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Transcriptionally active SV40 minichromosomes are restriction enzyme sensitive and contain a nucleosome-free origin region

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ABSTRACT

A nucleosome-free region or gap containing the origin of replication and the transcriptional promoter elements is observed on 20 to 25% of the SV40 minichromosomes isolated at physiological ionic strength late in infection. We used the preferential sensitivity of the gapped minichromosomes to restriction enzymes to obtain sucrose gradient fractions containing 50 to 80% of gapped molecules. The same fractions are also enriched in RNA polymerase B (II) molecules engaged in transcription. Using electron microscopy, we demonstrate here that the transcriptional complexes are preferentially sensitive to restriction enzyme digestion, which indicate that they represent a subpopulation of the gapped minichromosomes.

INTRODUCTION

Simian virus 40 (SV40) offers a model system to study the relationship between chromatin structure and gene expression. Late during the infection cycle, the SV40 genome can be extracted and purified from nuclei of infected cells in the form of a minichromosome (1), free of cellular chromatin (2-6). Electron microscopy has shown that 20 to 25% of the extracted minichromosomes contain a nucleosome-free region (gap) extending over approximately 400 bp (7, 8). The gap region is preferentially cut in the nuclei of infected cells by digestion with DNAse I and specific patterns of DNAse I hypersensitive sites within this region have been observed (9, 10). The region organised in an altered chromatin structure (the ORI region on the physical map) on gapped minichromosomes contains the origin of replication (11) and the DNA sequences involved in the regulation of both early and late transcription (1). As the gapped molecules and those which exhibit increased DNAse I sensitivity within the ORI region are well correlated (9, 12), it has been suggested that the generation of an altered chromatin structure could lead to the formation of an "open window" within the nucleosomal structure, thereby increasing the accessibility of DNA for binding of macromolecules involved in transcription (9, 13-15).

We have previously reported (16) that gapped minichromosomes represent a subpopulation of minichromosomes, which are preferentially accessible to restriction enzymes and particularly sensitive to simultaneous digestion with BglI and MspI. This observation offered the possibility to prepare sucrose gradient fractions enriched in gapped minichromosomes and thereby to further analyze the biological significance of the altered chromatin structure of the gapped molecules. We report here biochemical and electron microscopy studies which directly show that at least 60% of the viral transcriptional complexes extracted from nuclei late in infection at physiological ionic strength are minichromosomes with a nucleosome-free region.

MATERIALS AND METHODS

Digestion of SV40 minichromosomes and sucrose gradient centrifugation.

SV40 minichromosomes labeled *in vivo* with [^{14}C] thymidine (2) were extracted from infected cells 40 hours post-infection and purified at physiological ionic strength (3). The 75S peak fractions were pooled, concentrated and incubated with single cleavage site restriction enzymes (see Results) as described (16). The digestion was stopped by addition of EDTA (5 mM final concentration) and the samples were dialysed for 2 hours at 4°C against 10 mM Triethanolamine (TEA) pH 7.5, 50 mM NaCl, 2 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetate (EDTA) and 0.5 mM phenylmethylsulfonylfluoride (PMSF). At the end of dialysis, the material was loaded on a 5-30% (w/v) sucrose gradient in 10 mM TEA pH 7.5, 0.13 M NaCl, 2 mM DTT, 0.1 mM EDTA and 0.5 mM PMSF. After 3 hrs centrifugation in a SW41 rotor (Beckman) at 40.000 rpm and 4°C, the fractions (0.5 ml) were collected and 10 µl aliquots, after addition of SDS (0.5% final concentration), were analyzed by electrophoresis on 1% agarose gel.

Assay for RNA synthesis in vitro.

