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Barriers to Nuclease Bal31 Digestion Across Specific Sites in Simian Virus 40 Chromatin

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A portion of the nucleoprotein containing viral DNA extracted from cells infected by simian virus (SV40) is preferentially cleaved by endonucleases in a region of the genome encompassing the origin of replication and early and late promoters. To explore this nuclease-sensitive structure, we cleaved SV40 chromatin molecules with restriction enzymes and digested the exposed termini with nuclease Bal31. Digestion proceeded only a short distance in the late direction from the *Msp*I site, but some molecules were degraded 400 to 500 base pairs in the early direction. By comparison, *Bgl*I-cleaved chromatin was digested for only a short distance in the early direction, but some molecules were degraded 400 to 450 base pairs in the late direction. These barriers to Bal31 digestion (bracketing the *Bgl*I and the *Msp*I sites) define the borders of the same open region in SV40 chromatin that is preferentially digested by DNase I and other endonucleases. In a portion of the SV40 chromatin, Bal31 could not digest through the nuclease-sensitive region and reached barriers after digesting only 50 to 100 base pairs from one end or the other. Chromatin molecules that contain barriers in the *Bgl*I to *Msp*I region are physically distinct from molecules that are open in this region as evidenced by partial separation of the two populations on sucrose density gradients.

Viral nucleoprotein complex extracted from cells infected by simian virus 40 (SV40) resembles cellular chromatin (1, 10, 15, 27). A short segment of the viral genome (ca. 400 base pairs [bp] extending from the SV40 origin of replication in the late direction) is hypersensitive to endonuclease cleavage (33, 38–40) and appears, in the electron microscope, as a region which is free of nucleosomes (19, 32). Recently, more detailed studies have shown that, within the nuclease-sensitive site, there are subregions with differential sensitivity to DNase I (7, 8, 31, 32). Presumably, these features reflect the structural details of the nucleoprotein within this region.

Nuclease-hypersensitive sites usually occur over regions which contain genetic signals. Such a chromatin structure over a eucaryotic promoter may be a necessary (but not sufficient) condition for transcription from that promoter (6, 12, 43). In addition, hypersensitive sites have been reported which are probably not related to promoter function (3, 22, 28). SV40-infected cells provide a good opportunity to investigate the details of the nuclease-sensitive chromatin structure, since SV40 chromatin is present in high concentration by comparison with other genes and can easily be separated from bulk cellular chromatin.

We have developed a new approach for probing nucleoprotein structure which should be applicable to the study of chromatin structure in other minichromosomes. SV40 is cleaved with a restriction enzyme which cuts at a unique position, and the exposed termini are digested with Bal31, an exonuclease which can degrade from either 3′ or 5′ ends. The exonuclease digests until it reaches barriers at specific locations in different chromatin molecules and those barriers are mapped by restriction enzyme digestion. Information derived from this type of analysis should help identify the nucleoprotein structures which keep hypersensitive sites distinct from other regions of chromatin.

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MATERIALS AND METHODS

Growth of cells and virus infections. African green monkey kidney cells (BSC-1) were grown in minimal essential medium (Earle salts; MA Bioproducts) supplemented with 8% newborn calf serum (Biocell) and 2% fetal calf serum (KC Biologicals or Flow Laboratories, Inc.). Wild-type SV40 (strain 776) was used to infect cells at a multiplicity of 3, and incubation was continued in the same medium supplemented with 2% fetal calf serum but no newborn calf serum.

Labeling and isolation of SV40 chromatin. ^{3}H-labeled chromatin was prepared by adding [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear Corp.) to infected BSC-1 cells from 30 to 42 h after infection. ^{32}P-labeled chromatin was prepared by adding [^{32}P]phosphate (ICN) in the medium described above prepared without unlabeled phosphate from 24 to 42 h after infection. Nucleoprotein complexes were extracted 42 h after infection as described by Fernandez-Munoz et al. (13) and isolated by centrifugation into a sucrose density gradient (17). Since the ^{3}H label was introduced only to aid in the chromatin isolation, ^{3}H-labeled chromatin is referred to as "unlabeled" in the text.

Nuclease digestion and analysis of digest products. Isolated chromatin was dialyzed into TENT buffer (10 mM Tris-hydrochloride [pH 7.4], 0.5 mM Na_2EDTA, 50 mM NaCl, 0.17% Triton X-100). For all restriction enzyme digests of chromatin, the following conditions were used (final concentrations): 19 mM Tris-hydrochloride (pH 7.4), 44 mM NaCl, 37 mM KCl, 12 mM $MgCl_2$, 0.44 mM Na_2EDTA, 0.15% Triton X-100, 1.0 mM dithiothreitol, and 200 μg of bovine serum albumin per ml. Incubations were carried out at 37°C. Then, NaCl and $CaCl_2$ were added to final concentrations of 250 and 10 mM, respectively, and the mixture was equilibrated at 30°C. Bal31 was added (2.8 U/ml), and the incubation was continued at 30°C. After various incubation times, enzyme digestion was stopped by adding sodium dodecyl sulfate (to 1%) and Na_2EDTA (to 20 mM), and the mixture was fractionated by electrophoresis in 1.4% agarose.

Alternatively, the digest mixture was treated with RNase

A (10 μg/ml [Worthington Diagnostics] heated 20 min at 80°C and stored at −20°C) and incubated 30 min at 37°C. The mixture was then phenol extracted twice, reextracted with chloroform twice, and precipitated with ethanol. The resulting pellet was dissolved in 15 mM NaCl–1.5 mM sodium citrate and incubated with various restriction enzymes (New England Biolabs), using conditions suggested by the supplier.

Digest products were fractionated by electrophoresis in 1.4% agarose (4) or in 4% polyacrylamide (11). DNA fragments were detected by exposing the dried gel to Kodak XRP-1 X-ray film.

