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Chromatin structure and factor site occupancies in an *in vivo*-assembled transcription elongation complex

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ABSTRACT

The chromatin structure specific to the SV40 late transcription elongation complex as well as the occupancy of several sites that bind transcription factors have been examined. These features have been determined by assessing blockage to restriction enzyme digestion. Cleavage specific to the elongation complex has been quantified using ternary complex analysis. This method involves radioactively labeling the complex by *in vitro* transcription followed by determining the extent of linearization by electrophoresis in an agarose gel. It was found that not only is the origin region devoid of nucleosomes, but there is also no stable factor occupancy at the *Bgl*I, *Sph*I, *Kpn*I and *Msp*I restriction enzyme sites within this region. Thus these sites were cleaved to a high degree, meaning that the binding sites for a number of transcription factors, including OBP/TEF-1, TBP, DAP, as well as a proposed positioned nucleosome, are unoccupied in the native viral transcription elongation complex. The absence of these *trans*-acting factors from their respective binding sites in the elongation complex indicates that they bind only transiently, possibly cycling on and off during the transcription cycle. This finding implies that various forms of transcription complex are assembled and disassembled during transcription and thus supports a 'hit-and-run' model of factor function.

INTRODUCTION

Considerable progress has been made recently in the identification and characterization of *trans*-acting factors, which are protein factors that bind to specific sites in the DNA regulatory regions of genes and modulate expression from those genes (see 1–4 for reviews). However, while a substantial inventory of these factors has been compiled, the various mechanisms by which they stimulate transcription are still uncertain. Ultimately, a complete understanding of the process of transcription will require a description of the structures of the transcription complex in its various functional forms: initiation complex, elongation complex and termination complex. A first step in these descriptions is a characterization of the factor content and locations in the various complex forms.

We previously initiated such a characterization of the simian virus 40 (SV40) late viral transcription elongation complex (VTC) by assessing the occupancy of the replication origin binding site (Site II) for T-antigen (5), the product of the viral oncogene. This was accomplished by determining the extent of restriction enzyme cleavage at that site in the native complex. It was found that T-antigen is absent from the origin site in the elongation complex, a finding that was inconsistent with certain models for the role of T-antigen in the control of transcription. This in turn spurred efforts to evolve alternative models for the participation of T-antigen in late transcription (6–8).

A similar study characterized the occupancy of the VTC sites for the cellular transcription factor TEF-1 (8). In contrast to the T-antigen origin site, the TEF-1 site at nt 270 was found to be occupied in a fraction of the VTC population, a result that indicates there are at least two forms of elongation complex.

Additional studies of SV40 have led to the identification of many other cellular *trans*-acting factors and their binding sites (1–4,9 and references therein), as well as characterization of the chromatin structure within which these sites are located. Thus numerous studies have observed that the SV40 region containing the sites for *trans*-acting factors, located around the replication origin (see Fig. 1), exhibits a chromatin structure that is devoid of nucleosomes in a fraction of the minichromosomes (10–13 and references therein). This conclusion derives from the observation that both restriction and non-specific nucleases cleave preferentially in this region in a subpopulation of the viral minichromosomes; this accessible stretch of chromatin has been termed the open region. An important extension of these studies was the demonstration (5,8,14,15) that all of the VTC (which themselves comprise <5% of the total minichromosome population; 16,17) contain an open region.

However, this high level of cleavage raises a paradox. While it is reasonable that the regulatory region is free of nucleosomes to allow transcription factors to bind, the presence of the factors in the functional complex would be expected to prevent nuclease access to their respective sites. As discussed above, such protection has been observed at one of the TEF-1 sites in a subpopulation of the VTC (8) and partial protection at sites within the DNase I

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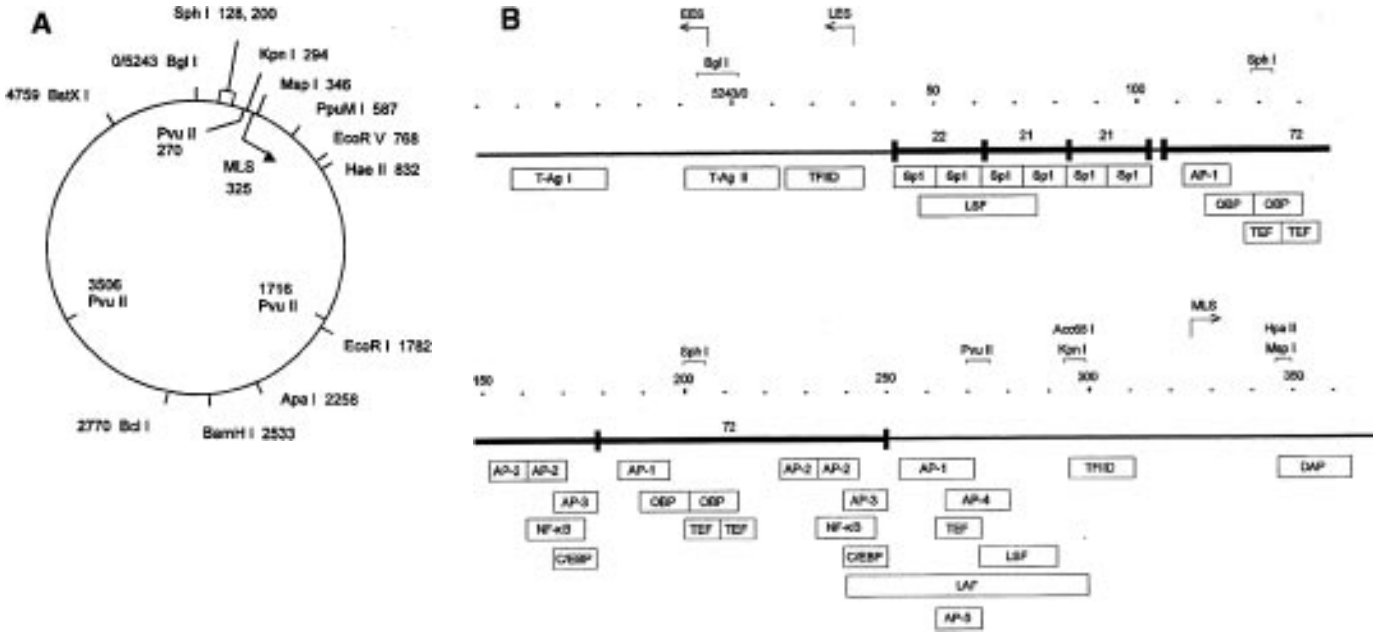