Assays for RNA synthesis *in vitro* were carried out with fraction aliquots from the sucrose gradient (see above). The samples (120 µl) were adjusted to 50 mM TEA pH 8.00, 2.2 mM $MnCl_2$, 0.1% Sarkosyl, 300 mM ammonium sulfate, 1 mM DTT, 0.5 mM each of ATP, GTP, CTP and 0.05 mM [^{3}H] UTP (11.8 Ci/mmole, Amersham) and incubated for 1 hour at 32°C. The reactions were stopped by addition of EDTA (5 mM, final concentration) and the samples stored at 4°C. The assay mixtures were adsorbed onto filters of DE-81 ion exchange paper (Whatman) and washed at least 10 times with 0.45 M Na_2HPO_4, twice with water and once with ethanol. After air drying, the filters were

counted as described (17). The relative transcriptional activity of the SV40 minichromosomes was calculated as the ratio between the [^{3}H]-labeled RNA counts and the [^{14}C]-labeled DNA radioactivity present in each fraction aliquot.

RNA-DNA hybridization.

[^{32}P]-labeled RNA was synthesized in vitro as described above with the following modifications. The reaction was carried out in presence of 0.5 mM each of ATP, GTP, UTP and 10 μM [α-^{32}P]-CTP (410 Ci/mmole, Amersham). After a 30 min incubation at 32°C, the SV40 minichromosome DNA was digested to completion with RNase-free DNAse I (Worthington) in presence of 5 mM $MgCl_2$ and yeast t-RNA (0.16 mg/ml). DNAse I was pretreated with 10 mM $CaCl_2$ and 1 mg/ml proteinase K (18). The reaction was stopped with EDTA (10 mM, final concentration) after a 30 min incubation at room temperature with EDTA and phenol-chloroform extracted. RNA was loaded on a 2 ml Sephadex G-15 (Pharmacia) column in 10 mM TEA pH 8.00, 1 mM EDTA to remove free nucleotides. The void volume was precipitated with ethanol in presence of 0.3 M Na Acetate.

SV40 DNA probes were cloned in M13 vectors. After Hirt extraction (19) and cesium chloride gradient purification, viral SV40 DNA was digested with the appropriate restriction enzymes (see Figure 3). The HindIII-HindIII (coordinates 1046 to 1493) and PstI-BamHI (coordinates 1988 to 2533) fragments were cloned in M13mp10 and M13mp11 vectors (20). The KpnI-EcoRV (coordinates 294 to 768) fragment was cloned in M13tg130 and M13tg131 vectors (21). Single stranded DNA was prepared as described (20).

For RNA-DNA hybridization, single-stranded DNA in 2 M NaCl, 0.1 M NaOH was dotted (3 μg/dot) directly on nitrocellulose filters (Schleicher and Schull, BA85). After air drying, the filters were baked 2 hrs at 80°C and prehybridized as described (22), in presence of 20 μM CTP. The [^{32}P]-labeled RNA were denatured for 10 min at 65°C in 50% formamide and hybridization was performed as described (22).

Electron microscopy.

RNA synthesis was carried out in vitro as described (see above) in presence of 0.5 mM each of ATP, GTP, CTP and UTP. After a 30 to 45 min incubation at 32°C, the reaction mixtures were adjusted to 5 mM EDTA, dialysed 1 hour at 4°C against 50 mM TEA pH 8.00 and precipitated with ethanol. The pelleted RNA-DNA complexes were resuspended in 10 mM TEA pH 8.00, 0.1 mM EDTA and processed for electron microscopy as described (23).

