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Effect of X-ray induced DNA damage on DNAase I hypersensitivity of SV40 chromatin: relation to elastic torsional strain in DNA

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ABSTRACT

The effect of X-irradiation on DNAase I hypersensitivity of SV40 minichromosomes within nuclei or free in solution was investigated. The susceptibility of the specific DNA sites in the control region of minichromosomes to DNAase I decreased in a dose dependent manner after irradiation of isolated nuclei. On the other hand, the irradiation of minichromosomes extracted from nuclei in 0.1 M NaCl-containing buffer almost did not affect the level of their hypersensitivity to DNAase I. This suggests that DNAase I hypersensitivity may be determined by two different mechanisms. One of them may be connected with elastic torsional strain within a fraction of minichromosomes and another seems to be determined by nucleosome free region. The first mechanism may be primarily responsible for the hypersensitivity of minichromosomes within nuclei. After irradiation of the intact cells, DNAase I hypersensitivity tested in nuclei substantially increased. This was connected with activation of endogeneous nucleases by X-irradiation which led to accumulation of single- and double-strand breaks superimposed to DNAase I induced breaks in the control region of SV40 DNA.

INTRODUCTION

According to some recent reports, the DNA within transcriptionally active chromatin may be elastically strained /1-3/. Another prominent feature of the active chromatin is its hypersensitivity to DNAase I, i.e. the appearance of DNA double-strand breaks in the areas of specific nucleotide sequences at early stages of hydrolysis of chromatin by DNAase I (for reviews see /4,5/). In this connection, the question arises whether the DNAase I hypersensitivity of transcriptionally active chromatin bears any relation to elastic torsional tension in DNA.

The irradiation of chromatin by X-rays may serve as an

instrument for relaxation of elastically strained DNA because single- and double-strand breaks in DNA induced by radiation lead to the loss of the torsional constraint in DNA. The ratio of single-strand to double-strand breaks produced by radiation depends on irradiation conditions and usually is within the range of 20:1 to 50:1. (Double-strand breaks are one-hit events when irradiation is performed in the buffered solutions.) It is important that both types of the breaks are induced in DNA at random, i.e. not depending on DNA sequence. Thus, the stochastic distribution of X-ray induced DNA breaks would not interfere with mapping of DNAase I hypersensitive sites which are sequence-specific.

In the present paper, we tried to investigate possible relation of torsional constraint in DNA to the DNAase I hypersensitivity using X-irradiation of SV40 infected cells, isolated nuclei or purified minichromosomes for relaxation of elastically strained DNA prior to DNAase I treatment. The results obtained suggest that the DNAase I hypersensitivity of intranuclear minichromosomes may at least partially be connected with elastic torsional tension in DNA.

Previously, we have shown that under specific conditions, i.e. extraction of minichromosomes with a buffer containing 0.4 M NaCl, all the DNAase I hypersensitivity was confined to elastically strained fraction of minichromosomes /6/. The elastically strained minichromosomes were identified by their relaxability with topoisomerase I. This approach, however, is difficult to apply to minichromosomes in nuclei or those isolated and purified in 0.1 M NaCl. The former may be hardly accessible to exogeneous topoisomerase, the latter could be exposed to endogeneous topoisomerase active in 0.1 M NaCl during extraction and purification procedures, thus eliminating elastic torsional tension.

MATERIALS AND METHODS

Cells and virus. Monolayer cultures of CV-1 monkey cells in roller bottles were infected with SV40, strain 776, and radioactively labeled as described /7/.

Isolation of nuclei and minichromosomes. Nuclei from infected cells (40 h postinfection) were obtained after cell lysis and washing of nuclei for three times with 0.1 M NaCl, 10 mM TEA-HCl, pH 7.6, 1 mM EDTA, 0.25% Triton X-100. Extraction of minichromosomes was performed in Dounce homogenizer in the same buffer by 20-30 strokes for 1 h at 4°C. Minichromosomes were purified through 10-30% sucrose gradient containing 0.1 M NaCl, 10 mM TEA-HCl, pH 7.6, 1 mM EDTA at 3°C, in SW27 rotor (20000 rpm, 10 h).