RESULTS

Bal31 digestion of SV40 chromatin cleaved by restriction enzymes. SV40 chromatin which had been isolated from a sucrose density gradient was cleaved rapidly by *Bgl*I (ca. 40% was cleaved in the first 4 min, increasing to 50 to 60% by 10 min and remaining constant thereafter). A similar time course was seen for digestion with *Msp*I, although a larger fraction of the chromatin remained resistant (ca. 40% was cleaved in the experiment whose results are shown in Fig. 1a). By contrast, *Bam*HI cleaved the chromatin more slowly; ca. 40% was cleaved after 40 min of incubation (Fig. 1e). In other experiments, cleavage occurred even more slowly. These results are in general agreement with those previously reported (35, 39).

Linear molecules produced in these reactions were shortened upon incubation with Bal31 (Fig. 1a, c, e), generating a nonrandom collection of digest products which were characteristic of the restriction enzyme used for the initial cleavage. Incubation of chromatin with Bal31 without prior cleavage by a restriction enzyme gave no digestion (no increase in the levels of form II or form III DNA [data not shown]). When chromatin which had been cut by one of these restriction enzymes was incubated in the absence of Bal31 but in the presence of the other reaction mixture components, the linear molecules were not shortened.

DNA isolated after restriction enzyme cleavage, followed by Bal31 digestion for various time intervals, was redigested with appropriate restriction enzymes (Fig. 1b, d, and f) to map the sites where Bal31 stopped. Results are summarized in Table 1, and Fig. 2 shows a map of SV40. Bal31 digested preferentially in the early direction from the *Msp*I site (Fig. 1b, Table 1), and it stopped abruptly after digesting only a short distance in the late direction. Chromatin was divided into two subpopulations with respect to the extent of Bal31 digestion in the early direction. In one population, the exonuclease traversed the nuclease-sensitive region (stopping after 380 to 500 bp); in the second population, Bal31 stopped after digesting only 60 to 150 bp in the early direction.

The analogous situation was observed with Bal31 digestion from the *Bgl*I site (Fig. 1d, Table 1). The exonuclease digested only a short distance in the early direction but digested different molecules to different extents in the late direction, with the most extensively degraded molecules being shortened ca. 350 to 500 bp. Chromatin molecules which had been cleaved by *Bam*HI were shortened to the same extent in either direction from the restriction site. Most molecules were digested only about 40 to 150 bp from either end; however, after longer incubation, additional bands appeared (Fig. 1e and f) which corresponded to digestion ca. 250 to 400 bp in either direction from the *Bam*HI site. Similar results were seen with chromatin which was cleaved by *Bcl*I.

Bal31 digestion of restriction enzyme-cleaved SV40 DNA.

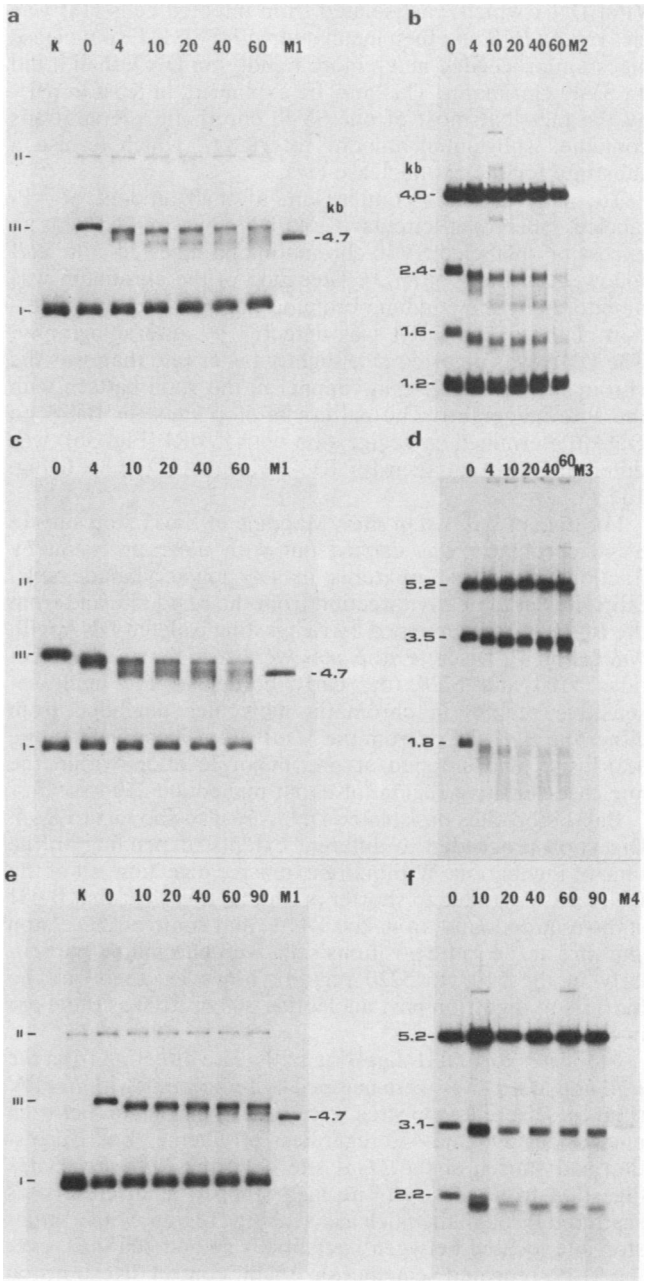

FIG. 1. Digestion of SV40 chromatin with Bal31 after incubation with single-cut restriction enzymes. ^{32}P-labeled SV40 chromatin was digested with *Msp*I (a and b) or *Bgl*I (c and d) for 10 min at 37°C or with *Bam*HI (e and f) for 40 min at 37°C. Then, incubation conditions were adjusted as described in the text, and the samples were incubated with Bal31 for the times indicated (in minutes) at the top of each channel. Samples in (a), (c), and (e) were fractionated by electrophoresis in 1.4% agarose without further treatment. An additional sample of each of the samples shown in (a) was treated with RNase; the DNA was isolated and digested to completion with *Pst*I (b). In a like manner, samples shown in (c) were digested with *Eco*RI (d), and samples shown in (e) were redigested with *Msp*I (f). Lanes labeled K contain samples of the chromatin preparation before digestion with restriction enzymes. Markers are ^{32}P-labeled SV40 DNA digested with *Bgl*I + *Taq*I (M1), *Msp*I + *Pst*I (M2), *Bgl*I + *Eco*RI (M3), or *Bam*HI + *Msp*I (M4). I, II, and III designate supercoiled circular, relaxed circular, and full-length linear DNA, respectively.