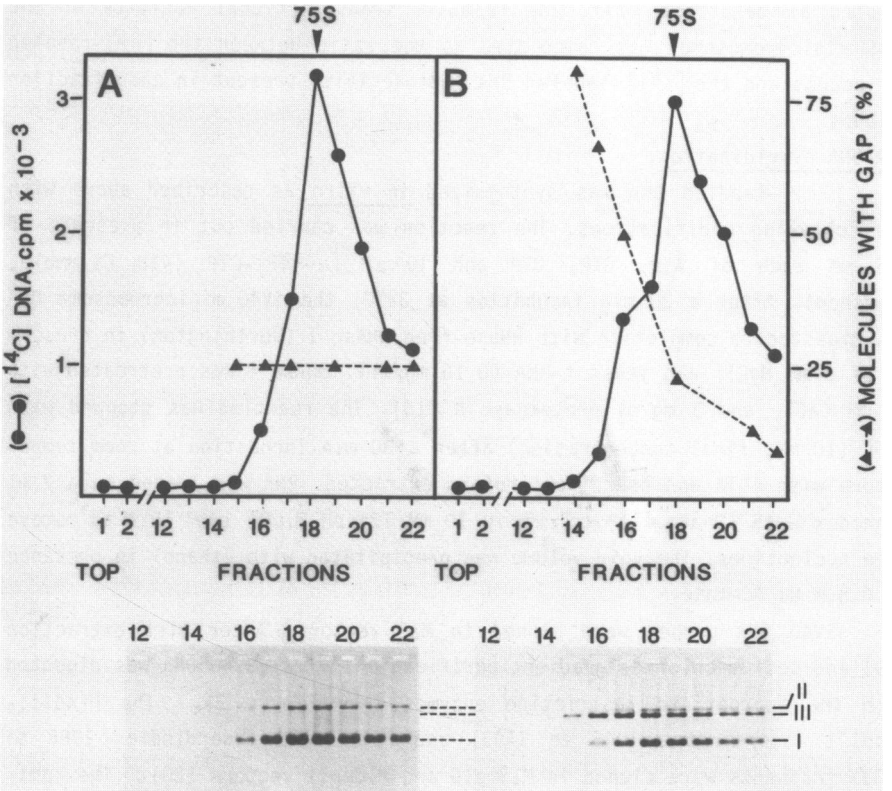

Fig. 1 : Analysis of purified SV40 minichromosomes after restriction enzyme digestion and sucrose gradient sedimentation.

[^{14}C] labeled SV40 minichromosomes were extracted from infected nuclei and purified on sucrose gradient (see Materials and Methods). The pooled 75S peak was concentrated (Materials and Methods) and incubated for 30 min in presence of 5 mM $MgCl_2$ at 37°C with (B) or without (A) KpnI restriction enzyme. The reactions were stopped by adding 5 mM EDTA (final concentration). After a 2 hour dialysis, the minichromosomes were fractionated on sucrose gradient (Materials and Methods). An aliquot (10 µl) of each fraction was analysed by electrophoresis on 1% agarose gel and the DNA visualized after ethidium bromide staining (lower part of the figure). The percentage of gapped molecules was determined by electron microscopy. Only molecules containing 20-24 nucleosomes were scored. Similar results were obtained in five independent experiments. ●, [^{14}C]-thymidine in DNA; Δ, percentage of molecules with gap.

RESULTS

1. Restriction enzyme digestion shifts the gapped minichromosomes to the slow sedimenting sucrose gradient fractions of the minichromosome peak.

In an attempt to isolate the subpopulation of minichromosomes containing a gap, the whole population of purified minichromosomes was fractio-