Irradiation. Cells, nuclei or isolated minichromosomes were irradiated in 0.1 M NaCl, 10 mM TEA-HCl, pH 7.6, 1 mM EDTA in X-ray apparatus RUM-13 (USSR) with 180 kV X-rays and a dose rate 45 Gy/min (4.5 Krad/min) at 0-2°C.

DNAase I digestion and mapping of hypersensitive sites. $MgCl_2$ was added to irradiation buffer to a final concentration of 6 mM. Immediately DNAase I was added and hydrolysis was performed for 5 min at 18°C (except incubation at 37°C in the case presented in Fig. 6b). DNAase I concentrations are indicated in legends to figures. Reactions were stopped by addition of EDTA to 10 mM and SDS to 0.1%. Isolation of DNA and mapping of DNAase I hypersensitive sites by indirect end labeling procedure were performed as described /8 /. Briefly, purified DNA samples were digested with EcoRI endonuclease and DNA fragments were fractionated on 1.5% agarose vertical gels in Tris-phosphate buffer, then transferred to nitrocellulose filters (Schleicher and Schuell BA85), and hybridized with nick-translated fragments to localize specific DNAase cuts.

Autoradiographs were scanned in Opton spectrophotometer in visible light.

RESULTS

Effect of irradiation on hypersensitivity of intranuclear minichromosomes to DNAase I

To study the mechanisms of DNAase I hypersensitivity with special reference to elastic torsional strain, one has to control the overall level of DNAase I digestion of DNA. We studied only those stages of the digestion when considerable proportion of superhelical DNA was still present along with