Figure 1. Restriction enzyme sites in SV40. The various restriction enzyme sites which are analyzed in this work are shown, as is the major late transcription start site (MLS). The late mRNA, encoding the viral capsid proteins, is transcribed from the right half of the genome as illustrated, while early mRNA, encoding the viral T-antigen, is transcribed from the left half of the genome in the opposite direction. The origin of replication is located at the*Bgl*I site (nt 1), while the region around this site contains early and late transcription regulatory sequences; this has also been termed the open region (nt 1 to~400). Shown below is an expanded view of this region, with binding sites of various *trans*-acting factors indicated. While it is apparent that the SV40 origin region contains sites for a large number of transcription factors, only a limited number of the factors shown have been implicated in control of SV40 late transcription (see text).

hypersensitive stretch encompassing the open region of the bulk population (18,19) has been observed. However, nuclease digestion studies of other factor binding sites in the open region of the VTC (5,8,14) have shown that these sites are vacant, raising a question as to how these factors regulate late transcription when they are absent from the VTC.

To investigate this question further, an expanded analysis of restriction enzyme accessibility of both the VTC regulatory region as well as the rest of the genome was performed. Samples of nuclear extract from infected cells were digested by 13 infrequent-cutting restriction enzymes, several of which cleave in the regulatory region in DNA binding sites of *trans*-acting factors known to be important for transcriptional activity. Cleavages made specifically in the VTC were quantified by extension labeling of ternary complexes (5,8,14). The results indicate that several factor binding sites are virtually unoccupied. These findings indicate that the factors bind to their sites only transiently during the transcription process, i.e. they function by a 'hit-and-run' mechanism.

MATERIALS AND METHODS

Cells and virus

BS-C-1 cells were used for all experiments. These cells were cultured and infected with SV40 wild-type strain 776 as described previously (20,21).

Restriction enzyme analysis of nuclear extracts

The isotonic extraction procedure previously described (8,20,21) was used to prepare nuclear extracts. When required, these extracts were concentrated and desalted using a Centricon 30 microconcentrator (Amicon). Typically 30–90 µl aliquots of 1–3× concentrated and desalted nuclear extract were combined with the appropriate 10× restriction enzyme buffer and 5 U/µg DNA of the indicated restriction enzyme. Either 10× M buffer (final concentrations 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10 mM Tris–HCl, pH 7.5) or 10× H buffer (as M buffer except that the final concentration of NaCl was 100 mM and that of Tris was 50 mM; both buffers supplied by Boehringer Mannheim Biochemicals) were used to adjust the salt concentrations of desalted nuclear extracts. Incubations in buffer with no added NaCl resulted in considerable nicking of VTC. Therefore, digestion with an enzyme with a low salt optimum (e.g.*Kpn*I) was performed at 100 mM NaCl to avoid nicking. Additional enzyme was added when necessary to compensate for any lower activity due to change in salt concentration. Digestions were performed for 40 min at 30°C except where indicated. At the end of the incubation, the digestion was halted by the addition of transcription mix or by the addition of EDTA to a final concentration of 20 mM. *In vitro* transcription and subsequent preparation of ternary complexes were performed as described previously (5,17,22). Samples were fractionated by electrophoresis through 1.0% agarose TBE gels, after which the gel was stained with ethidium bromide and photographed using 4x5 Tri-X film. The gel was then dried and autoradiographed using Kodak XAR film. Films were scanned and peaks integrated with an LKB Ultroscan densitometer (5,22,23).

VTC bands in the autoradiograph were completely sensitive to the presence of 1 µg/ml α-amanitin in the extension mix, while >90% of the incorporated radioactivity has been shown to be late strand specific (20).

Kinetic analysis of restriction enzyme digestion

Isotonic nuclear extract from SV40 strain 1x72 (24) was incubated with doubly end-labeled 610 bp bare DNA fragment (*Ava*II fragment nt 5118–557) from 1x72 SV40 (containing a single *Sph*I site). This mixture (10 µg total DNA in 50 µl) was incubated with *Sph*I (30 U) at 25°C in 1× M buffer as described above. Samples (6 µl) were removed at the indicated times and subjected to ternary complex analysis as described above.

Hypotonic nuclear extraction

Hypotonic nuclear extract was prepared by a modification of a method reported earlier (8,20). A 10 cm plate of cells was washed twice with phosphate-buffered saline, after which the cells were lysed with 0.5–1.0 ml lysis buffer containing 10 mM Tris–HCl, pH 6.8, 10 mM Na_2EDTA, 0.25% Triton X-100, 31 µg/ml phenylmethylsulfonyl fluoride, 5 µg/ml pepstatin A, 5 µg/ml leupeptin and 10 µg/ml aprotinin. The lysate was scraped into a centrifuge tube and nuclei were pelleted at 3000 *g* for 5 min. The nuclear pellet was resuspended in 60 µl extraction buffer containing 10 mM Na HEPES, pH 7.8, 1 mM Na_2EDTA and protease inhibitors as mentioned above. The nuclei were then treated with 60 strokes in a dounce homogenizer and incubated overnight at 4°C with rotation. The suspension was centrifuged for 10 min at 11 000 *g* to pellet the nuclear chromatin, leaving the supernatant which contained the SV40 minichromosomes and VTC.