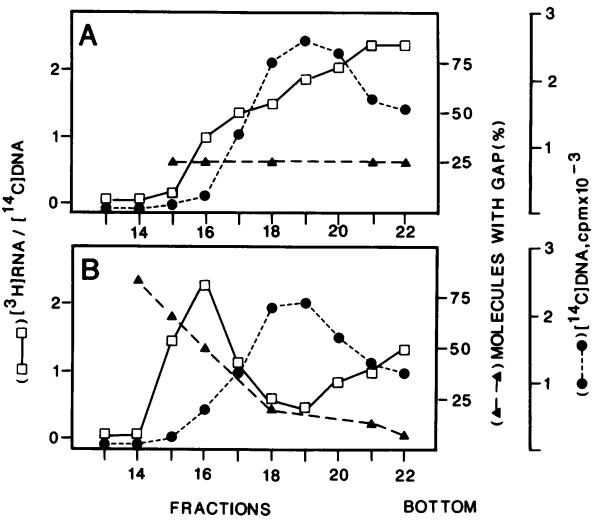

Fig. 2 : Sedimentation of SV40 transcriptional complexes.
Viral chromatin labeled in vivo with [^{14}C]-thymidine was obtained as described in Materials and Methods. After concentration, the minichromosomes were resuspended in an appropriate buffer (Materials and Methods) and incubated for 30 min : (A) at 4°C, (B) at 37°C in presence of 5 mM $MgCl_2$ and KpnI restriction enzyme. At the end of incubation, EDTA was added to 5 mM and the samples were sedimented in sucrose gradients after a 2 hour dialysis (Materials and Methods). In vitro RNA synthesis was performed using fraction aliquots as described in Materials and Methods. The relative in vitro SV40 transcriptional activity was calculated by dividing the [^{3}H] RNA cpm retained on DE-81 filters by the [^{14}C] DNA cpm present in each assay. Only fractions where [^{14}C] DNA could be detected are represented. □, in vitro transcriptional specific activity; ●, [^{14}C]-thymidine in DNA; Δ, percentage of molecules with gap, as determined by electron microscopy.

nated on sucrose gradient after KpnI restriction enzyme digestion. Since gapped minichromosomes are preferentially digested by restriction enzymes (16), we expected that the corresponding linearized molecules will sediment more slowly than the rest of the material on a 5-30% (w/v) sucrose gradient (24). When analysed by DNA gel electrophoresis, the slow sedimenting fractions contained predominantly form III DNA (see Figure 1B, fractions 14 to 16). The majority of linearized molecules present in these fractions were in fact minichromosomes containing a gap as observed by electron microscopy (Figure 1B), whereas gapped minichromosomes were evenly distributed throughout the 75S minichromosome peak in the absence of restriction enzyme digestion (Figure 1A).

A similar enrichment of linear minichromosomes containing a gap in

the slow sedimenting fractions on the sucrose gradient was obtained (not shown) by digestion with BglI, MspI, EcoRV, EcoRI and BamHI (see Figure 3). The average number of nucleosomes per minichromosome (20-24) was unchanged after the restriction enzyme digestion and additional gapped minichromosomes were not generated during this treatment, since the overall percentage of minichromosomes with gap (25%) did not vary, when taking into consideration all of the fractions of the sucrose gradient (result not shown). Finally the localization and the length of the gap were identical to those previously determined (7-9, 16): after BglI or MspI restriction enzyme digestion, a nucleosome-free region of approximatively 400 bp was always localized at one end of the minichromosome (not shown).

2. SV40 transcriptional complexes are also shifted after restriction enzyme digestion.

Late in infection, the SV40 transcriptional complexes which can be extracted from infected nuclei cosediment with a subset of the minichromosomes on a sucrose gradient (17, 25, 26). They are present on the heavy side of the minichromosome peak as demonstrated by in vitro RNA chain elongation in presence of ammonium sulfate and sarkosyl (which allow RNA chain elongation but no reinitiation) (17, 26, 27; Figure 2A). We determined the RNA polymerase activity present in the different fractions of the sucrose gradient of digested minichromosomes. After KpnI restriction enzyme digestion, the majority of in vitro RNA synthesizing activity was found in the slow sedimentating fractions of the sucrose gradient (Figure 2B). The same result was obtained when the minichromosomes were digested with either BglI or MspI before fractionation.

It is clear when comparing Figure 2A and B that the linearization of a portion of the minichromosomes before fractionation causes a displacement of the RNA polymerase peak on sucrose gradient. This preferential shift of the transcriptional complexes is not observed after incubation without restriction enzyme at 37°C in presence of $MgCl_2$ (not shown) and does not seem to be restricted to the action of enzyme digesting in the ORI region, since an identical displacement of the RNA polymerase activity was observed after BamHI digestion (not shown).