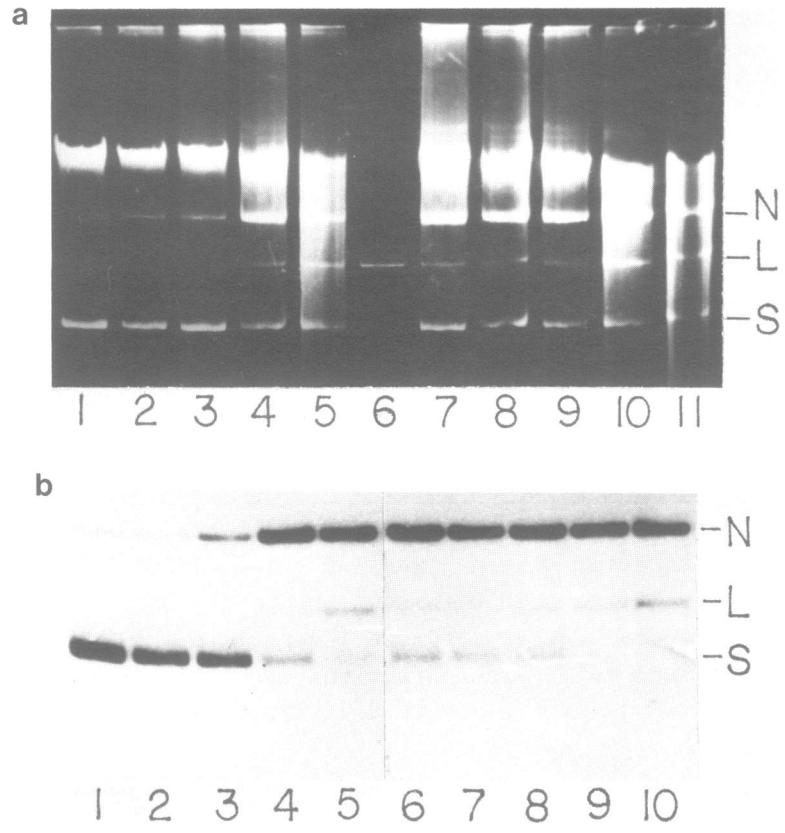


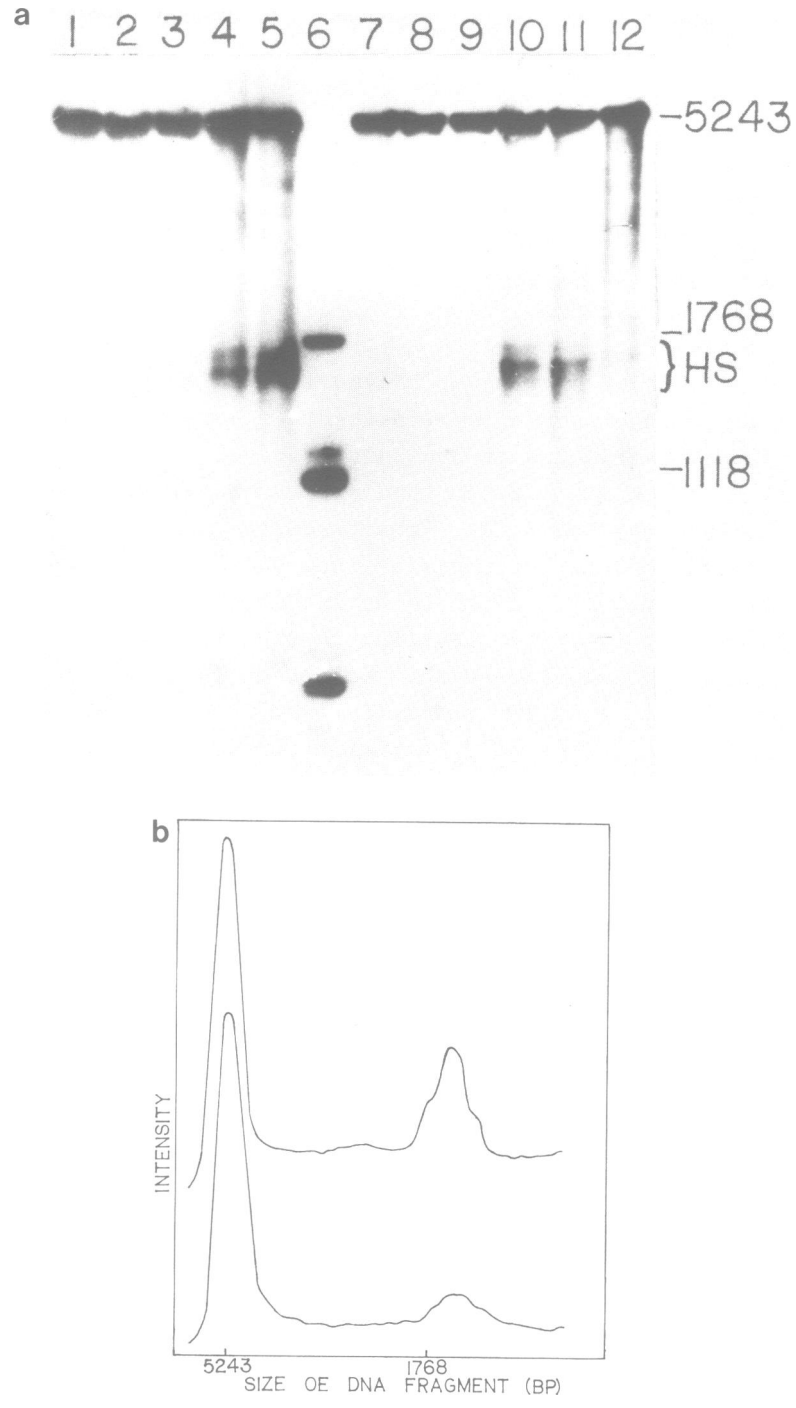
Fig. 1. DNAase I digestion of minichromosomal DNA before and after X-irradiation.
a - Digestion of control (lanes 1-5) or irradiated with 10 KGy of X-rays (lanes 7-11) minichromosomes within nuclei (10^7 nuclei/ml). 1, 7 - untreated; 2, 8 - incubated in the presence of 6 mM $MgCl_2$ at 18°C for 5 min; 3, 9 - 6.0 µg/ml of DNAase I; 4, 10 - 13.0 µg/ml of DNAase I; 5, 11 - 26.0 µg/ml of DNAase I. 6 - linearized SV40 DNA (endonuclease EcoRI digestion). S - superhelical, N - nicked, L - linear SV40 DNA.
b - Digestion of control (lanes 1-5) or irradiated with 30 KGy of X-rays (lanes 6-10) minichromosomes which were extracted from nuclei before irradiation and digestion. (Autoradiograph of *in vivo* ^{32}P-labeled DNA.) 1, 6 - untreated; 2, 7 - incubated in the presence of 6 mM $MgCl_2$ at 18°C for 5 min; 3, 8 - 0.15 µg/ml of DNAase I; 4, 9 - 0.5 µg/ml of DNAase I; 5, 10 - 1.5 µg/ml of DNAase I.