Viral DNA which was isolated from infected cells (18) was cleaved by *Bgl*I and then incubated with Bal31. Exonuclease digestion proceeded much more rapidly on DNA than it did on SV40 chromatin. This may be explained, at least in part, by the fact that most of our SV40 chromatin preparations contained substantial amounts of rRNA, which is also a substrate for the exonuclease (14).

To make a better comparison, a small amount of ^{32}P-labeled supercoiled circular SV40 DNA was mixed with an excess of unlabeled SV40 chromatin and digested with *Bgl*I followed by Bal31 (Fig. 3). Digestion of the chromatin was monitored by the ethidium bromide stain pattern, and digestion of the naked DNA was detected by autoradiography. The DNA was digested at a slightly faster rate than was the chromatin (cf. the 15-min channel in the stain pattern with the autoradiogram). The pattern of stop sites for Bal31 on DNA (determined by redigestion with *Eco*RI [Fig. 3b]) was different from that seen for SV40 chromatin (cf. with Fig. 1d).

Location of Bal31 stop sites. Mapping of Bal31 stop sites in SV40 chromatin was carried out with more precision by fractionating digest mixtures in 4% polyacrylamide gels. Stop sites in the early direction from the *Msp*I site and from the *Bgl*I site were mapped by redigesting isolated DNA with *Nde*I (Fig. 4). Discrete stop sites were seen between nucleotides 5100 and 5220 (the early border of the nuclease-sensitive region) in chromatin molecules degraded from either the *Bgl*I site or from the *Msp*I site. Digestion from the *Msp*I site also stopped at one major location within the nuclease-sensitive region (at about nucleotide 250 to 300).

Bal31 stop sites on isolated DNA are also shown in Fig. 4. Digestion proceeded to different extents, depending on the time of incubation. With more extensive digestion, all of the DNA was degraded to shorter products. It is clear that Bal31 stops nonrandomly on naked DNA, and some of these stop sites are at the same positions seen with chromatin (particularly in the 5100 to 5220 region); however, there are no barriers to digestion past nucleotide 300 or 5100 as there are in SV40 chromatin.

Stop sites for Bal31 digestion in the late direction from the *Bgl*I and *Msp*I sites were mapped by redigesting with *Eco*RV (Fig. 5). Discrete stop sites were seen in chromatin between nucleotides 390 and 450 regardless of whether Bal31 digestion had started at the *Msp*I site or at the *Bgl*I site. Bal31 digestion from the *Bgl*I site also stopped at discrete sites scattered through the nuclease-sensitive region, with a major stop site located between nucleotides 50 and 100 (i.e., over the 21-bp repeated sequences). Again, control digestions on isolated DNA show nonrandom stop sites for Bal31, some of which appear to be at the same locations where Bal31 stops in chromatin digestions.

TABLE 1. Barriers to Bal31 digestion of SV40 chromatin from various restriction sites[a]

Restriction enzyme	Stop sites for Bal31 digestion[b]	
	Early side (bp)	Late side (bp)
*Msp*I	60–150; 380–500	40–150
*Bgl*I	50–160	30–130; 350–500
*Bam*HI	40–150; 250–400	40–150; 250–400

[a] SV40 chromatin was cleaved by the indicated restriction enzymes and then incubated for various times with Bal31 as described in the legend to Fig. 1. DNA was isolated and redigested with appropriate restriction enzymes to map the stop sites for Bal31.

[b] Distances in bp from the restriction sites in the direction of early or late transcription were estimated from the data shown in Fig. 1.

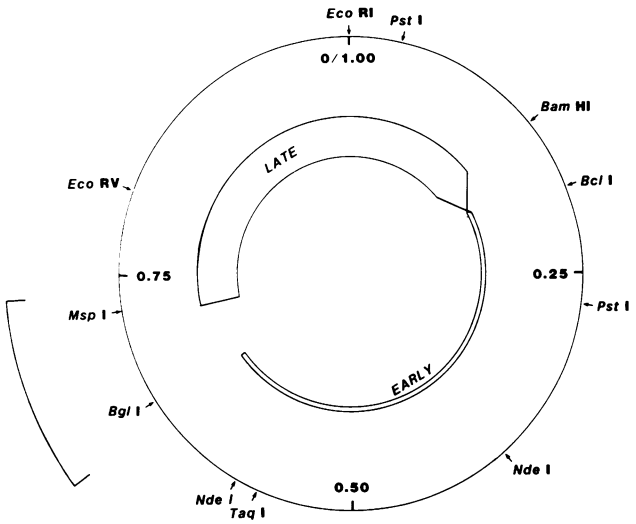

FIG. 2. Map of SV40 genome showing restriction enzyme cleavage sites used in this report. The region preferentially cleaved by DNase I or endogenous nuclease is indicated by a bracket (33, 38–40).