Restriction enzyme digestion of nuclei

The nuclei pellet from cells infected with SV40 was prepared as described for the hypotonic or isotonic nuclear extract preparations (see above). The pellet (representing 0.16 of a 15 cm plate of cells) was suspended in 40 µl of the appropriate buffer and digested with restriction enzyme for 40 min at 30°C. The digestion was terminated by the addition of 1 µl 40 mg/ml heparin, after which the sample was centrifuged for 10 min at 11 000 *g*. A sample of the supernatant, containing the SV40 ternary complexes, was then incubated with transcription mix and processed as described above for ternary complex analysis.

Fractionation of nuclear extract over glycerol gradients containing $(NH_4)_2SO_4$

A 1 ml sample of isotonic nuclear extract was sedimented through a 10–30% linear glycerol gradient containing either TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.9) or TE + 0.3 M $(NH_4)_2SO_4$ (20,21). The absorbance peak of the minichromosomes was then pooled, concentrated in a Centricon 100 microconcentrator and desalted by spin chromatography over Sephadex G-50 equilibrated with TE. Aliquots of the pools were adjusted to 1× H buffer (see above) and digested with the indicated restriction enzymes, after which they were extended *in vitro* with [α-^{32}P]UTP and analyzed as ternary complexes as described above.

RESULTS

Mapping the extent of the open region in the VTC by restriction enzyme cleavage and radioactive labeling of ternary complexes

Figure 1 illustrates a map of selected restriction enzyme cleavage sites in the SV40 genome. Even though VTCs comprise <5% of the SV40 minichromosome population in the nuclear extract of an infected cell, the extent of restriction enzyme cleavage of the VTC can be quantified by the radioactive extension labeling method (5,8,14,17). A brief description of the method follows. A sample of nuclear extract is incubated with a restriction enzyme, after which heparin is added to convert VTCs into ternary complexes. These are then labeled by extension *in vitro*, after which they are separated into linear, nicked and supercoiled forms by agarose gel electrophoresis. A photograph of the ethidium bromide stained gel reveals the relative levels of the three forms for the bulk population, while the autoradiograph reveals the relative levels for the VTC. The extent of exposure of the restriction enzyme site in the VTC is proportional to the fraction of linear molecules in the digested sample. When a restriction site lies within or near to a *trans*-acting factor binding site in the regulatory region near the SV40 origin, the presence of that factor is detected as a blockage to digestion. Moreover, if additional enzymes are chosen that have sites throughout the rest of the genome, information about the chromatin organization in various regions of the genome can be obtained. This is because DNA organized in randomly positioned nucleosomes is characterized by 30–50% protection from restriction enzyme digestion (reviewed in 12), so a sequence that is completely digested is likely to be nucleosome-free. Analysis of sites spanning the entire genome can therefore detect differences between the chromatin structures of bulk minichromosomes and VTC, which in turn can provide insight into the distinctive chromatin structural features necessary for transcriptional activity.

Figure 2 shows the results of such an experiment. An undigested sample (Fig. 2, UNCUT) exhibits the expected bands for supercoiled and nicked forms in both the ethidium bromide stained gel (representing bulk minichromosomes; MCs) and in the autoradiograph (representing VTCs). An initial examination of the samples incubated with various restriction enzymes reveals that the bulk minichromosome population tended to be linearized to a level of ~30–50% for all of the enzymes used (Fig. 2, MCs). As discussed above, this level of digestion is characteristic of a chromatin organization composed of nucleosomes positioned randomly over the respective sites (12).

However, this similarity in the levels of digestion of the bulk minichromosome sites contrasts with the levels observed for the VTC (Fig. 2, VTCs). The VTC cleavages, revealed by an autoradiograph of the same gel, show much greater variation among the different sites. Thus a minor fraction of the *Eco*RV, *Ppu*MI, *Bam*HI and *Bcl*I sites are linearized, i.e. cleavage similar to that of the bulk minichromosome and indicating nucleosome organization. In contrast, the *Sph*I, *Pvu*II (see also 8), *Kpn*I and *Hpa*II sites are cleaved to a significantly higher degree, with some levels approaching 100%. These highly cleaved sites are clustered along with the highly cleaved site *Bgl*I (5) in sequences surrounding the origin region (Fig. 1), while the sites cleaved to a lower extent are remote from the origin region. These results confirm previous studies that indicate the presence of this nucleosome-free open region surrounding the origin and extend those studies by providing a refined definition of the extent of that region in the VTC. The relationship between this high level of site exposure and nucleosomes is investigated further below.

Kinetic analysis of restriction enzyme digestion

The high levels of VTC digestion by the origin region enzymes in Figure 2 indicate that their sites are virtually bare. To further

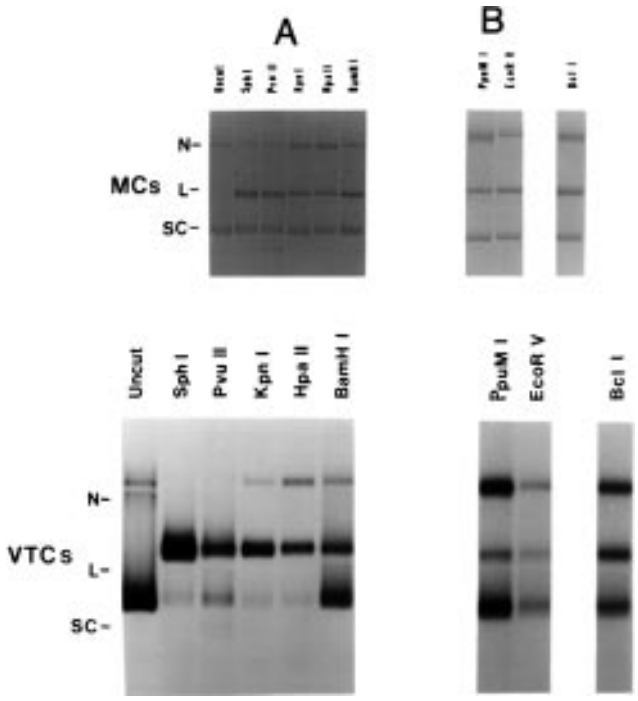

Figure 2. Restriction enzyme digestion of viral minichromosomes and transcription complexes. Aliquots of nuclear extract were digested with the indicated restriction enzymes, after which they were processed as described in Materials and Methods. DNA of the bulk minichromosomes was visualized by ethidium bromide staining (MCs; the photographic negative is shown here) and transcription complexes were visualized by autoradiography (VTCs). (**A** and **B**) Separate experiments performed on separate extracts. N, L and SC indicate the mobility of nicked, linear and supercoiled bare DNA.

