Deoxyribonuclease II as a probe to sequence-specific chromatin organization: preferential cleavage in the 72 bp modulator sequence of SV40 minichromosome

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ABSTRACT

To probe sequence-specific chromatin structure of SV40 minichromosome, we further modified previously described hybridization mapping. Actually (i) the digestion patterns by two nucleases (micrococcal and DNAase II) were compared and (ii) the kinetics of nuclease digestion was analyzed from early time points when only a fraction of minichromosomes was cleaved once to longer digestions when oligo- and mononucleosomal bands appeared. DNAase II is shown to possess certain sequence specificity different from that of micrococcal nuclease. The major finding is that DNAase II preferentially cleaves the SV40 minichromosome at a distinct region of the genome known as 72 bp modulator element. Other hypersensitive sites are located near the replication origin and T-ag binding site II and also near BamHI site where termination of replication and "late" transcription occurs. Micrococcal nuclease splits the BgII-HpaII region in a different manner.

INTRODUCTION

Specific chromatin organization of certain genomic regions can be analyzed by hybridization techniques. The indirect end labeling protocol is applicable for studying both the nucleosome positioning and the distribution of hypersensitive sites in chromatin (see /1,2/ for reviews).

Micrococcal nuclease and DNAase I used in previous studies revealed a strong sequence specificity when analyzed on pure DNA /3,4/. Looking for a better biochemical probe, we have introduced DNAase II which can act in different ionic conditions. Although this enzyme also turned out to be sequence specific, together with micrococcal nuclease analysis it gave less ambiguous results. We have also extended the indirect end labeling procedure to different stages of nuclease digestion to discriminate between the most preferential sites and those which appear later in digestion.

In this paper, we describe the mode of DNAase II action at very early stages of digestion when several hypersensitive sites appear. The same sites are attacked on pure SV40 DNA with much lower probability. Other results relevant to nucleosome positioning on SV40 DNA will be publushed elsewhere.

MATERIALS AND METHODS

Preparative procedures. CV1 monkey kidney cells were grown and infected with SV40 virus (strain Rh 911) as described earlier /5, 6/. Viral minichromosomes were extracted from nuclei 36-40 hours postinfection in a buffer containing 0.1 M NaCl, 10 mM TEA-HCl, pH 7.8, 0.1 mM EDTA, 0.5% NP40, 0.5 mM PMSF /7, 8/. The extraction was performed in a Blaessig homogenizer (2-3 strokes on ice). The overall isolation procedure usually did not exceed 1 hour. Minichromosomes were separated from other nucleoprotein complexes by velocity sedimentation /5/. The virion to minichromosome ratio at these stages of infection ranged from 1.5 to 2.0 /5, 8/, implying that virion degradation is not occurring, as can be found when lower ratios are obtained.

Minichromosomes from assembled virions were prepared by disrupture of intranuclear viral particles /5-8/ by a procedure modified from Brady <u>et al.</u> /9/.

SV40 DNA was prepared according to Hirt procedure /10/.

<u>Nuclease digestion of SV40 minichromosomes and DNA.</u> DNAase II (15000 units/mg, Worthington) digestion was performed at 4° C or 37° C after two-fold dilution of the sucrose gradient buffer (0.1 M NaCl, 5 mM TEA-HCl, pH 7.5, 0.1 mM Na-EDTA, ~ 10% sucrose) with 20 mM TEA-HCl, pH 6.9. The reaction was terminated by SDS and Na-EDTA addition to final concentrations of 0.1% and 20 mM, respectively.

Micrococcal nuclease (15000 u/mg, Worthington) digestion was performed at 4° C or 37° C directly in the sucrose gradient buffer (see above) after CaCl₂ addition to 1 mM. Purified SV40 DNA (~80% of form I) was digested and analyzed under identical conditions.