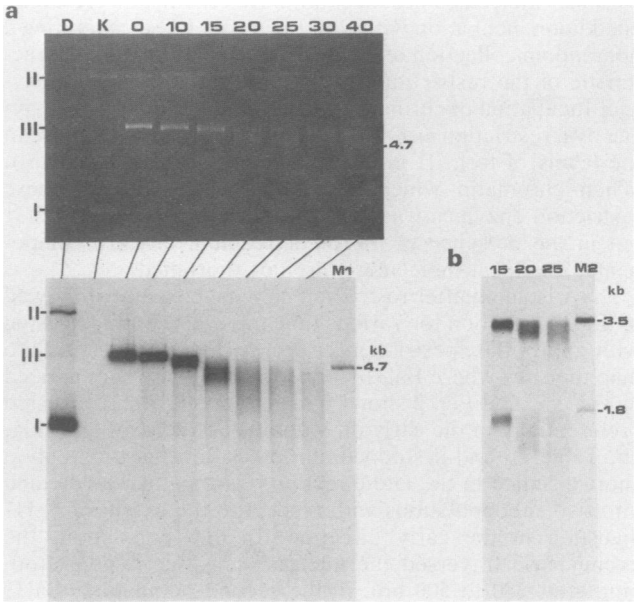

FIG. 3. Digestion of isolated SV40 DNA by Bal31 in the presence of unlabeled SV40 chromatin. ^{32}P-labeled supercoiled circular SV40 DNA (prepared by centrifugation to equilibrium in CsCl-ethidium bromide) was added to unlabeled SV40 chromatin (isolated from a sucrose gradient). The mixture was digested with *Bgl*I for 10 min and then with Bal31 for various times (indicated in minutes at the top of the figure) as described in the legend to Fig. 1. Digest mixtures were fractionated by electrophoresis on 1.4% agarose, and the gel was stained with ethidium bromide, dried, and processed for autoradiography (a). Lane D contains a sample of ^{32}P-labeled DNA alone (equal in amount to that in the mixture in the other lanes). Lane K contains a sample of unlabeled chromatin alone (also equal in amount to that in the mixtures in the other lanes). Samples of three samples shown in (a) were redigested with *Eco*RI (b). Markers are ^{32}P-labeled SV40 DNA digested with *Bgl*I + *Taq*I (M1) or with *Bgl*I + *Eco*RI (M2). I, II, and III are described in the legend to Fig. 1.

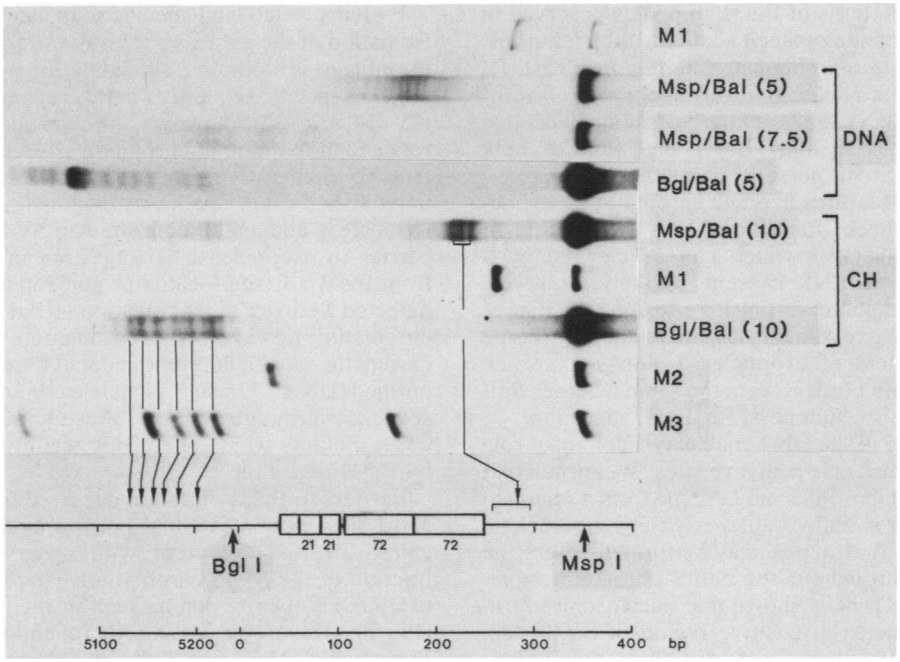

FIG. 4. Stop sites for Bal31 digestion in the early direction from the *Msp*I and *Bgl*I sites. The top of the figure shows ^{32}P-labeled supercoiled circular SV40 DNA (purified by centrifugation to equilibrium in CsCl-ethidium bromide) or ^{32}P-labeled SV40 chromatin (CH) (isolated from a sucrose gradient) digested with either *Msp*I or *Bgl*I for 10 min and then with Bal31 for the times indicated in parentheses (in minutes). The incubation mixtures were treated as described in the text, digested to completion with *Nde*I, and fractionated by electrophoresis in 4% polyacrylamide. Markers are ^{32}P-labeled SV40 DNA digested with *Nde*I + *Msp*I (M1), *Nde*I + *Bgl*I (M2), or *Hae*III (M3). The bottom depicts a segment of the SV40 map on which the major stop sites for Bal31 digestion of SV40 chromatin in the early direction from the *Msp*I and *Bgl*I sites are shown. The 21- and 72-bp repeated segments of the SV40 genome are shown by boxes. The SV40 sequence is numbered according to the Buchman-Burnett-Berg system (5).

Bal31 stop sites in different chromatin subpopulations. Chromatin fractions recovered from the leading and trailing edges of the nucleoprotein peak in a sucrose gradient were subjected to cleavage by *Msp*I followed by Bal31 digestion (Fig. 6). In a fraction from the trailing edge of the peak (fraction 10, Fig. 6a), most of the chromatin molecules were rapidly digested across the nuclease-sensitive region. (Locations of the stop sites in these experiments were confirmed by redigesting with restriction enzymes.) By contrast, in a fraction from the more rapidly sedimenting material (fraction 7, Fig. 6b), Bal31 paused after digestion only a short distance in either direction from the *Msp*I site. Similar results have been obtained with *Bgl*I-digested chromatin (data not shown). After 90 min of incubation in this experiment, Bal31 was able to pass the barriers to its digestion.