5-20% of linear DNA produced by DNAase I (Fig. 1a, lanes 4, 5; Fig. 1b, lanes 4, 5). DNAase I concentrations sufficient to begin nicking and double-strand breakage were quite different

for nuclei and purified minichromosomes (compare panels a and b in Fig. 1). X-irradiation did not affect overall level of digestion of chromatin by DNAase I either in nuclei (Fig. 1a) or in isolated minichromosomes (Fig. 1b). In the latter case, this was confirmed by densitometry of autoradiograph. The extraction and sedimentation characteristics of both irradiated and unirradiated minichromosomes were virtually the same. This suggested that overall conformation and solubility of minichromosomes were not severely affected by radiation under conditions used.

The results of mapping of DNAase I hypersensitive sites in SV40 minichromosomes after treatment of purified nuclei with 30 KGy of X-rays are presented in Fig. 2. The position of the main hypersensitive sites in minichromosomes within irradiated nuclei does not differ from those in unirradiated nuclei (~1700 bp counterclockwise from EcoRI site). However, the relative intensity of the bands is substantially decreased in irradiated nuclei. The quantitation of the difference by densitometry of underexposed autoradiograph is presented in Fig. 2B. The extent of hypersensitivity of SV40 DNA in irradiated nuclei is decreased 3-4-fold in comparison with the control level. (High intensity of hypersensitive bands in comparison to the full length SV40 DNA band is due to better transfer to nitrocellulose of shorter DNA fragments. This, however, is not a drawback for studying of the effect of irradiation on hypersensitivity.) Thus, the X-rays substantially alter the DNAase I hypersensitivity of minichromosomes within isolated nuclei. This may be explained by the decrease in the percentage of minichromosomes specifically hypersensitive to DNAase I.

To find out more about the mechanism of the observed effect, we studied the X-ray dose-response of DNAase I hypersensitivity. The extent of DNAase I hypersensitivity decreased with X-ray dose (Fig. 3, closed circles). The percentage of SV40 DNA that remained superhelical also decreased depending on the dose though somewhat more sharply than the hypersensitivity (Fig. 3, open circles). Nevertheless, the extent of DNAase I hypersensitivity roughly correlates with the per-


a
1 2 3 4 5 6 7 8 9 10 11 12
-5243
-1768
}HS
-1118
b
INTENSITY
5243
1768
SIZE OE DNA FRAGMENT (BP)

centage of DNA remaining superhelical. The hypersensitivity remaining after the highest dose applied (40 KGy) may at least partially be explained by the remaining fraction of superhelical DNA. This argues that the intact (covalently closed) structure of DNA within minichromosomes may be relevant for its hypersensitivity to DNAase I. The possibility that the remaining hypersensitivity may be determined by some other reasons, i.e. nucleosome-free region or specific proteins is substantiated below.

Irradiation of isolated minichromosomes

Analogous experiments were performed with extracted minichromosomes purified through sucrose gradient, and then irradiated with X-rays. The results are shown in Fig. 4. The extent of DNAase I hypersensitivity in isolated minichromosomes is only slightly altered by X-irradiation. The observed decrease in DNAase I hypersensitivity usually did not exceed 30% from control level even at maximal X-ray doses leading to relaxation of more than 80% of DNA (Fig. 4b). The position of double-strand breaks induced by DNAase (hypersensitive region) was the same as in nuclei (within the limits of accuracy of our mapping procedure). The hypersensitive area, however, seems to be more broad than in intranuclear minichromosomes. Also, some non-specific cuts scattered all around minichromosomal DNA create higher background than in the case of nuclear digestion.

These results were confirmed in experiments with minichromosomes labeled *in vivo* by ^{32}P-orthophosphate. Such minichromosomes were isolated, irradiated and treated with DNAase I.