Mapping of nuclease cuts on SV40 genome. The procedure for hybridization mapping was described previously /11, 12/. In this study, the indirect end labeling was performed for a range of digestion kinetics. Briefly, each sample (corresponding to a different stage of nuclease digestion of minichromosomes) was deproteinized by a proteinase-phenol procedure. then purified DNA was redigested by one of the restriction enzymes which cleaved SV40 DNA once. Redigested DNA was fractionated by agarose electrophoresis, transferred onto nitrocellulose filters /13/ and hybridized to DNA probes adjacent to this restriction nuclease site. In particular, after EcoRI redigestion EcoRI-BamHI 752 bp fragment was used for clockwise direction and HaeIII fragments I, G or J for counterclockwise direction (see Fig. 4). Thus, after two subsequent hybridizations of the same gel, mapping in both directions could be done.

RESULTS

DNAase II splits SV40 minichromosomes in a highly specific manner

SV40 minichromosomes purified from the nuclear extract late in the infection were mildly digested with DNAase II in the conditions favouring its internucleosomal action (see Materials and Methods). As shown in Fig. 1A, only a part of form I DNA was converted into form III (see legend to Fig.1). The absence of significant background below form I DNA showed that a fraction of the minichromosomes cut with DNAase II more than once was negligible.

The double-stranded cuts were mapped after EcoRI redigestion of the purified DNA by hybridization with certain radioactive probes (Fig. 1 B-E). When total SV40 DNA (SV40 probe) was used, two prominent hypersensitive bands appeared which corresponded to 1.57 kb and ~3.6 kb long fragments. (Fig. 1 B, indicated by the arrows). In addition, 3-4 less intensive, but also preferential sites could be observed.

To map these sites, we employed an indirect end labeling



procedure /11, 12/ with the <u>I</u>-probe for the counterclockwise direction and the Bam-RI-probe for the clockwise direction (see legend to Fig. 1). In both cases one major band was revealed (Fig. 1 C, D) corresponding to cleavage 1.57 kb counterclockwise to the EcoRI site. The use of longer gel (Fig. 2 B) allowed one to map it more precisely as $1.560^{\pm}10$ bp from the EcoRI just inside a single copy of 72 bp repeat of SV40 DNA (strain Rh 911). One additional site could be seen in Fig. 1 C (small arrow) which was mapped 0.74 kb clockwise to the EcoRI site. Also some additional sites could be observed in Fig. 1 (D, E), one of them being located near the major hypersensitive site (small arrow).

When more extensive digestion with DNAase II was performed, the relative intensity of the 1.56 kb band could often be lower (for example, lane 2 in Fig. 2 B corresponds to the stage when a significant fraction of the minichromosomes is cleaved more than once). The same is true for later stages when oligonucleosomal bands appear (not shown). However, it is not clear from our results, whether all minichromosomes are digested with identical patterns. Preferential cleavage of a specific subfraction of the minichromosome population

Fig. 1. Specific DNAase II cleavage of isolated SV40 chromosomes.

SV40 minichromosomes were purified and mildly digested with DNAase II as indicated in the Materials and Methods. Isolated SV40 DNA was redigested with EcoRI (B,C,D,E), fractionated in a 1.6% agarose 15 cm gel, blotted and hybridized with ^{J2}P-labeled DNA probes. A. Mild digestion of minichromosomes with DNAase II. The arrow indicates the direction from "zero" point (no enzyme added) to later stages of digestion. Hybridization with total SV40 DNA (SV40-probe). The positions of superhelical (I) and linear (III) forms of SV40 DNA are indicated (form II DNA is not shown). (Note that form I DNA is underrepresented 3-4 fold due to losses during the blotting procedure.) B. The same DNA samples shown in A, but redigested with EcoRI and hybridized with the SV40 probe. The positions of HaeIII fragments and their lengths are shown on the left slot. C. The same as in B, but indirectly labeled by BamHI-EcoRI 751 bp fragment. D. The same as C, but labeled by <u>I</u>-probe (<u>I</u> fragment of SV40 HaeIII digest, see Fig. 4). E. The same as D, but after longer exposure. See Fig. 4 for the summary of the results.



Fig. 2. Mapping of DNAase II cleavages in the vicinity of the Bgll-Hpall genome region for SV40 minichromosomes and bare DNA.

And bare DNA. A. Mild digestion of naked SV40 DNA by DNAase II at $4^{\circ}C$ (from the left to the right). Cleavages revealed after EcoRI redigestion and indirect end labeling with <u>I</u> probe (electrophoresis in a 2%, 25 cm long gel). The arrow indicates cleavage inside 72 bp element.