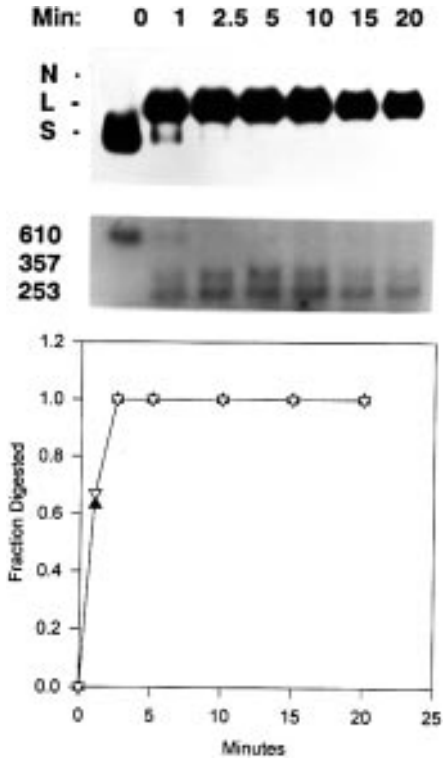

Figure 3. Kinetics of *Sph*I digestion of 1x72 VTC and bare DNA. Nuclear extract isolated from cells infected with 1x72 SV40 was mixed with an end-labeled DNA fragment containing the 1x72 *Sph*I site (see Materials and Methods). This mixture was digested with *Sph*I for the indicated times. An autoradiograph of a ternary complex gel is shown at the top and the bare DNA probe in the same gel lane is shown in the middle. Autoradiographs were exposed for different lengths of time to compensate for the different levels of radioactivity in the ternary complex and bare DNA probe bands. The 610 bp fragment is cleaved into the 253 and 357 bp fragments by *Sph*I. Results of densitometric quantitation of the bands are shown at the bottom. The open triangles represent digestion of the end-labeled bare DNA fragment, while the closed triangles represent digestion of the VTC.

investigate this, a representative origin region enzyme was examined in more detail. We had previously shown that *Sph*I digestion is in fact blocked when its site is occupied by the *trans*-acting factor TEF-1 (8), so a kinetic analysis of cleavage was performed with this enzyme (Fig. 3). Since there is an ambiguity in interpretation of the *Sph*I cleavage due to the presence of two closely spaced *Sph*I sites in the duplicated 72 bp repeat of wild-type SV40, this analysis was performed on a variant of SV40 (1x72; 25) that contains only one copy of the 72 bp repeat sequence. To provide a direct comparison to the digestion of bare DNA, an end-labeled fragment of bare SV40 DNA containing a single *Sph*I site was added to 1x72 nuclear extract. A time course of *Sph*I digestion was performed on this mixture, followed by ternary complex analysis. The results in Figure 3 show that both the bare DNA and the VTC were digested to completion. The plot of the results of densitometric quantitation (Fig. 3, bottom) reveals that the VTC was digested at the same rate as bare DNA present in the same tube. These results indicate that the single *Sph*I site in the 1x72 VTC is entirely vacant.

Occupancy of individual *trans*-acting factor binding sites in the origin region of the VTC

The high accessibility of the VTC origin to *Sph*I, *Hpa*II and *Kpn*I (Fig. 2) as well as *Bgl*I (5) raises a paradox. On the one hand, it is reasonable to have this region free of nucleosomes so that transcription regulatory factors can bind. On the other hand, these factors should be present in the functional complex, in which case restriction enzyme access to their sites would be blocked. For example, *Sph*I and *Hpa*II/*Msp*I cleave in the binding sites of the transcriptional activators OBP/TEF-1 and DAP respectively and mutational analysis indicates that the activation mechanism of these factors requires binding to their sites (26–28). Similarly, *Bgl*I (29) and *Kpn*I (30,31) cleave in sequences that are potential binding sites for late transcription stimulatory factors.

One consideration in interpretation of these results is the possibility that the factor was bound to its VTC site in the cell, but was lost from the site upon preparation of the isotonic nuclear extract. To investigate this, two variations of the procedure were developed. First, to control for the possibility that even isotonic salt in the extraction may perhaps be removing some weakly bound proteins during extract preparation, the analysis was performed on a hypotonically prepared sample. Second, the analysis was performed at an earlier stage in the preparation: digestion was carried out directly in nuclei.

The results of digestion of hypotonic and isotonic nuclear extracts are shown in Figure 4. The VTC in the isotonically prepared extract were linearized to a high degree for all the origin region enzymes used, consistent with the results in Figures 2 and 3.