Fig. 2. DNAase I hypersensitivity of control (lanes 1-5) or irradiated with 30 KGy of X-rays (lanes 7-11) minichromosomes within nuclei.
a - Autoradiograph of nitrocellulose filter hybridized to ^{32}P-labeled EcoRI-MspI fragment of SV40 DNA. 1, 7 - untreated nuclei; 2, 8 - incubation with 6 mM $MgCl_2$ for 5 min at 18°C; 3, 9 - 6.0 µg/ml of DNAase I; 4, 10 - 13.0 µg/ml of DNAase I; 5, 11 - 26.0 µg/ml of DNAase I; 6 - HindIII fragments of SV40 DNA; 12 - naked SV40 DNA after limited digestion with DNAase I (0.15 µg/ml of DNAase I); HS - fragments excised by DNAase I (hypersensitivity).
b - Densitograms of lanes 5 (upper line) and 11 (lower line) from panel a.

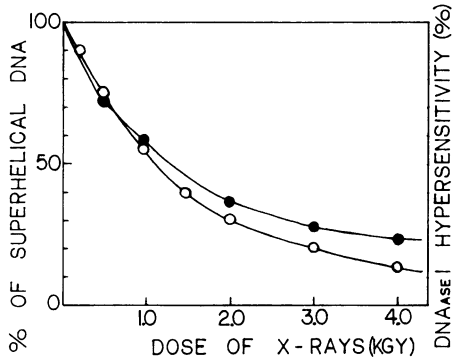


Fig. 3. X-ray dose-response of percentage of SV40 DNA remaining superhelical (open circles) or percentage of DNAaseI hypersensitivity relative to its level in unirradiated cells (closed circles). Percentage of superhelical DNA was determined after irradiation of isolated, in vivo ^{32}P-labeled SV40 minichromosomes, isolation of DNA, electrophoresis, autoradiography, and densitometry of X-ray film on Opton spectrophotometer. An example of such an autoradiograph is shown in Fig. 1b. Relative percentage of DNAase I hypersensitivity was determined by densitometry of autoradiographs after end-labeling procedure, an example shown in Fig. 2.

Then purified DNA was cut with EcoRI restriction endonuclease and electrophoretically separated in 1% agarose gel. The dried gel was autoradiographed without transferring the DNA onto nitrocellulose filter (Fig. 5). Although this experiment could not resolve whether hypersensitive sites were located to the left or to the right from EcoRI site, it gave quantitative estimation of DNAase I hypersensitivity provided the exact location of hypersensitive sites was established earlier (Figs.2 and 4). The accurate quantitation in blotting experiments was not possible because of unequal effectivity of transfer of different DNA fragments to nitrocellulose filter. The maximal level of DNAase I hypersensitivity in the experiment presented in Fig. 5 was 22% for control minichromosomes and 16% for those treated with 3.0 KGy of X-rays (the values obtained after scanning the lanes corresponding to maximal DNAase I doses). Hence, DNAase I hypersensitivity in minichromosomes isolated in 0.1 M NaCl mainly is not connected with elastic torsional strain in DNA.