B. Mapping of the major cleavage site on the SV40 genome (electrophoresis in a 1.6%, 30 cm gel). (1) Naked SV40 DNA mildly digested by DNAsse II (form III to form I ratio 2.0) and redigested with EcoRI. Labeling with <u>G</u> probe. (2) The same, but for SV40 minichromosomes extracted late in the infection. (3) The same as (2), but after longer exposure. early in the digestion with DNAase II is not excluded.

Some previous studies on nuclease hypersensitivity were performed on minichromosome preparations partially derived from disrupted viral particles. In experimental protocol used here free minichromosomes and assembled virions were separated by velocity sedimentation /5, 7, 8/. We have probed intravirion minichromosomes prepared by a procedure modified from Brady <u>et al.</u> /9/ with DNAase II. In this case, much less prominent sensitivity in 72 bp repeat can be observed (unpublished observation).

DNAase II cutting of naked SV40 DNA is also non-random

Specific cleavage of SV40 DNA in viral minichromosomes could be due to DNAase II sequence specificity. In this case, essentially the same sites would be attacked on naked DNA whereas nucleosomal organization of SV40 minichromosome would only modulate the cleavage probability found on pure DNA. Thus, identical digestion and analysis procedures were applied to SV40 DNA preparations.

We analyzed the location of DNAase II cleavages on naked DNA in the vicinity of the genome region where the major hypersensitive site on the minichromosomes was found. DNA samples at similar stages of the digestion (judged by DNA III to DNA I ratio) were redigested with EcoRI and indirectly end labeled with <u>I</u> or <u>G</u> probe. Hybridization pattern (Fig. 2A) did not reveal strong hypersensitivity. All bands labeled by <u>I</u>-probe are of comparable intensity in contrast to the results obtained on the minichromosomes (Fig. 2 B). Thus, the same region of SV4O genome in the minichromosomes was differently attacked by DNAase II depending apparently on the peculiarities in chromatin structure.

In general, the sites attacked by DNAase II in the minichromosome can be also detected among those attacked on free DNA. However, either some "potential" DNA sites were not observed in the minichromosome digestions or the corresponding bands were much less intensive.

Thus, DNAase II is a sequence specific nuclease with cleavage sites differing from those for micrococcal nuclease (see below).

Micrococcal nuclease cleaves the same region in SV40 minichromosomes in a different manner

We have compared patterns of DNAase II cleavage of the SV40 minichromosome and naked SV40 DNA with those obtained after micrococcal nuclease digestion. It was shown previously that micrococcal nuclease cleaved SV40 DNA in both cases in a specific manner. Mapping of the most preferential sites (corresponding to a primary double-stranded cut) showed their distribution through the whole SV40 genome (/10/, see also /14/). The preferential cleavage in the 300-400 bp long segment adjacent to the Bgl1 site was not so evident as in the case of DNAase II. On the other hand, structural alterations in this particular region of SV40 chromatin have been established by a number of biochemical and physical methods /15-20/. Therefore, it was interesting to detect some preferential sensitivity of this "open" region against a background of prominent sequence specificity of micrococcal nuclease.

It turned out that altered structure was reflected in the cleavage pattern after extensive digestion when each minichromosome had been split several times (Fig. 3). Hybridization mapping from the EcoRI site revealed 5-6 multiple cleavages through the whole BglI-HpaII segment, most of them being also found on naked DNA (data not shown). Three sites were mapped inside or immediately adjacent to 72 bp repeat, but in contrast to DNAase II digestion there was no single prominent cleavage. In fact, a site ~ 1 kb counterclockwise to the EcoRI site was attacked more frequently than those located between the BglI and the HpaII sites (see Fig. 3).

DISCUSSION

Nucleases as probes to specific DNA-protein interactions in chromatin

The specificity of chromatin structure at defined regions of the eukaryotic genome has been under intensive investigation during the last several years (see /1,2,21/ for reviews). Two main problems are of particular importance: (1) the peculiar chromatin structure involving the elements which control gene activity (promoters, terminators, modulators,

517 5.2 1.6 Bgl I Hpa Il 1.4

Fig. 3. Mapping of micrococcal nuclease cleavages in the vicinity of the BglI-HpaII region. A. Kinetics of micrococcal nuclease digestion (at 37°C) of the SV40 minichromosomes (from the right to the left) after EcoRI redigestion of the purified DNA. End labeling by I-probe.