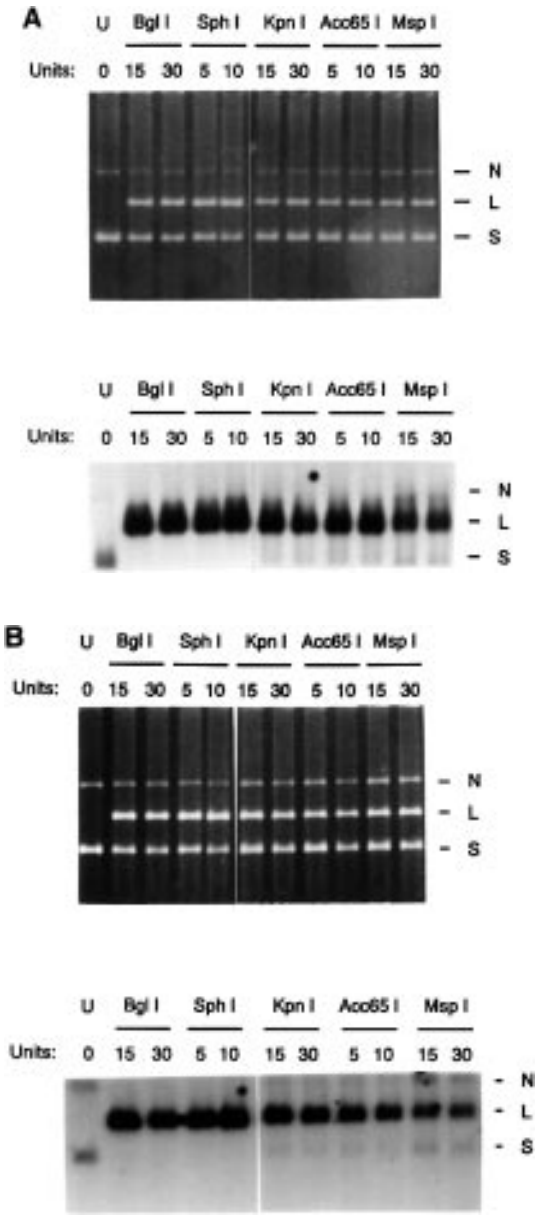

Figure 4. Restriction enzyme cleavage of viral minichromosomes and transcription complexes in isotonic and hypotonic nuclear extract preparations. Isotonic nuclear extract (**A**) and hypotonic nuclear extract (**B**) were prepared as described in Materials and Methods from a single infection of cells. Samples were digested with restriction enzymes and processed as described in Materials and Methods. The restriction enzymes used are indicated, as are the number of units used per 10 µl sample (~1 µg DNA). A photograph of the ethidium stained gel (representing cleavage of the bulk minichromosome) is shown in the upper panel, with N, L and S indicating the mobilities of nicked, linear and supercoiled DNA respectively. An autoradiograph of the same gel (representing cleavage of the VTC) is shown in the lower panel, with N, L and S indicating the mobilities of nicked, linear and supercoiled ternary complexes respectively.

When the hypotonically prepared sample was analyzed, high levels of linearization similar to those observed for the isotonically prepared sample were obtained. The results of densitometric quantitation (Table 1, Nuclear extract) reveal that there was no significant difference between the levels of linearization in the isotonic and hypotonic samples for the five origin region enzymes analyzed. This indicates that the lack of occupancy at these sites is not due to removal of bound factor by isotonic salt during extract preparation.

The second procedural variation involved cleavage in nuclei, i.e. at an earlier stage in the preparation. This controls for the possibility that a factor may be lost from its site during the preparation of the extract from the nuclei. The results are shown in Figure 5, where it can be seen that the VTC in both isotonically (Fig. 5A) and hypotonically (Fig. 5B) prepared nuclei were again cleaved to a high degree by the origin region enzymes. The quantitation data in Table 1 demonstrate that the level of cleavage in nuclei was not significantly different from the respective values in nuclear extract; cleavage was very high in both samples. To summarize, the data in Figures 4 and 5 and Table 1 demonstrate that the high cleavage at the four VTC origin sites analyzed was not due to loss of factors as a result of washing with isotonic salt or of preparation of extract from nuclei. Thus the results indicate that the sites are not occupied by factors in the elongating transcription complex in the cell. In contrast, the bulk minichromosome population exhibits a partial cleavage pattern indicative of the presence of randomly located nucleosomes, regardless of the restriction enzyme used (Figs 4 and 5).

Table 1. Percent linearization of VTC

	Nuclear extract		Nuclei	
	Isotonic	Hypotonic	Isotonic	Hypotonic
*Bgl*I	(9)[a] 96[b] ± 4	(9) 98 ± 3	(7) 97 ± 5	(6) 95 ± 7
*Sph*I	(9) 96 ± 4	(9) 98 ± 4	(7) 94 ± 5	(6) 99 ± 2
*Kpn*I	(4) 92 ± 2	(5) 87 ± 5	(9) 94 ± 7	(8) 90 ± 10
*Acc*65I	(9) 86 ± 3	(9) 89 ± 2	(9) 91 ± 5	(9) 91 ± 2
*Msp*I	(5) 81 ± 10	(3) 75 ± 5	(7) 83 ± 9	(6) 76 ± 2

[a]Values in parentheses are numbers of determinations.
[b]Percent linearization ± SD.

Chromatin structure of bulk minichromosomes and VTC

The percent cleavages for bulk minichromosomes and VTC for 13 enzymes were determined by densitometric scanning and a summary of the results is presented in Figure 6. This quantitative representation of the data bears out the conclusions reached above. Figure 6B shows that the variation of linearization with different restriction enzymes is somewhat limited for the bulk minichromosome population, ranging between ~30 and 50%, with no particular part of the genome showing systematically distinctive digestion. However, Figure 6A shows that sites in the VTC origin region are digested to a markedly higher level than are sites in the remainder of the genome. The distinction between the two forms of complex is particularly clear in a difference plot for the VTC versus bulk minichromosome (Fig. 6C).