3. Characterization of RNA synthesized by the SV40 transcriptional complexes.

The lack of total conversion from the fast sedimenting transcriptional complexes to the slow sedimenting transcriptional complexes after restriction enzyme digestion (see Figure 2B) may suggest that we were

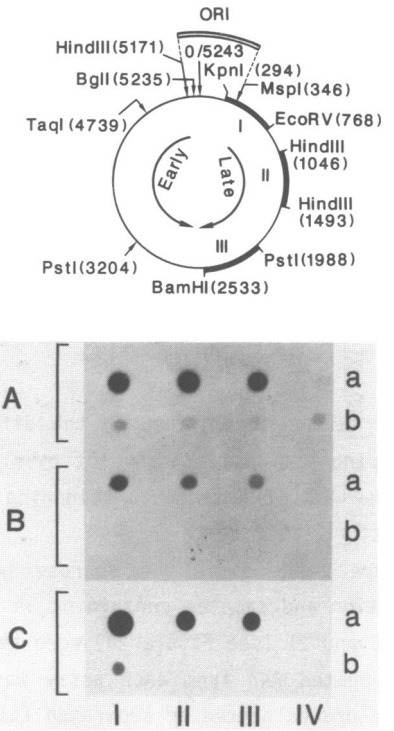

Fig. 3 : Characterization of restriction enzyme-digested transcriptional complexes by dot-blot hybridization.

Purified SV40 minichromosomes were digested with KpnI restriction enzyme and fractionated on sucrose gradient as described in legend to Figure 2. Aliquots (120 µl) containing identical amounts of SV40 DNA (0.3 µg) from fractions 16 and 21 (see Figures 1 and 2) were incubated at 32°C, in parallel with undigested bulk minichromosomes (8 µg SV40 DNA), for in vitro RNA synthesis during 30 min in presence of [α-^{32}P]CTP (Materials and Methods). At the end of the incubation period, the RNA was purified and hybridized to different single stranded probes dotted on nitrocellulose filters (Materials and Methods). The upper part of the figure indicates the localization of the DNA probes on the SV40 genome (solid bars) [coordinates according to the BBB system (1)]. The lower part of the figure shows the autoradiograms of the in vitro synthesized RNA hybridized to the different probes, using fraction 16 (A), fraction 21 (B) and undigested minichromosomes (C). The late and early coding strand of the probes are dotted in lanes a and b, respectively. Probe IV (a and b), used as control, correspond to the isolated strands of the EcoRI-BamHI fragment of wild-type pBR322. All probes are roughly 500 nucleotides in length. Filters A and B were exposed to X-ray film for 12 hours and filter C was exposed 2 hours.

dealing with at least two different subpopulations of transcribing minichromosomes. To demonstrate that the slow and the fast sedimenting transcriptional complexes have the same characteristics, we have mapped the

	I	II	III	IV
A	326	314	146	BL
	22	BL	BL	10
B	94	53	47	BL
	BL	BL	BL	BL
C	1035	204	139	BL
	36	BL	BL	12

Table 1 : Quantitation of the dot-blot hybridization.
The [^{32}P] labeled RNA hybridized to the probes I, II, III and IV in Figure 3 (A, B and C) was cut out from the filters and counted. [^{32}P] radioactivity (in cpm) was normalized to the same amount of SV40 DNA (0.3 μg) in each experiment (A, B and C), and is given after substraction of the background. Similar results were obtained in three independent experiments. BL, background level.

regions of the SV40 late genome transcribed in the different sucrose gradient fractions. The mapping was done by dot-blot hybridization, using the separated strands of three SV40 DNA segments belonging to the late region (see Figure 3, upper part).

Minichromosomes were fractionated on sucrose gradient after KpnI restriction enzyme digestion and samples containing an identical amount of SV40 DNA from fraction 16 and 21 (see Figure 2B) were assayed for RNA polymerase activity. The elongated RNA from each assay was hybridised to the different single stranded probes dotted on separated filters (Materials and Methods). As shown in Figure 3, the in vitro elongated RNA chains obtained with fraction 16 (Figure 3A) and fraction 21 (Figure 3B) were essentially complementary to the late coding strand (a), as well as the in vitro elongated RNA chains obtained with undigested bulk minichromosomes (Figure 3C). However, more RNA was synthesized in fraction 16 (Figure 3A) than in fraction 21 (Figure 3B) confirming the above results (Figure 2B).