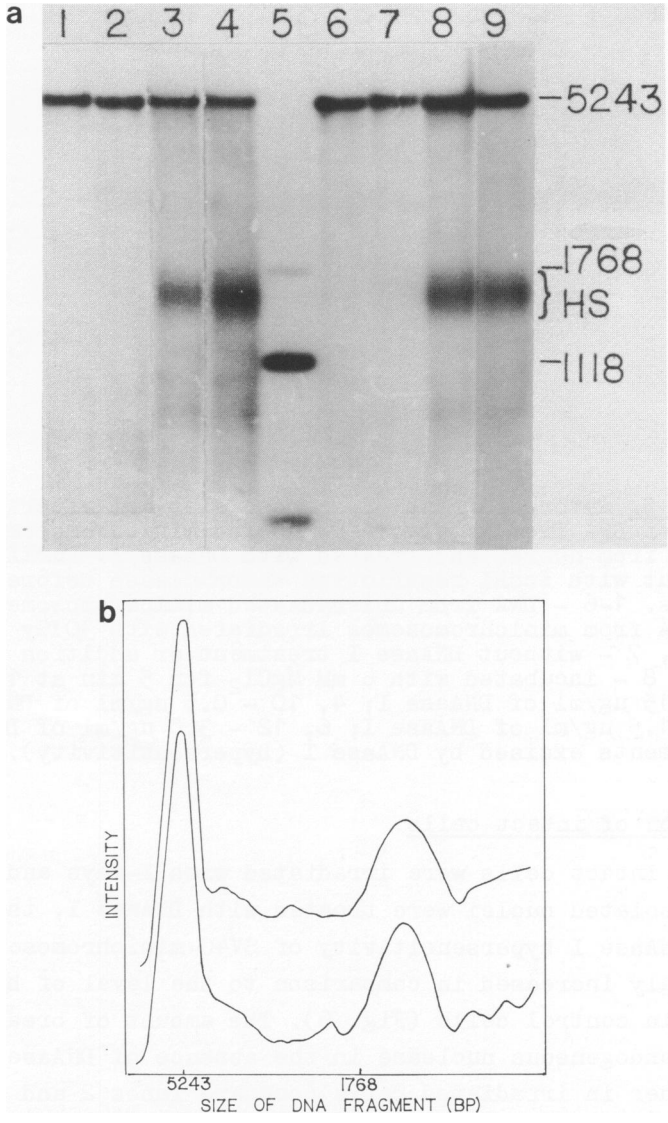


Fig. 4. DNAase I hypersensitivity of control (lanes 1-4) or irradiated with 4.0 KGy of X-rays (lanes 6-9) minichromosomes free in solution.

a - Autoradiograph of nitrocellulose filter hybridized to ^{32}P-labeled EcoRI-MspI fragment of SV40 DNA. 1, 6 - untreated minichromosomes; 2, 7 - incubation with 6 mM $MgCl_2$ for 5 min at 18°C; 3, 8 - 0.5 µg/ml of DNAase I; 4, 9 - 1.5 µg/ml of DNAase I; 5 - HindIII fragments of SV40 DNA. HS - fragments excised by DNAase I (hypersensitivity).

b - Densitograms of lanes 4 (upper line) and 9 (lower line) from panel a.

Fig. 5. Autoradiograph of agarose slab gel after electrophoresis of DNA from in vivo ^{32}P-labeled minichromosomes extracted from nuclei and treated with DNAase I. Purified DNA was cut with EcoRI restriction endonuclease before electrophoresis. 1-6 - DNA from unirradiated minichromosomes; 7-12 - DNA from minichromosomes irradiated with 30 KGy of X-rays. 1, 7 - without DNAase I treatment or addition of $MgCl_2$; 2, 8 - incubated with 6 mM $MgCl_2$ for 5 min at 18°C; 3, 9 - 0.15 µg/ml of DNAase I; 4, 10 - 0.5 µg/ml of DNAase I; 5, 11 - 1.5 µg/ml of DNAase I; 6, 12 - 3.0 µg/ml of DNAase I; HS - fragments excised by DNAase I (hypersensitivity).

Irradiation of intact cells

When intact cells were irradiated with X-rays and subsequently isolated nuclei were treated with DNAase I, the extent of DNAase I hypersensitivity of SV40 minichromosomes surprisingly increased in comparison to the level of hypersensitivity in control cells (Fig. 6). The amount of breaks induced by endogeneous nuclease in the absence of DNAase I was also higher in irradiated cells (compare lanes 2 and 6).(It is even more prominent on overexposed gels.) The effect of endogeneous nuclease activated by radiation was more pronounced after incubation of nuclei from irradiated cells for 5 min at 37°C in the digestion buffer (Fig. 6b). The localization of single-strand breaks induced by endogeneous nuclease by means of S1 treatment of DNA and subsequent routine mapping of double-strand breaks revealed many cuts scattered on SV40

minichromosome including bands at hypersensitive region (not shown). It is conceivable that single-strand breaks occurring in hypersensitive region interact with those induced by DNAase I preferentially at nucleosome-free region, thus elevating the overall level of DNAase I hypersensitivity.

This argues that irradiation of the whole cells is not an appropriate model for studying the effect of X-rays on DNAase I hypersensitivity.