The effect of exposure to elevated ionic strength on site accessibility

Numerous studies observe increased enzyme accessibility (32–35) in the regulatory region of the SV40 bulk minichromosome population and this region of accessibility has been termed the open region of SV40. Electron microscopy indicates that this

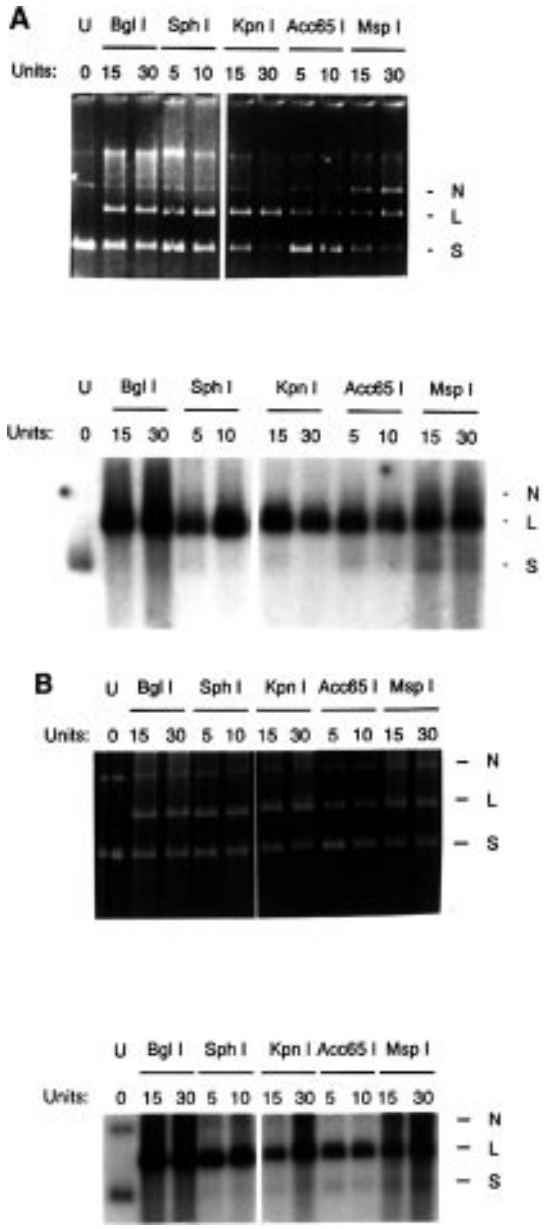

Figure 5. Restriction enzyme cleavage of viral minichromosomes and transcription complexes in nuclei. Nuclei were prepared isotonically (**A**) or hypotonically (**B**), after which they were digested with the indicated restriction enzymes as described in Materials and Methods. The number of units of enzyme added per 10 µl sample (~1 µg DNA) is indicated. Labeling is as in Figure 4.

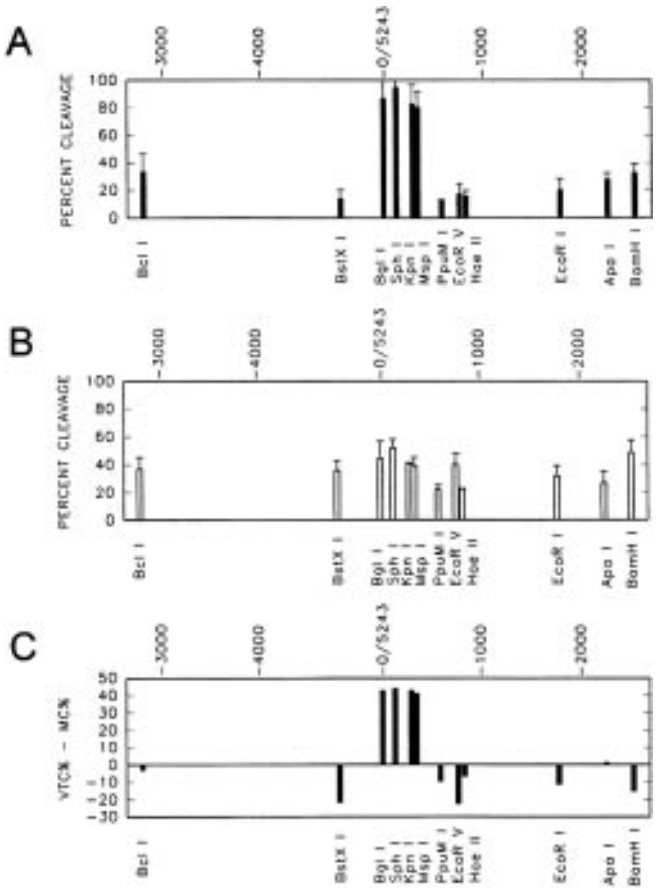

Figure 6. Summary of restriction enzyme digestion of SV40 minichromosomes and transcription complexes from isotonic nuclear extract. (**A**) The average level of cleavage of VTCs by the indicated restriction enzymes. Error bars represent standard error about the mean for the following number of determinations: *Bgl*I, 21; *Sph*I, 13; *Kpn*I, 7; *Msp*I, 5; *Ppu*MI, 2; *Eco*RV, 5; *Hae*II, 2; *Eco*RI, 5; *Apa*I, 2; *Bam*HI, 7; *Bcl*I, 3; *Bst*XI, 6. (**B**) The average level of cleavage of the bulk minichromosomes in the same experiments. (**C**) A difference map in which the percent MC cleavage is subtracted from the percent VTC cleavage at the same site. Thus a positive value means that the VTC was cleaved to a greater degree than the bulk MC at that site.

accessibility reflects an absence of nucleosomes in this region (32,34). While the data in Figure 6B show no significant indication of an open origin region in the bulk minichromosomes of our preparation, the apparent coincidence between the extent of the open region of the VTC (Fig. 6A) and that reported by others (see references above) for the bulk minichromosome implies that nucleosomes may be responsible for the VTC open region as well.

To investigate this, samples of nuclear extract were analyzed following exposure to an ionic strength [300 mM $(NH_4)_2SO_4$] at which nucleosome sliding is known to occur (12,36,37). Thus nuclear extract samples were sedimented through glycerol gradients containing different ionic strengths, after which accessibilities to various restriction enzymes were determined.