According to these data, the displacement of the transcriptional complexes on a sucrose gradient after restriction enzyme digestion do not correspond to a selection of a subpopulation of transcriptional complexes. However, it appears that there is a selective loss of transcriptional activity during restriction enzyme digestion, since, for the same amount of SV40 DNA there is relatively more RNA hybridised to probe I (KpnI-EcoRV fragment) with undigested minichromosomes (Table 1C) than with digested minichromosomes (Table 1A, B).

4. The SV40 transcriptional complexes correspond to gapped minichromosomes.

To demonstrate that the in vitro synthesized RNA originates from minichromosomes containing a nucleosome-free region, minichromosomes were fractionated after a double BglI + MspI restriction enzyme digestion. We

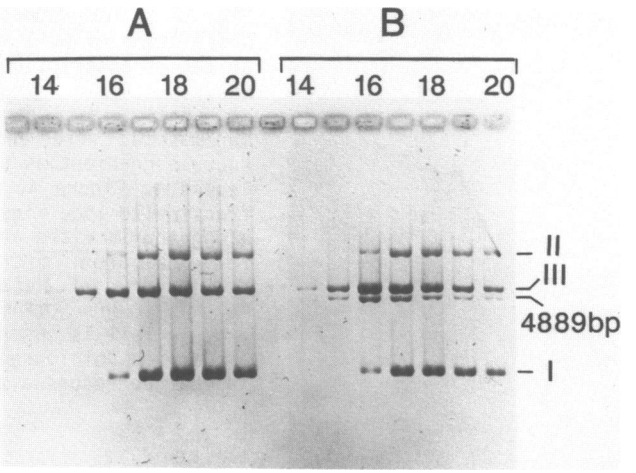

Fig. 4 : DNA analysis of sucrose gradient fractions containing BglI or BglI + MspI digested minichromosomes.

Purified SV40 minichromosomes were obtained as described in legend to Figure 1. After digestion either with BglI (A) or with both BglI and MspI (B) restriction enzymes, the nucleoprotein complexes were fractionated on sucrose gradient (Materials and Methods). Aliquots (10 µl) of the fractions from the "light" and middle part of DNA peak (14 to 20; see Figure 1) were analyzed by electrophoresis in presence of SDS on 1% agarose gel. DNA was visualized after ethidium bromide staining. I, II and III represent SV40 DNA in superhelical, relaxed circular and linear forms, respectively. The 4889 bp DNA corresponds to the large BglI-MspI fragment after excision of most of the ORI region.

have reported previously (16) that BglI + MspI restriction enzyme digestion of minichromosomes yields a 354 bp and a 4889 bp chromatin fragment which result from digestion of gapped molecules. Minichromosomes were digested with BglI and MspI restriction enzymes and fractionated on sucrose gradient. The DNA present in each fraction of the sucrose gradient was analysed by electrophoresis on agarose gel. The light fraction (14-16) contained essentially linearized minichromosomes together with minichromosomes from which the BglI-MspI segment of 354 bp was excised (Figure 4B), since the 4889 bp band was absent when minichromosomes were cleaved with BglI alone (Figure 4A). When aliquots of each fraction of the sucrose gradient containing the BglI + MspI restricted minichromosomes were tested for their ability to synthesize RNA in vitro, the majority of RNA polymerase activity was found in the slow sedimenting fractions (15 and 16; not shown), as observed above after digestion with BglI, KpnI or MspI restriction enzymes alone.