The difference in the patterns of Bal31 stop sites between gradient fractions was not due to the fractionation of materials (e.g., ribosomes) which alter the activity of the exonuclease. Bal31 digestion of *Msp*I-cleaved fraction 7 chromatin gave the same result even in the presence of an excess of unlabeled fraction 10 chromatin (Fig. 6c), and *Msp*I-cleaved fraction 10 chromatin gave the same pattern after Bal31 digestion even in the presence of an excess of unlabeled fraction 7 (Fig. 6d).

Chromatin from fractions 7 and 10 was also digested with DNase I, and the frequency of cleavage within the nuclease-sensitive region was determined as described previously (33, 44). DNase I cleaved preferentially within the 400-bp segment of the late side of the origin of replication in both chromatin subfractions; however, the preference was substantially greater in the more slowly sedimenting chromatin (ca. 40% of the molecules in fraction 10 were cleaved in this region as compared with ca. 20% for fraction 7).

DISCUSSION

Major barriers to Bal31 digestion appear to define the outer borders of the region of SV40 chromatin previously shown to be preferentially cleaved by endonucleases (7, 8, 31–33, 38–40). These borders appear as clusters of Bal31 stop sites located between nucleotides 390 and 450 on the late side (Fig. 5) and between nucleotides 5100 and 5220 on the early side (Fig. 4). These results are in good agreement with the region of DNase I sensitivity (mapped to extend between nucleotides 5212 and 360 [7, 31]) if we suppose that Bal31 can digest for a short distance into each border of the nuclease-sensitive region into sequences which are not accessible to DNase I.

We cannot distinguish whether any given band on a gel was determined by the preference for the exonuclease to stop at that DNA sequence (see the control digestions in Fig. 4 and 5), by some feature of the nucleoprotein structure at these barriers, or more likely, by a combination of both sequence specificity and nucleoprotein configuration. What is clear from these experiments is that Bal31 pauses at positions in the chromatin which it would have passed much more readily had it been digesting naked DNA. Some aspect of the chromatin structure in the neighborhood of these stop sites (if not at the actual location on the sequence) must account for these observations.

It is interesting to speculate that the nuclease-sensitive region may be created by nucleosomes positioned precisely

at each border. The patterns of Bal31 stop sites observed in Fig. 4 and 5 (clusters of sites spaced at about 10-bp intervals) are reminiscent of patterns obtained when exonuclease III was used to digest bulk nucleosomes from chicken erythrocyte chromatin (29, 30). A similar pattern of subnucleosomal fragments has been seen after Bal31 digestion of bulk nucleosomes (21; A. Stein, personal communication).

Mapping structural features in SV40 chromatin by exonuclease digestion is subject to artifacts due to selection of subpopulations of molecules which can be cleaved by a given restriction enzyme (24). It seems unlikely, however, that the borders of the nuclease-sensitive region which have been detected in these experiments are the result of this type of artifact. The clusters of exonuclease stop sites which define the early and late borders were the same whether *BglI* or *MspI* provided the starting point for Bal31 digestion.

Secondary structure of the DNA may play a dominant role in the appearance of nuclease-sensitive sites. Weintraub (42) has suggested that nucleosomes may escape from a region in which their formation is unfavorable, releasing superhelical strain. An altered DNA structure may be formed before the strain is dissipated throughout the entire chromatin molecule. Wasylyk et al. (41) have shown that nucleosomes form less readily in the nuclease-sensitive region of SV40 than they do elsewhere. If superhelical tension is required to produce the nuclease-sensitive region in SV40 chromatin, it is necessary to explain why the borders of the nuclease-sensitive region can be identified by Bal31 digestion of molecules which have been cleaved by restriction enzymes. It is possible that an altered DNA structure is formed as a result of supercoil tension and prevents nucleosomes from entering this region. Once the tension is relieved, nucleosomes may only migrate in very slowly.

Proteins which bind specific sequences may be involved in formation of the nuclease-sensitive site. SV40 tumor antigen (T antigen) is a possible candidate for such a protein since it binds to specific sequences in this region of the viral genome (25, 34, 36, 37); however, the nuclease-sensitive site is retained in *tsA* mutant chromatin after incubation at nonpermissive temperature (31; J. P. Hartmann and W. A. Scott, unpublished data). Shalloway et al. (34) used exonuclease III to locate T-antigen-binding sites on SV40 DNA and found a barrier to exonuclease III digestion in the early direction from the *MspI* site located at nucleotide 118. We have not detected a barrier at that location to Bal31 digestion of SV40 chromatin; however, the association of T antigen with chromatin may be different in detail than its association with purified DNA. Further evidence about the role of DNA sequence in the definition of these borders will be obtained when mutants which affect this region have been analyzed by Bal31 digestion.

Barriers to Bal31 digestion occurred between the *BglI* and *MspI* sites in a substantial portion of the chromatin molecules. This is consistent with observations that only a fraction of the SV40 chromatin isolated from infected cells exhibits an open region as seen in the electron microscope (19, 20, 32) or by sensitivity to endonucleases (32, 33). Presumably, *MspI* and *BglI* are able to cleave some molecules which do not contain an open region, but Bal31 cannot digest across this region in those molecules. By sucrose gradient sedimentation, we were able to obtain partial separation between molecules that are open and molecules that contain barriers to exonuclease in this region. We have not correlated these populations with any known subpopulations of SV40 chromatin. Chromatin species sedimenting in this region of the gradient include transcription and replication