The results are shown in Figure 7. VTCs sedimented through a gradient containing low ionic strength showed higher cleavage at the *Bgl*I site, a representative origin region enzyme, than at the other sites in the genome (Fig. 7A, TE). This result is similar to those from less processed samples (Figs 2, 4 and 5). However, exposure to 300 mM $(NH_4)_2SO_4$ in the glycerol gradient decreased the relative level of digestion of the *Bgl*I site to where it approximated the levels of the other sites [Fig. 7A, 300 mM $(NH_4)_2SO_4$]. Figure 7B shows the results of quantitation of the data for both the VTC and the bulk minichromosomes. While exposure to elevated ionic strength caused little change in the pattern of exposure of sites in the bulk minichromosomes, the pattern of the VTC sites changed significantly, becoming more like the bulk pattern. Specifically, the *Bgl*I site has changed from being highly accessible in the low salt-treated sample to being only partially accessible and similar to the other sites in the high

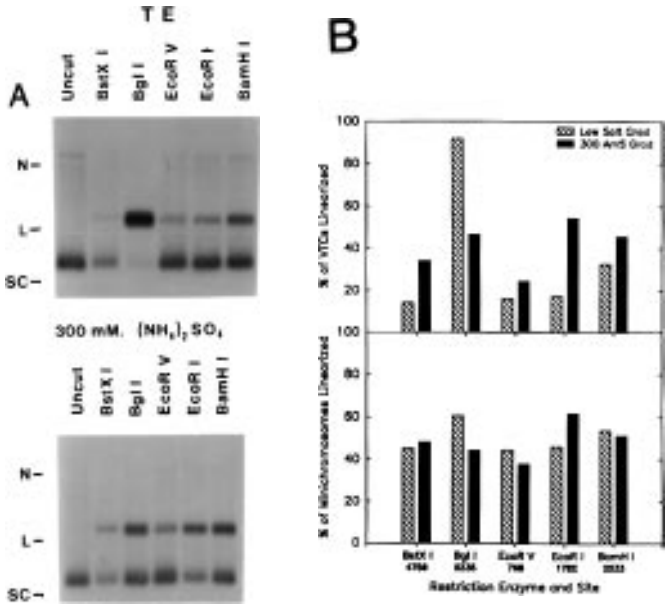

Figure 7. Effect of elevated $(NH_4)_2SO_4$ on the extent of digestion of various sites of transcription complexes. Nuclear extract was sedimented through 10–30% linear glycerol gradients containing either TE or TE + 300 mM $(NH_4)_2SO_4$, after which the minichromosomes were pooled, concentrated and desalted. Aliquots of the pools were digested with the indicated restriction enzymes and processed as described in Figure 2 (see Materials and Methods). SC, L and N indicate the mobilities of supercoiled, linear and nicked bare SV40 DNA. (**A**) An autoradiograph of the gel, depicting the ternary complexes. (**B**) Quantitation of the extent of linearization of the VTCs and of the bulk minichromosomes. Solid bars represent percent linearization of samples sedimented in the presence of $(NH_4)_2SO_4$, while the cross-hatched bars represent that of samples sedimented in the presence of TE only.

salt-treated sample. Thus the results are consistent with elevated ionic strength having caused nucleosomes to invade the open region VTC by sliding, partially blocking the previously exposed *Bgl*I site. This suggests that nucleosomes are indeed responsible for the open region in the VTC and further supports the conclusion that the VTC contains features which are both similar to and different from those of the bulk population (22).

DISCUSSION

SV40 has proven to be a useful model in the study of both viral and cellular gene expression. In an effort to gain insight into mechanisms by which *trans*-acting factors regulate transcription, we have examined the structure of SV40 late transcription complex. Two related features of VTC structure have been characterized: the open region in the chromatin of the VTC and the occupancy of *trans*-acting factor binding sites in that open region.

We have found that the extent of the open region in the VTC corresponds to that reported for the bulk minichromosome population. Thus Varshavsky *et al.* (35) and Scott *et al.* (19) observed in bulk minichromosome studies that the open region extends from nt ~1 to 400. The high levels of cleavage observed here for *Bgl*I, *Sph*I, *Kpn*I and *Msp*I in the origin region define an open region from nt 1 to 346. In contrast, *Ppu*MI (nt 587) and *Bst*XI (nt 4759) cleave to a low level and this low cleavage, along with the similarly low cleavages at the *Bcl*I, *Bam*HI, *Eco*RI, *Hae*II and *Eco*RV sites, define limits to the extent of the open region. It should be noted that while the open region is quite apparent in the data for the VTC (Fig. 6A), the data here for bulk minichromosomes (Fig. 6B) do not show a strong indication of an open region. This variation in the observation of the open region in the bulk population has been reported by other groups (see for example 38,39) and may reflect a variation in the composition of the minichromosome population in different nuclear extract preparations.

It was demonstrated that hypertonic treatment of nuclear extract caused digestion of the VTC origin region to change from a high level of cleavage to one characteristic of nucleosome organization (Fig. 7), suggesting that the high salt caused the nucleosomes to slide into the origin region. An additional feature of VTC nucleosome organization is revealed by *Msp*I digestion (Figs 4–6 and Table 1). The high level of cleavage by *Msp*I indicates that the nucleosome, which has been proposed to be positioned over that site in the bulk population (40,41), is not present in the VTC. If such a positioned nucleosome is important for transcription, as the authors propose (40,41), then it is possible that the nucleosome is present in the preinitiation state and then displaced once transcription ensues. Alternatively, rather than being a component of a transcriptionally active complex, perhaps a more likely role for such a nucleosome would be to repress late transcription, since the data here indicate that it is absent from the VTC.

A nucleosome has also been proposed to be specifically positioned over the *Bam*HI site and has been assigned a role in transcription termination (42,43). This would predict that the *Bam*HI site in the VTC should be quantitatively blocked. The data in Figures 2, 6 and 7 demonstrate that such is not the case, a finding which makes the proposal unlikely.

The open region contains binding sites for numerous *trans*-acting factors that stimulate transcription from the late promoter (6,7,28–30,44–49). In order to gain insight into mechanisms by which *trans*-acting factors function, we determined the level of occupancy of several *trans*-acting factor binding sites in the VTC origin region. This was accomplished by analysis of cleavage sites for *Bgl*I, *Kpn*I, *Msp*I and *Sph*I in the VTC.