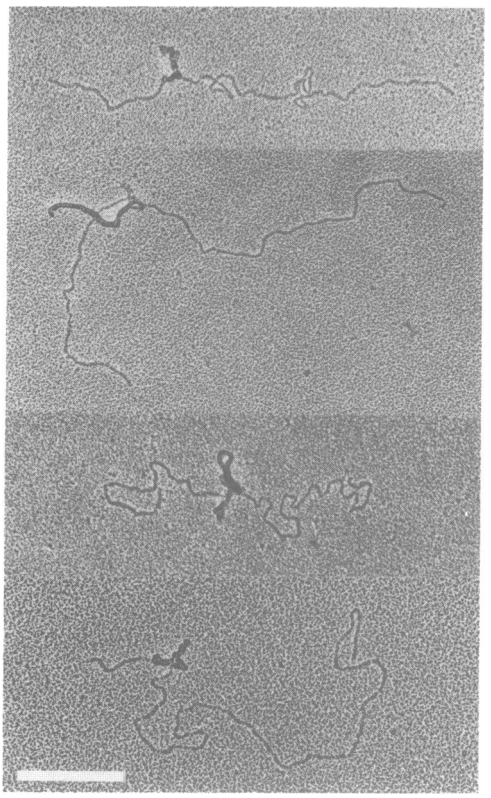

Fig. 5 : Electron microscopic observation of BglI + MspI linearized transcriptional complexes.

BglI + MspI digested minichromosomes were sedimented on sucrose gradient as described in legend to Figure 4. Aliquots of fraction 16 (see Figure 4B) were elongated in vitro and processed for electron microscopy as described in Materials and Methods. One RNA-T4 gene 32 protein tail is observed on each linearized minichromosome DNA. The bar corresponds to 0.25 μm.

To determine if the RNA polymerase molecules were present on the linearized or the double cleaved minichromosomes (minichromosomes with gap), aliquots of different fractions from the sucrose gradient containing the BglI + MspI restricted minichromosomes were subjected to in vitro RNA synthesis and examined by electron microscopy after incubation with T4 phage gene 32 protein which binds to single-stranded nucleic acid (23). Transcriptionally inactive molecules were visualized as naked DNA filaments, since histones are totally dissociated by Sarkosyl from the minichromosomes (28). Transcriptional complexes are characterized by the presence of a thick RNA-T4 gene 32 protein tail connected to the DNA strand (23). 95% of the transcriptional complexes (molecules with RNA-T4 gene 32 protein tail) present in fraction 16 and 21 were linear molecules. The transcriptional complexes were not observed in absence of in vitro elongation or after DNAse-free RNAse treatment (not shown). They represent 2 to 4% of the molecules present in fraction 16 and less than 1% of those pre-

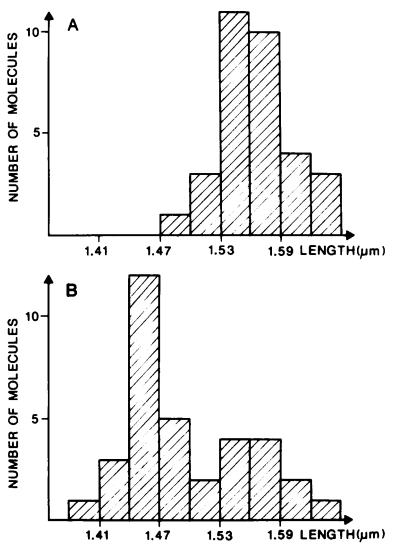

Fig. 6 : Distribution of the DNA length of the transcriptional complexes after BglI or BglI + MspI digestion as determined by electron microscopy.

SV40 minichromosomes were digested either with BglI only (A) or with BglI + MspI (B) restriction enzymes and processed as indicated in legend to Plate 1. The DNA length of the transcriptional complexes was determined on electron microscopic micrographs, similar to those shown in Plate 1.

sent in fraction 21. Only one RNA-T4 gene 32 protein tail is generally present per SV40 genome (Figure 5), confirming our previous observations (17).