<u>B</u>. Low resolution gel indicating DNA fragments obtained after micrococcal nuclease digestion of pure SV40 DNA and redigestion with EcoRI. End labeling with <u>F</u>-probe.

etc.), and (ii) the specificity of nucleosome location with respect to DNA sequence with its possible (if any) relation to replication and transcription. In both cases, nucleases are usually exploited as primary biochemical probes to chromatin structure. After introducing secondary cleavages into purified DNA by restriction nucleases, these primary cuts could be unambiguously mapped on genomic DNA by the simple hybridization procedure termed as indirect end labeling /11, 12/. In ideal situation the primary nuclease cleavages would be solely determined by the specificity of the chromatin structure. However, micrococcal nuclease widely used in the study of chromatin has been shown to be highly sequence specific /3,4,11,29/. As shown here, DNAase II which in certain conditions cuts DNA between nucleosomes and gives rise to a 200 bp ladder characteristic of micrococcal nuclease /37-39/ is also sequence specific. These two nucleases cut naked SV40 DNA mostly at different sites of the genome (not shown). One could suggest that the chromatin structure only modulated the probabilities of cutting in those potential sites which are cleaved on naked DNA.

This specificity of nucleases complicates the analysis of the nucleosome positioning along certain DNA sequences (see /1-4, 22-29/). On the other hand, the use of several nucleases differing in their sequence specificity can give less ambiguous results and is being used for nucleosome mapping on the SV40 DNA /36/.

Another type of specific chromatin organization is reflected in hypersensitivity to nucleases of certain genomic regions, in particular, of the upstream sequences for some genes /12,15,21,30,31/. For example, the pattern of hypersensitivity to DNAase I sites has been shown to be specifically changed during differentiation and correlates remarkably well with transcriptional switch and changes in DNA methylation /32,33,34/.

The existence of a DNAase I hypersensitive region in SV40 minichromosomes had been reported /15,18/. The same region mapped between the BglI and the HpaII sites on the SV40 genome has also been shown to be preferentially cleaved by other nucleases /16,17/. In this paper, we analyze the cleavage pattern of this genome region in detail, using indirect end labeling of EcoRI redigested DNA.

On the chromatin structure around BglI-HpaII region as probed by nucleases

Previous studies have shown that the region of the SV40 genome comprising approximately 400 bp clockwise to the replication origin has an altered chromatin structure in the viral minichromosome /15-20/. Among other possibilities, absence of nucleosomes in this region has been suggested /18,19/. Recently we have shown, that labeled <u>H</u> HaeIII fragment (mapped for Rh 911 strain inside the BglI-HpaII segment, see legend to Fig. 4) hybridizes to oligo- and mononucleosomal bands of nuclease digested SV40 chromatin /50/. These data, however, do not exclude that a certain (probably, functionally distinct) subfraction of the SV40 minichromosomes isolated late in the infection lack nucleosomes in this particular region. In this respect, our most recent data are hardly consistent with the existence of the <u>maked</u> DNA stretch even in a subfraction of SV40 minichromosomes. In fact, there are at least several potential DNAase II specific "sites" inside the BglI-HpaII segment (Fig. 2). However, only one of them is hypersensitive in the SV40 minichromosome.

From the data presented in Figures 1 and 2, the most prominent cleavage site for DNAase II can be mapped 1560-1570 bp counterclockwise to the EcoRI site (which is located for Rh 911 strain used here 1711 nucleotides from the BglI site). Thus, the cleavage occurs ~140-150 nucleotides clockwise to the BglI site just inside a single copy of the 72 bp repeat. Another preferentially attacked site can be mapped inside T-antigen binding site II /49/ (see Fig. 4) just near the origin of DNA replication. In this respect, it should be noted that T-antigen binding could influence the chromatin structure of the whole BglI-HpaII segment. Our preliminary results with strain 776 minichromosomes imply that both copies of 72 bp repeat are equally cut by DNAase II. The cleavage near T-ag binding site II (located in this case 1.8 kb from the EcoRI site) is also observed (data not shown).