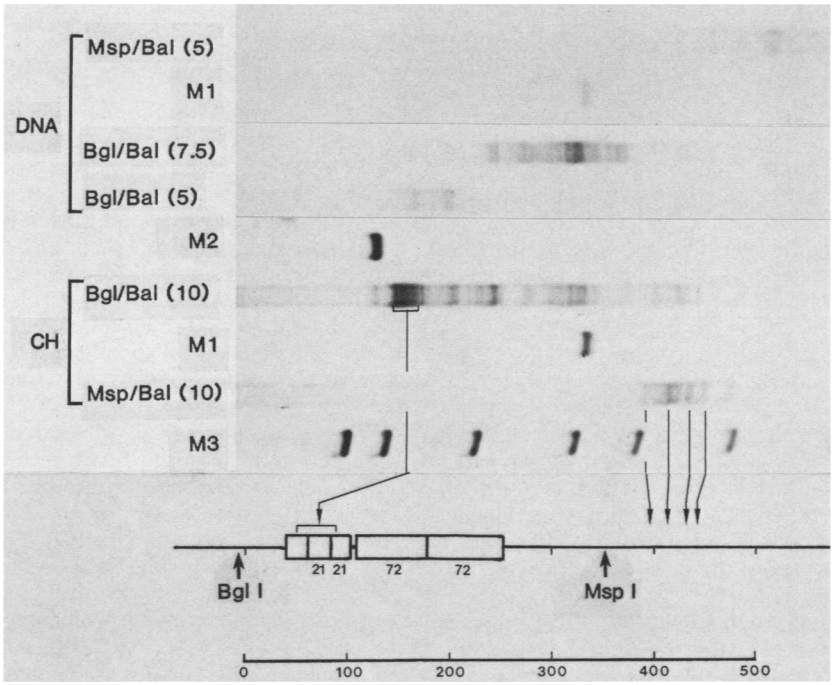

FIG. 5. Stop sites for Bal31 digestion in the late direction from the *MspI* and *BglI* sites. Portions of the Bal31 digest mixtures described in the legend to Fig. 4 were redigested with *Eco*RV and fractionated by electrophoresis in 4% polyacrylamide. Markers are ^{32}P-labeled SV40 DNA digested wtih *Eco*RV + *MspI* (M1), *Eco*RV + *BglI* (M2), or *Bst*NI (M3). The major stop sites for Bal31 digestion of SV40 chromatin in the late direction from the *MspI* and *BglI* sites are shown on the map at the bottom.

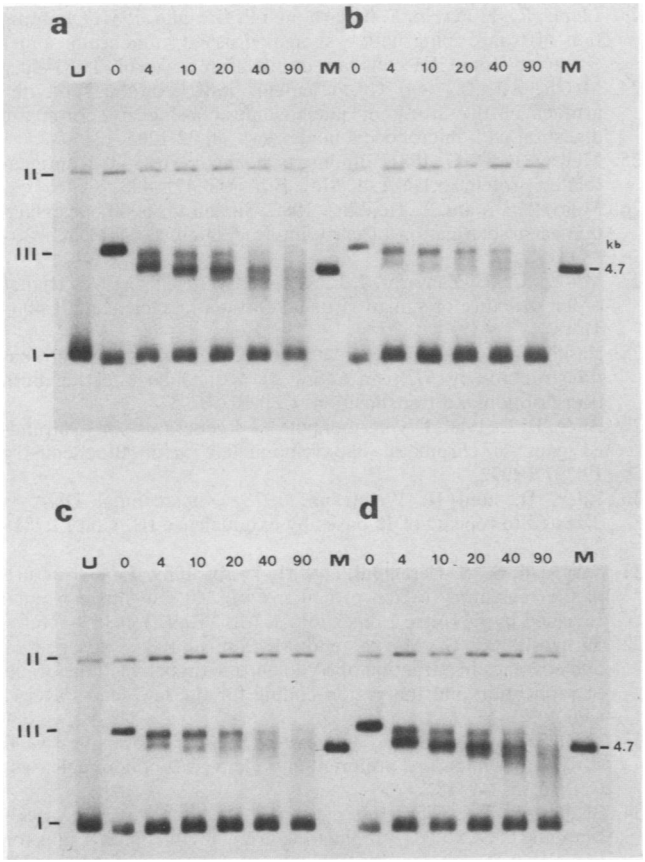

FIG. 6. Bal31 digestion of SV40 chromatin subfractions with different sedimentation properties after cleavage by *Msp*I. ^{32}P-labeled SV40 chromatin was fractionated by centrifugation into a 5 to 20% sucrose gradient, and the location of nucleoproteins containing SV40 DNA was determined by electrophoresis of a deproteinized sample from each gradient fraction followed by ethidium bromide staining. The peak of SV40 chromatin was in fractions 8 and 9 (out of 22 total fractions). Fraction 10, which sedimented less rapidly than the peak, was mixed with an eightfold excess of the same fraction from a parallel gradient run on unlabeled chromatin. (a) shows the mixture incubated with *Msp*I, digested with Bal31 for the times indicated (in minutes), and fractionated by electrophoresis as described in the legend to Fig. 1a. (b) shows fraction 7 from the gradient containing ^{32}P-labeled chromatin mixed with an eightfold excess of fraction 7 from the gradient containing unlabeled chromatin and digested with *Msp*I and Bal31 as described above. (c) shows the same experiment performed on a mixture of ^{32}P-labeled fraction 7 and unlabeled fraction 10; (d) was performed on a mixture of ^{32}P-labeled fraction 10 and unlabeled fraction 7. I, II, and III are described in the legend to Fig. 1. U, Sample of chromatin before digestion with *Msp*I or Bal31. M, Size standard generated by digestion of ^{32}P-labeled SV40 DNA with *Bgl*I + *Taq*I.

intermediates (which are only present as small components of the nucleoprotein population [9, 23, 35]) and certain early assembly intermediates (26).