DISCUSSION

Evidence for the two mechanisms of DNAase I hypersensitivity

In the present study, we have found that X-irradiation decreased DNAase I hypersensitivity of SV40 minichromosomes after irradiation of nuclei, but hardly affected the hypersensitivity of minichromosomes isolated from nuclei in 0.1 M NaCl. The difference in response of DNAase I hypersensitivity to X-irradiation can be explained by (1) the absence of extraction of minichromosomes which possess torsional strain in DNA under given conditions, and (2) the loss of elastic strain during extraction and purification procedures in 0.1 M NaCl, the conditions where topoisomerase I is active. Whatever the explanation is, it is clear that hypersensitivity to DNAase I observed in isolated minichromosomes is not related to torsional stress in DNA. Thus, we suppose that virtually all hypersensitivity observed in minichromosomes isolated in 0.1 M NaCl may be connected to nucleosome-free region or some other structural properties of nucleoprotein. The nucleosome-free region in the control area of SV40 minichromosomes was repeatedly demonstrated by means of electron microscopy of minichromosomal preparations containing histone H1 / 9, 10, 11/.

Then, what could be the basis of decreased DNAase I hypersensitivity after irradiation of isolated nuclei? We consider the most reasonable explanation to be the loss of torsional constraint in response to random DNA nicking by X-rays. This explanation suggests that a portion of hypersensitivity related to elastic torsional strain exhibits itself at earlier stages of digestion than that determined by nucleosome-free

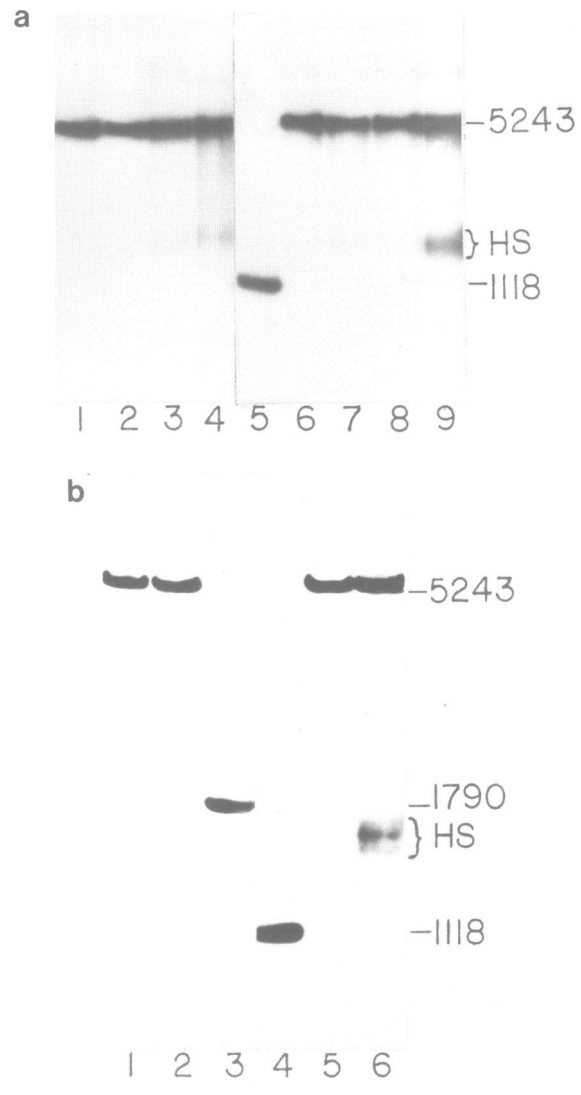


Fig. 6. Effect of X-irradiation of the whole cells on DNAase I hypersensitivity of SV40 minichromosomes within isolated nuclei (a) and detection of radiation activated endogeneous nuclease by incubation of nuclei at 37°C (b). Nitrocellulose filters were hybridized to EcoRI-MspI ^{32}P-labeled fragment of SV40 DNA.

a: 1-4 - unirradiated cells; 6-9 - cells irradiated with 30 KGy of X-rays; 1, 6 - without treatment with DNAase I or magnesium; 2,7 - incubation of nuclei in 6 mM $MgCl_2$ for 5 min at 18°C; 3, 8 - 6 µg/ml of DNAase I; 4, 9 - 13 µg/ml of

DNAase I. Lane 5 - 1118 bp(HindIII) fragment of SV40 DNA.
b: 1, 2 - unirradiated cells; 5, 6 - cells irradiated with 30 KGy of X-rays; 1, 5 - without incubation; 2, 6 - nuclei incubated at 37° for 5 min in 6 mM $MgCl_2$; 3, 4 - marker fragments of SV40 DNA.

region during DNAase I digestion, provided the nucleosome-free region exists in transcriptionally active chromatin in the nuclei /12/. This implies that DNA structural features created by torsional tension are more readily recognized by DNAase I than naked DNA itself. Our previous studies gave evidence that this could be indeed the case /6/.

