Structural Changes in Simian Virus 40 Chromatin as Probed by Restriction Endonucleases

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The structure of simian virus 40 (SV40) chromatin was probed by treatment with single- and multiple-site bacterial restriction endonucleases. Approximately the same fraction of the chromatin DNA was cleaved by each of three different single-site endonucleases, indicating that the nucleosomes do not have unique positions with regard to specific nucleotide sequences within the population of chromatin molecules. However, the extent of digestion was found to be strongly influenced by salt concentration. At 100 mM NaCl-5 mM MgCl₂, only about 20% of the simian virus 40 (SV40) DNA I in chromatin was converted to linear SV40 DNA III. In contrast, at lower concentrations of NaCl (0.05 or 0.01 M), an additional 20 to 30% of the DNA was cleaved. These results suggest that at 100 mM NaCl only the DNA between nucleosomes was accessible to the restriction enzymes, whereas at the lower salt concentrations, DNA within the nucleosome regions became available for cleavage. Surprisingly, when SV40 chromatin was digested with multiple-site restriction enzymes, less than 2% of the DNA was digested to limit digest fragments, whereas only a small fraction (9 to 15%) received two or more cuts. Instead, the principal digest fragment was full-length linear SV40 DNA III. The failure to generate limit digest fragments was not a consequence of reduced enzyme activity in the reaction mixtures or of histone exchange. When the position of the principal cleavage site was mapped after *Hpa*I digestion, it was found that this site was not unique. Nevertheless, all sites were not cleaved with equal probability. An additional finding was that SV40 chromatin containing nicked-circular DNA II produced by random nicking of DNA I was also resistant to digestion by restriction enzymes. These results suggest that the initial cut which causes relaxation of topological constraint in SV40 chromatin DNA imparts resistance to further digestion by restriction enzymes. We propose that this may be accomplished by either "winding" of the internucleosomal DNA into the body of the nucleosome, or as suggested by others, by successive right-hand rotation of nucleosomes.

Polyoma and simian virus 40 (SV40) DNA can be isolated from infected cells and purified virus in association with cellular histones (13, 16, 17, 19, 26, 30, 32-34). In the electron microscope, these nucleoprotein complexes are seen as circular molecules having nucleosomes characteristic of cellular chromatin (11, 21, 58). These molecules have been designated as SV40 mini-chromosomes or, more recently, SV40 chromatin.

To understand the arrangement of nucleosomes in relation to the DNA base sequences, SV40 chromatin has been used as a substrate for

bacterial restriction endonucleases. The assumption inherent in this approach has been that DNA cleavage sites which are covered or are strongly associated with histones will be resistant to the endonucleases. Several groups have studied SV40 chromatin, but the conclusions arrived at by the various workers have not always been in agreement. Polisky and McCarthy (42) found that only 20% of the DNA in SV40 chromatin was protected from nuclease digestion and that the protected regions were randomly distributed. However, the SV40 nucleoprotein complex which they studied was prepared by alkaline degradation of virions, and this treatment has been shown to cause partial removal of nucleosomes and degradation of histones (3, 7, 34, 56). In contrast, Cremisi et al. (11), who studied the more "native" intracellular

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form of SV40 chromatin, found that 73 to 85% of the DNA was resistant to digestion by *EcoRI*. They suggested that the cleavage was occurring only in the internucleosomal DNA regions. Nevertheless, Persico-Dilauro et al. (41) found that 50% of DNA within intracellular SV40 chromatin was cleaved by both staphylococcal nuclease and *EcoRI* nuclease. From their analysis of the *HindIII* digest patterns, they proposed that the nucleosomes are distributed randomly in the population of chromatin, but are not randomly arranged within the SV40 chromatin molecule. Ponder and Crawford (44) on the other hand, proposed that the arrangement of nucleosomes is not completely random within the whole population, but that each nucleosome can occupy one of a limited number of positions.

In this paper, we describe the results of our experiments using intracellular SV40 chromatin purified by hydroxyapatite chromatography (34) as a substrate for bacterial restriction enzymes. The SV40 chromatin purified by this procedure has a ratio of protein to DNA greater than one (22, 34) and has about the same complement of histones as is found in whole virions and in H1-depleted cellular chromatin. In our study, we found that the susceptibility of SV40 chromatin DNA to restriction endonucleases was strongly affected by the exact experimental conditions, particularly the salt concentration, and this finding may provide a partial explanation for the discrepancies in some of the previous studies. An additional significant finding was that relaxation of the topological constraints in SV40 chromatin DNA altered that structure of the SV40 chromatin such that the chromatin DNA became highly resistant to the action of the restriction enzymes.

MATERIALS AND METHODS

Cells and viruses. Plaque-purified SV40 virus (strain RH911) was propagated in the TC7 subline of CV-1 as previously described (22, 34).