The major barriers to Bal31 within the nuclease-sensitive region lie at each border of the 72-bp repeated sequences. It would be of particular interest to find a nucleoprotein structure in which Bal31 is prevented from crossing into the 72-bp repeated sequences, since this might lead to the isolation of a nucleoprotein complex involved in the function of that region as a transcriptional enhancer (2, 16). To determine whether these barriers occur on the same nucleoprotein molecules, we incubated SV40 chromatin with a mixture of *Bgl*I and *Msp*I and analyzed the products after Bal31 digestion. Twenty percent of the chromatin was cleaved by both enzymes; however, the 354-bp fragment generated by double digestion was quickly reduced to fragments too small to detect when incubated with Bal31 (data not shown), suggesting that the barriers were not preserved in the double-digest nucleoprotein product. One possible explanation for this result is that molecules which contain barriers to Bal31 at the borders of the 72-bp repeated region cannot be cut by both restriction enzymes. This might be the case if, for example, these molecules contained nucleosomes in this region spaced at 200-bp intervals. If the barrier to Bal31 digestion from the *Msp*I site at nucleotides 250 to 300 were defined by a nucleosome, an adjacent nucleosome on the early side would obscure the *Bgl*I site. Likewise, if Bal31 digestion in the late direction from the *Bgl*I site reached a nucleosome at nucleotides 50 to 100, an adjacent nucleosome on the late side would block the *Msp*I site. The only molecules cut by both restriction enzymes would be those which had a region free of nucleosomes. Consequently, the double-digest product would be degraded by Bal31. Molecules which have two additional nucleosomes might sediment more rapidly than those which lack nucleosomes in this region, explaining the results in Fig. 6. Since only those molecules which are cleaved by a restriction enzyme are degraded by Bal31, there would be a strong selection for a particular nucleosome distribution in the situation described above. Consequently, it is not necessary to postulate strict phasing of nucleosomes to obtain the observed results.

At face value, the results in Fig. 1e and f suggest that a subset of SV40 chromatin molecules has nucleosomes which are phased with respect to the *Bam*HI site, and another subset has nucleosomes which are phased with respect to the *Bcl*I site. The existence of such phasing is not proven by this experiment since it is also possible that those molecules which are cleaved by *Bam*HI (or by *Bcl*I) represent a selected subpopulation in which the *Bam*HI site lies in a restricted portion of the internucleosomal DNA (24). If this were the case, nucleosomes would lie, on the average, a certain distance on either side of the restriction site in those molecules which have been cut by that restriction enzyme, even if the nucleosomes were randomly distributed in the total chromatin population. The next nucleosome would be ca. 200 bp farther away on each side, giving the impression of nucleosome phasing.

The demonstration of barriers to Bal31 degradation from more than one restriction site provides evidence for structural discontinuities at discrete locations in the SV40 genome. We can use these features to identify chromatin subpopulations in our attempts to relate structural heterogeneity to specific chromatin functions.

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LITERATURE CITED

1. **Bellard, M., P. Oudet, J.-E. Germond, and P. Chambon.** 1976. Subunit structure of simian-virus 40 minichromosome. Eur. J. Biochem. **70**:543–553.
2. **Benoist, C., and P. Chambon.** 1981. In vivo sequence require-