The hypersensitivity remaining after the highest dose (40 KGy) of X-rays (20-30% from the control level) does not seem to be completely explained by the remaining fraction of superhelical DNA (13%) provided the irradiation breaks the DNA both in transcriptionally active and inactive regions with equal effectivity /13/. So, we suppose that nucleosome-free region or some prominent structural feature of DNA created by specific proteins located at the control region of minichromosome may give their contribution to DNAase I hypersensitivity exhibiting itself after the nuclei were exposed to higher X-ray dose given to nuclei.

The alternative explanations for the effect of X-rays on DNAase I hypersensitivity within nuclei, not connected to the elastic torsional strain, must also be considered. Among them could be the influence of DNA single- or double-strand breaks or DNA-protein cross-links induced by radiation. The average amount of nicks per SV40 DNA circle induced at a highest dose (40 KGy) is 2.35 in both DNA strands (in circles hit by radiation). The value is calculated from Poisson distribution of nicks if we know the fraction of molecules that are not nicked (13%). It is clear that such low amount of nicks randomly distributed in the molecule hardly can affect the DNAase I hypersensitivity found in the narrow area of the molecule comprising less than 10% of its length. The amount of random double-strand breakage induced by radiation is even lower. The level of DNA-protein cross-links at a given doses of X-rays does not exceed the number of single-strand breaks, so they also are unlikely

to influence the DNAase I hypersensitivity. The third explanation could be that some protein factors can shield the control region after irradiation of the nuclei. This hypothesis is not consistent with the data obtained after irradiation of the whole cells (Fig. 6). In this case, the exposure of the control region to DNAase I (or endogeneous nuclease) does not decrease after irradiation. So, we consider the relaxation of elastic torsional strain in DNA within a fraction of intranuclear minichromosomes as the best explanation for the observed effect of X-rays.

Earlier, we have found that DNAase I hypersensitivity in minichromosomes extracted in 0.4 M NaCl is abolished after topoisomerase I treatment /6/. Also, it was found that limited exposure to DNAase I preferentially induces nicks and double-strand breaks into elastically strained fraction of minichromosomes - the latter was no longer detectable after subsequent topoisomerase I treatment. That approach was difficult to apply to intact nuclei or minichromosomes isolated in 0.1 M NaCl because both of them contain plenty of topoisomerase I. This enzyme, however, may be temporarily inhibited in transcriptionally active chromatin or compartmentalized in intact nuclei. On the other hand, topoisomerase I was fully active during extraction and purification of minichromosomes in 0.1 M NaCl, probably eliminating all the torsional strain in DNA. For this reason in the present work, we used X-rays to relax DNA in nuclei or in free minichromosomes isolated in 0.1 M NaCl. The results show that X-irradiation strongly diminishes DNAase I hypersensitivity of intranuclear minichromosomes, but hardly affects the level of hypersensitivity in isolated minichromosomes. We consider this result in favour of the existence of elastic torsional strain in intranuclear minichromosomes hypersensitive to DNAase I. We do not exclude the existence of the elastic torsional strain in DNA within a small fraction of minichromosomes isolated in 0.1 M NaCl for the following reasons. In the minichromosomes extracted in 0.4 M NaCl, the elastically strained (and hypersensitive to DNAase I) minichromosomes comprised ca. 2% of their total amount. The level of DNAase I hypersensitivity in minichromo-

somes isolated in 0.1 M NaCl was ca. 20%. It is noteworthy in this connection that nucleosome-free region can hardly be found in minichromosomes isolated in 0.4 M NaCl. So, we do not exclude that minor portion of DNAase I hypersensitivity in minichromosomes extracted in 0.1 M NaCl may be connected to elastic torsional strain, but is masked by the excess of non-elastic molecules containing nucleosome-free region. However, the possibility of the existence of torsional tension in minichromosomes isolated in 0.1 M NaCl may be questioned simply because the endogeneous topoisomerase I is fully active under these conditions.

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