The 72 bp sequence has been shown to be an obligatory <u>cis</u>-acting element for SV40 "early" gene expression <u>in vivo</u> /40,41/ and probably similar to modulator elements found in other genes /42-47/. Similar element from polyoma virus has been reported to control replication of the viral DNA /43/. Interestingly, this 72 bp element can be replaced by a non--homologous sequence of 73 bp repeat from the long terminal repeat (LTR) of murine leukemia virus (P.Gruss, personal commun.and /54/). On the other hand, nuclease hypersensitivity in-



Fig. 4. Summary of the results: DNAase II hypersensitive sites on the SV40 genome map.

A. SV40 genome (strain Rh 911). Sites for several restriction nucleases are indicated. A-J correspond to HaeIII fragments, some of them used for indirect end labeling. Note that this DNA contains a deletion (nucleotides 180 to 252) which includes one complete copy of the two direct 72 bp repeats /52,53/. As a result, the pattern of HaeIII digest in the gel differs from that of strain 776 DNA /52, 53/ by the appearance of 253 bp long H fragment (mapped just inside the short BglI-HpaII segment). Moreover, each of the ten A-J Hae III fragments could be separated in 6% polyacrylamide non--denaturing gel (for 776 DNA several HaeIII fragments comigrate, see /17/). The positions of the fragments on the map were verified by hybridization of the purified D, E, F, G, H, and I probes with fractionated Hinf I and Hind III digests of SV40 DNA (strains Rh 911 and 776, not shown).

Arrows indicate the major DNAase II hypersensitive site in 72 bp element, two additional sites (firstly, near the replication origin where 5'-ends of early mRNA and T-ag binding site II have been mapped, secondly, near BamHI site where termination of the replication and the late transcription occur) and some minor sites (see Fig. 1).

B. A close-up of the BglI-HpaII region. One 72 bp repeat (solid bar) is shown as deleted. T-ag binding sites, DNAase II (D) and micrococcal nuclease cleavage sites (M) are shown below. Open bar corresponds to 16 bp segment homologous to the LTR of the avian retroviruses /49/.

side the LTR of transcriptionally active retroviral genome has been demonstrated /33/. In this particular case, the LTR contains a short 16 bp sequence common with a part of 72 bp element of SV40 /33, 48/. As shown in Fig. 4, the major DNAase II cleavage site is located just near this 16 bp sequence indicated above 72 bp element.

One could imagine that 72 bp repeat and immediately adjacent DNA sequence are involved in chromatin attachment to the nuclear matrix to provide **proper structural state** of the template (P.Gruss, W.Schaffner and R.Kamen, personal communications). Another interesting possibility has been suggested by Moreau <u>et al.</u> /46/ and postulates the role of 72 bp element as bidirectional entry site for RNA polymerase or some other component of the transcription machinery. We believe that both of these mechanisms would be reflected at chromatin level as some alterations in a regular nucleosome structure. In addition, our most recent results indicate against a direct contribution of 72 bp sequence to DNA superhelicity (in preparation).

We have found that DNAase II action at early stages of the digestion is different to that of micrococcal nuclease or DNAase I. In fact, although multiple micrococcal nuclease cleavages between the BglI and the HpaII sites could imply enhanced level of sensitivity of the whole 300 bp segment, there is no hypersensitivity inside and around 72 bp element (Fig. 3, 4). As shown recently, DNAase I preferentially cleaves the BglI-HpaII segment on the SV40 minichromosome at several distinct points, one of them being mapped inside or immediately adjacent to 72 bp element (/51/. and C.Cremisi cited in /33/). In this case, however, there are several other sites which are attacked with the same probability. As shown here, DNAase II in contrast to micrococcal nuclease and DNAase I, recognizes 72 bp modulator as a major hypersensitive site on the SV40 genome. This fact could be due to a very local alterations in chromatin structure which involves only DNAase II potential site. The role of ionic conditions specific for DNAase II digestion is also not excluded. In any case, this remarkable feature of DNAase II could be helpful for studying the peculiarities of chromatin structure in specific regions. We are now applying the same procedure to probe integrated SV40 DNA in transformed cells and other modulator sequences of eukaryotic genome.

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