Purification of SV40 chromatin. The procedure used to purify SV40 chromatin from Triton lysates of infected cells was essentially that described in detail in a previous publication (34), with the following modifications. SV40 chromatin was eluted from hydroxyapatite columns with 0.28 M phosphate buffer, pH 6.8–0.1% Triton X-100 after washing extensively with 0.22 M phosphate buffer, pH 6.8–0.1% Triton X-100. Columns and buffers were maintained at 8 to 10°C. The SV40 chromatin which eluted from the columns was concentrated and then sedimented at 36,000 rpm in a 5 to 20% (wt/wt) sucrose gradient containing 0.1 M NaCl–0.025% Triton X-100–0.001 M EDTA–0.01 M Tris-hydrochloride (pH 7.9) for 2.5 h at 4°C. Only the leading two-thirds portion of the SV40 chromatin peak was pooled. These fractions were concentrated by

vacuum dialysis and then dialyzed into the appropriate reaction buffer.

The average buoyant density in CsCl gradients of the SV40 chromatin purified by the above procedure and fixed with glutaraldehyde was 1.44 g/cm³. The histone complement of this form of SV40 chromatin is similar to that found in virions in that it contains about equimolar amounts of H2A, H2B, H3, and H4 histones but is devoid of H1 histone (34). Nonhistone proteins could not be detected in the SV40 chromatin even after the proteins were labeled in vitro with ¹²⁵I to a high specific activity (G. L. Liggins and D. A. Goldstein, unpublished data).

Radioactive labeling of SV40 chromatin DNA.

For the routine preparation of SV40 chromatin DNA, the DNA was labeled at low specific activity to assist in the identification and quantitation of the chromatin during purification. [¹⁴C]thymidine, (0.05 to 0.1 μ Ci/ml; New England Nuclear Corp.) was added at 24 h after infection and not removed until the cells were lysed at 72 to 90 h after infection.

For labeling of SV40 chromatin DNA to high specific activity with ³²P, [³²P]orthophosphate (25–50 μ Ci/ml; New England Nuclear) was added in phosphate-free growth medium (Eagle minimum essential medium containing 2.5% dialyzed horse serum) at 24 h, and the radioactivity was removed just before Triton lysis of cells at 72 h after infection. Naked ³²P-labeled SV40 DNA was prepared from purified SV40 chromatin by removal of protein. For this purpose, ³²P-labeled SV40 chromatin was incubated at 40°C in 0.1% sodium dodecyl sulfate (SDS) with 50 μ g of pronase per ml for 1 h. Thereafter, the DNA was sedimented for 2 h at 45,000 rpm in a 5 to 20% sucrose gradient at room temperature in an SW 50.1 rotor (22). ³²P-labeled SV40 DNA prepared in this way had a specific activity of 1×10^5 to 2×10^5 cpm/ μ g of DNA.

Bacterial restriction endonucleases. Restriction endonucleases *R. EcoRI*, *R. HpaI*, *R. HpaII*, *R. BamHI* and *R. HindIII* were purchased from Bethesda Research Laboratories, Bethesda, Md. *R. HindII* + *III* was obtained from Mary Gutai and Michael Chen. The locations of the cleavage sites for the various enzymes used in this study are depicted on the SV40 DNA map in Fig. 1. Enzymes were stored at –20°C. Unless otherwise specified in the text or in the legends to the figures, SV40 DNA or chromatin was incubated with the enzymes at 37°C in the “standard” reaction mixture containing 10 mM Tris-hydrochloride (pH 7.5), 0.025% Triton X-100, 100 mM NaCl, 5 mM MgCl₂ and 2 mM β -mercaptoethanol. All buffers were autoclaved before use, and all reaction were carried out in sterile vials and tubes. Reactions were terminated by SDS (final concentration, 0.1%)–EDTA (0.05 M) and heated at 56°C for 5 min.

Polyacrylamide and agarose gel electrophoresis. For the separation of the DNA fragments generated by digestion of SV40 chromatin with restriction enzymes, the protein was first removed by heating the chromatin at 56°C for 10 min in 0.35% SDS, and the DNA was electrophoresed on vertical agarose or polyacrylamide slab gels. Samples were adjusted to a concentration of 5% glycerol and 0.02% bromophenol blue in a volume of 50 μ l and loaded onto slots of slab gels.

FIG. 1. Restriction enzyme cleavage map of SV40 DNA. The cleavage map positions assigned to the various enzymes are shown relative to the single cleavage site for *EcoRI*. There are 4 cleavage sites for *HpaI* (12, 50, 66), 6 sites for *HindIII* (66), 13 sites for *HindII* + *III* (12, 66), 1 site for *HpaII* (50, 66), and 1 site for *BamHI* (R. Roberts, personal communication).

The agarose gels were 1.4% agarose in 40 mM Tris, 5 mM sodium acetate, and 1 mM EDTA (pH 7.8). The polyacrylamide gel electrophoresis buffer was 40 mM Tris–20 mM sodium acetate–2 mM sodium EDTA (pH 7.8). The dimensions of the gels, the time of the runs, and other conditions varied for each enzyme reaction. These details are given in the text or in the legends to the figures. In all experiments, buffers were circulated between the reservoirs during the runs.