ments of the SV40 early promoter region. Nature (London) **290**:304–310.
3. **Bloom, K. S., and J. Carbon.** 1982. Yeast centromere DNA is in a unique and highly ordered structure in chromosomes and small circular minichromosomes. Cell **29**:305–317.
4. **Brockman, W. W., and D. Nathans.** 1974. The isolation of simian virus 40 variants with specifically altered genomes. Proc. Natl. Acad. Sci. U.S.A. **71**:942–946.
5. **Buchman, A. R., L. Burnett, and P. Berg.** 1980. The SV40 nucleotide sequence, p. 799–829. *In* J. Tooze (ed.), DNA tumor viruses: molecular biology of tumor viruses, (2nd ed.), part 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Cartwright, I. L., S. M. Abmayr, G. Fleischmann, K. Lowenhaupt, S. C. R. Elgin, M. A. Keene, and G. C. Howard.** 1982. Chromatin structure and gene activity: the role of nonhistone chromosomal proteins. CRC Crit. Rev. Biochem. **13**:1–86.
7. **Cereghini, S., P. Herbomel, J. Jouanneau, S. Saragosti, M. Katinka, B. Bourachot, B. DeCrombrugghe, and M. Yaniv.** 1983. Structure and function of the promoter-enhancer region of polyoma and SV40. Cold Spring Harbor Symp. Quant. Biol. **47**:935–944.
8. **Crémisi, C.** 1981. The appearance of DNase I hypersensitive sites at the 5′ end of the late SV40 genes is correlated with the transcriptional switch. Nucleic Acids Res. **9**:5949–5964.
9. **Crémisi, C., A. Chestier, and M. Yaniv.** 1977. Preferential association of newly synthesized histones with replicating SV40 DNA. Cell **12**:947–951.
10. **Crémisi, C., P. F. Pignatti, and M. Yaniv.** 1976. Random location and absence of movement of the nucleosomes on SV40 nucleoprotein complex isolated from infected cells. Biochem. Biophys. Res. Commun. **73**:548–554.
11. **Danna, K. J., G. H. Sack, and D. Nathans.** 1973. Studies of SV40 DNA. VII. A cleavage map of the SV40 genome. J. Mol. Biol. **78**:363–376.
12. **Elgin, S. C. R.** 1981. DNAase I-hypersensitive sites of chromatin. Cell **27**:413–415.
13. **Fernandez-Munoz, R., M. Coca-Prados, and M.-T. Hsu.** 1979. Intracellular forms of simian virus 40 nucleoprotein complexes. I. Methods of isolation and characterization in CV-1 cells. J. Virol. **29**:612–623.
14. **Gray, H. B., T. P. Winston, J. L. Hodnett, R. J. Legerski, D. W. Nees, C.-F. Wei, and D. L. Robberson.** 1981. The extracellular nuclease from *Alteromonas espejiana*: an enzyme highly specific for non-duplex structure in nominally duplex DNAs, p. 169–203. *In* J. G. Chirikjain and T. S. Papas (ed.). Gene analysis and amplification, vol 2. Elsevier/North-Holland Publishing Co., New York.
15. **Griffith, J. D.** 1975. Chromatin structure: deduced from a minichromosome. Science **187**:1202–1203.
16. **Gruss, P., R. Dhar, and G. Khoury.** 1981. Simian virus 40 tandem repeated sequences as an element of the early promoter. Proc. Natl. Acad. Sci. U.S.A. **78**:943–947.
17. **Hartmann, J. P., and W. A. Scott.** 1983. Nuclease-sensitive sites in the two major intracellular simian virus 40 nucleoproteins. J. Virol. **46**:1034–1038.
18. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
19. **Jakobovits, E. B., S. Bratosin, and Y. Aloni.** 1980. A nucleosome-free region in SV40 minichromosomes. Nature (London) **285**:263–265.
20. **Jakobovits, E. B., S. Bratosin, and Y. Aloni.** 1982. Formation of a nucleosome-free region in SV40 minichromosomes is dependent upon a restricted segment of DNA. Virology **120**:340–348.
21. **Künzler, P., and A. Stein.** 1983. Histone H5 can increase the internucleosome spacing in dinucleosomes to nativelike values. Biochemistry **22**:1783–1789.
22. **Livingston, D. M.** 1982. A sequence of the yeast 2 μm DNA plasmid chromosome near the origin of replication is exposed to restriction endonuclease digestion. J. Mol. Biol. **160**:397–410.
23. **Llopis, R., F. Perrin, F. Bellard, and P. Gariglio.** 1981. Quantitation of transcribing native simian virus 40 minichromosomes extracted from CV1 cells late in infection. J. Virol. **38**:82–90.
24. **McGhee, J. D., and G. Felsenfeld.** 1983. Another potential artifact in the study of nucleosome phasing by chromatin digestion with micrococcal nuclease. Cell **32**:1205–1215.
25. **McKay, R. D. G.** 1981. Binding of a simian virus 40 T antigen-related protein to DNA. J. Mol. Biol. **145**:471–488.
26. **Milavetz, B., and T. Hopkins.** 1982. Simian virus 40 encapsidation: characterization of early intermediates. J. Virol. **43**:830–839.
27. **Müller, U., H. Zentgraf, I. Eicken, and W. Keller.** 1978. Higher order structure of simian virus 40 chromatin. Science **201**:406–415.
28. **Nasmyth, K.** 1982. The regulation of yeast mating-type chromatin structure by *SIR*: an action at a distance affecting both transcription and transposition. Cell **30**:567–578.
29. **Riley, D. E.** 1980. Deoxyribonuclease I generates single-stranded gaps in chromatin deoxyribonucleic acid. Biochemistry **19**:2977–2992.
30. **Riley, D., and H. Weintraub.** 1978. Nucleosomal DNA is digested to repeats of 10 bases by exonuclease III. Cell **13**:281–293.
31. **Saragosti, S., S. Cereghini, and M. Yaniv.** 1982. Fine structure of the regulatory region of simian virus 40 minichromosomes revealed by DNAase I digestion. J. Mol. Biol. **160**:133–146.
32. **Saragosti, S., G. Moyne, and M. Yaniv.** 1980. Absence of nucleosomes in a fraction of SV40 chromatin between the origin of replication and the region coding for the late leader RNA. Cell **20**:65–73.
33. **Scott, W. A., and D. J. Wigmore.** 1978. Sites in simian virus 40 chromatin which are preferentially cleaved by endonucleases. Cell **15**:1511–1518.
34. **Shalloway, D., T. Kleinberger, and D. M. Livingston.** 1980. Mapping of SV40 DNA replication origin region binding sites for the SV40 T antigen by protection against exonuclease III digestion. Cell **20**:411–422.
35. **Tack, L. C., P. M. Wassarman, and M. L. DePamphilis.** 1981. Chromatin assembly. Relationship of chromatin structure to DNA sequence during simian virus 40 replication. J. Biol. Chem. **256**:8821–8828.
36. **Tegtmeyer, P., B. Anderson, S. B. Shaw, and V. G. Wilson.** 1981. Alternative interactions of the SV40 A protein with DNA. Virology **115**:75–87.
37. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen-related protein. Cell **13**:165–179.
38. **Varshavsky, A. J., O. Sundin, and M. Bohn.** 1979. A stretch of "late" SV40 viral DNA about 400 bp long which includes the origin of replication is specifically exposed in SV40 minichromosomes. Cell **16**:453–466.
39. **Varshavsky, A. J., O. H. Sundin, and M. J. Bohn.** 1978. SV40 viral minichromosome: preferential exposure of the origin of replication as probed by restriction endonucleases. Nucleic Acids Res. **5**:3469–3477.
40. **Waldeck, W., B. Föhring, K. Chowdhury, P. Gruss, and G. Sauer.** 1978. Origin of DNA replication in papovavirus chromatin is recognized by endogenous endonuclease. Proc. Natl. Acad. Sci. U.S.A. **75**:5964–5968.
41. **Wasylyk, B., P. Oudet, and P. Chambon.** 1979. Preferential in vitro assembly of nucleosome cores on some AT-rich regions of SV40 DNA. Nucleic Acids Res. **7**:705–713.
42. **Weintraub, H.** 1983. A dominant role for DNA secondary structure in forming hypersensitive structures in chromatin. Cell **32**:1191–1203.
43. **Weisbrod, S.** 1982. Active chromatin. Nature (London) **297**:289–295.
44. **Wigmore, D. J., R. W. Eaton, and W. A. Scott.** 1980. Endonuclease-sensitive regions in SV40 chromatin from cells infected with duplicated mutants. Virology **104**:462–473.