DNA length measurements of the population of transcriptional complexes in fraction 16 of BglI + MspI digested minichromosomes revealed a minor and a major peak, at 1.55 μm and 1.45 μm (Figure 6B). The peak at 1.55 μm represents the minichromosomes cleaved only once, since it corresponds to the population of linearized transcriptional complexes cut with BglI or MspI alone (Figure 6A). This peak of DNA length is also obtained after linearization of purified SV40 DNA with either of these enzymes (not shown). The peak at 1.45 μm represents the double cut minichromosomes, since it is not present when minichromosomes are cut only once (Figure 6A) and corresponds to the length of the BglI-MspI SV40 DNA fragment of 4889 bp (not shown). The molecules present in the double cut peak represent 66% of the total population of characterized transcriptional complexes. Since fraction 16 contains three to four times less double cut than single cut SV40 DNA molecules (see Figure 4B), most of the transcriptional complexes are preferentially sensitive to simultaneous digestion with BglI and MspI restriction enzymes, and therefore correspond to gapped minichromosomes which are known to be preferentially sensitive to simultaneous digestion by BglI and MspI (see above and 16).

DISCUSSION

1. SV40 transcriptional complexes are more sensitive to restriction enzyme digestion than bulk minichromosomes.

Late in infection, SV40 transcriptional complexes can be extracted from infected nuclei and sediment somewhat faster than the bulk of minichromosomes on sucrose gradient (17, 25, 26; see also Figure 2A). They consist of minichromosomes associated with RNA polymerase B (17, 29) and represent almost 1% of the minichromosomes present late in infection (30). We show here that, after restriction enzyme digestion, the extracted transcriptional complexes sediment slower than the bulk of minichromosomes on sucrose gradient and that most of the *in vitro* RNA synthesizing activity is associated with linearized minichromosomes as evidenced by electron microscopy, demonstrating that the SV40 transcriptional complexes are preferentially digested by restriction enzymes.

Luchnick et al. (31) have reported that a fraction (2.5%) of the minichromosomes in which the DNA is under torsional strain is enriched in endogeneous RNA polymerase B. They have subsequently reported that the same fraction of minichromosomes, which can be relaxed by topoisomerase I, is also hypersensitive to DNAse I digestion (32). It is possible that we are dealing with the same subpopulation of minichromosomes. In this case, the "torsionally strained" minichromosomes will also be preferentially cut by restriction enzymes under our conditions of digestion.

2. Characterization of restriction enzyme digested transcriptional complexes.

The small percentage (1-2%) of transcriptional complexes that we have observed after restriction enzyme digestion is in agreement with previous studies (17, 25, 26). However, we cannot rule out that some of the characterized transcriptional complexes do not correspond to actively transcribing complexes *in vivo*. "Pausing" RNA polymerase molecules are indeed activated under transcriptional conditions (27, 33, 34).

As mentioned in the results section, some loss of RNA polymerase activity occurs during restriction enzyme digestion (see also 35). This loss appears to be not random, since the RNA polymerase molecules present in the 5' region of the late transcription unit between the KpnI and the EcoRV restriction sites (see Figure 3) are preferentially concerned. It is interesting that this region corresponds to that where premature termination of transcription has been found late in infection (33, 34). It is possible that the RNA polymerase molecules present in this region of "attenuation"

are preferentially inhibited or released during the restriction enzyme treatment at 37°C.

3. Relationship between transcriptional complexes and gapped molecules.

A report published while the present study was in progress (12) has shown that all transcriptionally active minichromosomes exhibit hypersensitivity to both DNAse I and restriction enzymes, and that the nuclease hypersensitive molecules are well correlated with gapped molecules, suggesting that all transcribing SV40 minichromosomes possess a nucleosome-free ORI region. Our results demonstrate directly that at least 60% of the SV40 transcriptional complexes correspond to gapped minichromosomes. We cannot exclude that some of the transcriptional complexes do not have a nucleosome-free region. In fact, not all minichromosomes with gap are preferentially sensitive to restriction enzymes even in large excess of enzyme, and some minichromosomes without gap are also digested (16).

The SV40 transcriptional complexes represent not more than 1% of the total minichromosome population (30) and approximately 25% of the minichromosomes containing a nucleosome-free region (a gap). Therefore, it is possible that the majority of the gapped minichromosomes represent potential transcriptional complexes which display no RNA polymerase activity due to limiting amounts of specific transcriptional factors present in the nuclei (36).

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