The *Bgl*I site of the VTC was cleaved to a high degree in both extract as well as nuclei (Figs 3, 4, 6 and 7 and Table 1), a result which confirms and extends our earlier findings (5). The *Bgl*I cleavage site lies within T-antigen Site II in the origin of replication, a region that has been identified to be important for T-antigen-dependent stimulation of late transcription activity (29). Our findings indicate that neither T-antigen nor any other factor is bound to this site in the VTC.

The sequence surrounding the *Kpn*I site (nt 294) has been assigned significance as a *trans*-acting factor binding site (30,31) and a second study reports that the DNA double helix is melted in this region in the bulk minichromosome population (41). However, the data here indicate that this site is unoccupied and in the normal DNA B-form in the VTC.

The sequence surrounding the *Msp*I site (nt 345) is the binding site for IBP-s, a repressor protein that is proposed to be bound to the early transcription complex in order to maintain late transcription repression (50). The shift from early to late transcription has been proposed to involve removal of this IBP-s. Our finding that the *Msp*I site is vacant therefore supports this proposal. A study by Ayer and Dynan (28) proposes that the *Msp*I site is the binding site for downstream activator protein (DAP). The authors suggest that DAP may stimulate late transcription by

facilitating assembly of the preinitiation complex. Again, our data indicate that this site is vacant in the VTC, so if DAP participates in the preinitiation complex, it would need to dissociate from the complex once transcription elongation begins. This will be discussed in more detail below.

This finding that the binding sites for numerous *trans*-acting factors are unoccupied in the VTC raises an important question: are these sites in fact vacant in the cell or is the apparent lack of occupancy due to the procedure used to analyze them? We feel that the site vacancy observed is not a procedural artifact, for the following reasons.

First, the same high level of cleavage was observed whether the experiment was performed on nuclear extract or in nuclei (Figs 4 and 5 and Table 1), indicating that the factors were not lost during the preparation of extract from nuclei. The nuclei sample was prepared with minimal manipulation and is similar to what has been termed an '*in vivo*' state of chromatin used in another analysis (51).

Second, high cleavage was observed when hypotonic conditions were used for preparation of nuclei and extract (Figs 4 and 5 and Table 1), indicating that salt-induced removal of factors had not occurred. Indeed, as described above, reduced cleavage occurred with hypertonic treatment of extract, but this can be attributed to salt-induced nucleosome sliding over the open region.

Third, the high level of digestion could be accomplished within minutes for *Sph*I (Fig. 3) as well as *Msp*I and *Kpn*I (data not shown). This digestion time is typical of methods used to detect factor occupancy that employ non-specific footprinting agents such as DNase I and dimethyl sulfate (51–53). Complete digestion with such short digestion times makes the possibility of the putative bound factor cycling off its binding site during digestion unlikely.

Fourth, different enzymes that cleave at the same site each gave equally high levels of digestion, indicating that the cleavage level at a site reflects VTC structure and not the particular preferences of the enzyme used (see 54 for a discussion of this point). Enzymes analyzed were *Acc*65I and *Kpn*I for the site at nt 294 (Figs 4 and 5 and Table 1), *Hpa*II and *Msp*I for the site at nt 346 (Figs 2, 4 and 5) and *Sph*I and *Nsi*I for the site at nt 128 (data not shown).

Fifth, protein complexes that form at each of the sites are typically very stable, as indicated by band shift and footprint data for proteins which bind at the *Sph*I site [TEF-1 (26), OBP (55), 'band A' factors (56)], the *Bgl*I site [T-antigen (5,57)] and the *Msp*I site [DAP (28), positioned nucleosome (41)]. Thus, if these proteins were bound to their respective sites in the VTC *in vivo*, the demonstrated *in vitro* stability of each indicates that they would remain bound under the comparatively gentle conditions of isolation and analysis used here.

Sixth, while the VTC origin sites are quantitatively cleaved, these sites are partially blocked in the bulk population, even after extended digestion times (Figs 2 and 4–7). This provides evidence for the presence of another protein structure, the nucleosome, on an alternative functional form of the same DNA sequence and constitutes a general positive control that bound protein will block digestion at each of the sites.

Seventh, this approach demonstrated factor occupancy at nt 270 under comparable conditions (8).

The procedure described here determines the occupancy level of several *trans*-acting factor binding sites in a complex that is in a specific stage of transcription, elongation. As such it provides insight into the mechanism of factor function that goes beyond that which is readily available from current methods. Thus such transcription stage-specific information is not generally available from mutation-based methods; while this genetic approach has proven to be extremely powerful in the functional identification of *trans*-acting factors, it is less useful for elucidating the mechanism of action of factors. Details of mechanisms are better addressed by an *in vitro* strategy involving reconstitution, but a significant concern in this approach is the uncertainty of whether proper chromatin substrates have been reconstructed. Consequently, the method here that characterizes factor content in an *in vivo*-assembled transcription elongation complex serves as an important complement to the genetic and *in vitro* reconstitution approaches for factor analysis.

A major conclusion of this study is that factors which bind to the *Sph*I, *Bgl*I, *Msp*I and *Kpn*I sites are not directly involved in maintaining the transcription elongation state, because they are not present on the native elongation complex. This indicates that the transcription activation roles identified by the mutational analysis of their respective binding sites must occur at some other stage of the transcription process, such as initiation complex formation. Thus, construction of a functional transcription initiation complex may require the presence of the large number of factors implicated by the genetic studies. However, entry into the elongation state may then require disassembly of this complex, resulting in opening of the sites to restriction enzyme digestion as observed. Following termination, these sites would then be reoccupied in the construction of a new initiation complex. This is reminiscent of the 'hit-and-run' mechanism of transcription activation proposed for the glucocorticoid receptor (51). However, such transient occupancy by factors contrasts with the situation in the 5S rRNA gene, where factors remain bound to sites through multiple rounds of transcription (reviewed in 58). This different mechanism of factor action may in turn represent that of the factor bound to the *Pvu*II site at nt 270 (8). Thus both types of occupancy characteristics appear to be present, as revealed by our study on the elongating transcription complex, and the system described here can serve to classify factors according to mechanism of action.

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