradient of formaldehyde-fixed minichromosomes taken from fractions 14–16 of the sucrose gradient in Figure 1a.

of SV40 chromatin in comparison to naked SV40 DNA. We found that the appearance of linear DNA in chromatin followed a dose-response quite different from that in pure superhelical SV40 DNA (Figure 4). The former looks like the one-hit response, the first hits appearing almost simultaneously with the first nicks. The dose dependence for appearance of the linear form in pure SV40 DNA was obviously two-hit with double cuts resulting from two neighbouring nicks in the opposite strands of DNA (Figure 4). It is noteworthy that, in our digestion conditions, the rate of nicking of pure SV40 DNA by DNAase I was only slightly higher than that for SV40 minichromosomes (Figure 4). This finding indicates that the early appearance of the linear form in minichromosome digestion cannot result from two-hit breaking of some naked SV40 DNA mixed with minichromosomes. Similar results were obtained in the experiment in which traces of $^{32}$P-labeled free SV40 DNA were added to non-labeled minichromosomes, then the mixture was digested by DNAase I, and the digestion products were analysed in agarose gels by $^{32}$P-radioautography and ethidium staining (not shown).

Second, we mapped DNAase I hypersensitive sites in chromatin and pure SV40 DNA by hybridization of $^{32}$P-labeled SV40 restriction fragments to blots containing EcoRI restricted DNA from minichromosomes digested with DNAase I. The hypersensitive
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sites were mapped in the regulatory region of SV40 minichromosomes essentially in accordance with the data of other authors (24, 25). The digestion of pure SV40 DNA revealed numerous and weaker cuts distributed along the DNA (not shown).

Third, the CsCl density gradient equilibrium centrifugation of formaldehyde-fixed minichromosomes revealed that free DNA was absent from the population of SV40 minichromosomes enriched with transcription complexes (Figure 5). RNAase treatment did not change the homogeneity of minichromosomes as determined by CsCl centrifugation (not shown).

DISCUSSION

Evidence that DNAase I hypersensitivity in transcriptionally active chromatin is a function of torsional strain

We have shown above that DNAase I cuts and topoisomerase I relaxes one and the same fraction of SV40 chromatin. We may be certain that, at least under the experimental conditions employed, all DNAase I hypersensitive chromatin possesses a torsional strain. The reverse statement is not completely correct since not all the torsionally strained minichromosomes seem to catch double-strand scissions. Some are nicked and hence also lose a torsional strain. The ratio of nicking to double-strand breakage can simply depend on the probability of specific double-strand breaks at the hypersensitive region and that of nicking (possibly non-specific) somewhere else in the chromosome.

In the previous paper, we argued that torsionally-strained minichromosomes were transcriptionally active because they were heavily enriched with endogenous RNA polymerase activity (20). Now this statement is further supported by their hypersensitivity to DNAase I, the feature intrinsic to transcriptionally active chromatin (see refs. in Introduction).

It seems likely that the torsional strain itself is needed to create DNAase I hypersensitivity. In fact, the relaxation of transcriptionally active minichromosomes by topoisomerase I immediately abolishes their hypersensitivity to DNAase I.

The hypersensitivity induced by torsional strain can be obtained with free DNA if to perform the DNAase I digestion in
the presence of Mn$^{2+}$ (26). Therefore, a torsional strain is required for hypersensitivity but some other specific conditions may also be required. Also, the extent of DNA torsional strain within transcriptionally active chromatin is unknown and it can be far greater than the strain in naked superhelical SV40 DNA.

Recently, we have found that nicking of minichromosomal DNA by irradiation of nuclei with X-rays strongly suppresses the DNAsase I hypersensitivity (27). This provides independent evidence for the involvement of elastic torsional strain in creation of DNAsase I hypersensitivity.

The evidence has been published that DNAsase I hypersensitivity may be related to nucleosome-free regions (28, and refs. therein). We have also found that major portion of DNAsase I hypersensitivity may be connected with nucleosome-free region in minichromosomes isolated in 0.1 M NaCl (27). However, similar to the presently described situation, elastic torsional strain seems to be the major component of DNAsase I hypersensitivity tested in nuclei (27).

**DNA secondary structure in transcriptionally active chromatin**

Torsional strain in naked superhelical DNA induces several kinds of sequence-dependent conformational transitions in the DNA secondary structure. Among them are Z-DNA (29–32), cruciform structures (33–35) and possibly A-form (36, 37).

Either Z-form, cruciform structures or some yet unknown conformations may also arise in transcriptionally active chromatin in the result of torsional strain and possibly may be recognized by DNAsase I.

Such local conformational transitions sometimes located far from the start site of transcription can play a regulatory role in the transcription process by competing for energy of torsional strain with those conformations in the region of initiation of transcription (A-form, melting?) which may be a prerequisite for transcription. It is noteworthy that transcription of some superhelical plasmids by yeast RNA polymerase drops down sharply at certain superhelical densities and increases again at either higher or lower superhelical densities (38). This seems to be due to competing conformational tran-
sitions in superhelical DNA.

Relation to cellular chromatin

It is generally accepted now that eukaryotic chromatin is organized in loops or domains 10-100 kb long with the ends fixed to some skeletal elements of the nucleus (39-46). Closed superhelical DNA loop does not differ topologically from a closed DNA circle. Circular viral minichromosomes may be considered therefore as a model for the domain of host nuclear chromatin.

If such extrapolation is correct, our results on the nature of DNAase I hypersensitivity in SV40 may be relevant to the mechanism of DNAase I hypersensitivity in cellular chromatin.

Another piece of evidence for elastic torsional strain in transcriptionally active chromatin comes from its hypersensitivity to S1 nuclease. This enzyme recognizes the same poly-nucleotide sequences in naked DNA if only the later is elastically strained (47, 48).

Thus, we consider it plausible that transcriptionally active loops of cellular chromatin possess a torsional strain in DNA and that this strain at least partially may be a prerequisite for DNAase I hypersensitivity. However, if the strain is necessary, it may be not sufficient for creation of all DNAase I hypersensitive sites in the area of a given gene because different sets of hypersensitive sites are found in the vicinity of a gene in different tissues expressing the gene (49). This argues that some other factors such as specific proteins may also be involved.

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