For radioautographic analysis of ^{32}P -labeled DNA digest fragments, slab gels were removed from the glass plates at the conclusion of the electrophoresis, the wet gels were wrapped tightly in Saran Wrap to prevent dehydration, and the gels were placed in contact with medical X-ray film. Films were developed 8 h to 5 days after exposure. For quantitative analysis, the exposed films were scanned with a recording densitometer. For analysis of DNA digests not labeled with ^{32}P , gels were placed in an aqueous solution of ethidium bromide (2 $\mu\text{g}/\text{ml}$) at 4°C for at least 4 h. The wet gels were illuminated with UV light, and the fluorescent DNA bands were photographed with a Polaroid camera.

For removal of DNA fragments from agarose gels, gel slices were dissolved in 2 volumes of 5 M sodium perchlorate at 65°C, and the dissolved agarose was passed through small columns of hydroxyapatite at 65°C. Columns were washed with 10 volumes of 0.01 M sodium phosphate buffer (pH 6.9), and the DNA was eluted with 0.4 M sodium phosphate buffer (pH 6.9). DNA was dialyzed against 0.1 M NaCl–0.01 M Tris (pH 7.9)–0.001 M EDTA, and the DNA was concentrated by ethanol precipitation.

RESULTS

Theoretical considerations. The generally accepted notion that histones do not bind to DNA with base sequence specificity has a theoretical basis in the fact that the histones as an evolutionary class of proteins are very highly conserved. Nonetheless, this does not rule out

the possibility that in vivo there is “phasing” of nucleosomes with respect to a specific DNA base sequence as, for example, the sequence at the origin of replication. If nucleosomes do indeed associate with the DNA at a region such as the origin of replication (20, 49), each subsequent nucleosome could be “in phase” with the corresponding nucleosome in all other SV40 chromatin molecules. The assumptions in this argument are that (i) nucleosomes do not move laterally along the DNA backbone in vivo and (ii) the first nucleosome added to the DNA after initiation of replication establishes the natural spacing of nucleosomes inherent in chromatin.

In a case where the nucleosomes would be in phase, digestion by a restriction endonuclease with a single cleavage site should result in either cleavage of all the molecules (e.g., site not protected by a nucleosome) or complete resistance (site protected by a nucleosome). If the nucleosomes are not in phase within such a population but randomly arranged with respect to a given base sequence, then an intermediate degree of digestion would be predicted. In this case, the resistant fraction would reflect the degree to which the histones protect the DNA. Nevertheless, partial digestion might result from other causes such as reassortment during isolation or digestion or both, or during other perturbations which would allow access to sites in some, but not all molecules. Of course, the above predictions are justified provided no unforeseen interactions take place between the endonuclease and the histones during treatment of the SV40 chromatin. However, if the same fraction of chromatin molecules was resistant to cleavage after digestion by other single-site restriction enzymes, then a strong argument could be made for a nonspecific arrangement of histones. Furthermore, chromatin with such a distribution of histones should yield equimolar amounts of each limit digest DNA fragment after cleavage by multiple-site restriction enzymes. It should be pointed out that others (11, 41–44) have presented similar arguments in previous studies on the arrangement of histones in SV40 and polyoma chromatin.

Digestion of naked SV40 DNA and SV40 chromatin with single-site restriction endonucleases. SV40 chromatin and naked SV40 DNA were incubated separately with *EcoRI* and treated with SDS to stop the reaction and to remove the protein from the DNA, and the reaction products were analyzed by electrophoresis on agarose slab gels (Fig. 2). Bands are observed at three positions; the top, middle, and lower bands correspond to nicked-circular SV40 DNA (DNA II), linear DNA (DNA III), and

supercoiled DNA (DNA I), respectively.

Treatment of naked control supercoiled DNA was characterized by the conversion of DNA I to linear DNA III molecules by way of a short-lived, nicked-circular DNA II intermediate (Fig. 2a). The conversion sequence has been noted by others (37, 47). The intermediate does not result from endonuclease contamination, since prolonged incubation with *EcoRI* did not result in further degradation of the linear form and alkali denaturation of the linear molecule failed to reveal significant amounts of single strands shorter than unit-length SV40 DNA.

In contrast to naked SV40 DNA I, which was entirely converted to DNA III, SV40 DNA I in chromatin was partially resistant to digestion even after prolonged incubation with the enzyme

(Fig. 2b). A large fraction remained as DNA II and was not converted to DNA III. To demonstrate that the partial digestion pattern observed with DNA from chromatin was not due to limiting enzyme or interaction of the histones with enzyme, additional reactions were carried out in the presence of a large excess of *EcoRI*. Even under these reaction conditions, only 23% of the DNA in chromatin was converted to DNA III (Table 1), whereas control naked SV40 DNA I was entirely converted to DNA III within 1 min (data not shown). In addition, the amount of DNA II initially present in SV40 chromatin did not decrease, but actually increased slightly (Table 1).

In view of the finding that treatment with *EcoRI* resulted in only partial cleavage of the SV40 chromatin, the tentative conclusion would be that nucleosomes are not in phase within the population. However, the experiments described above give little information as to the distribution or coverage by nucleosomes within any given SV40 chromatin molecule, since as pointed out above, partial cleavage could be a consequence of a number of factors.

To further investigate this distribution, SV40 chromatin was digested with two other restriction enzymes, *Bam*HI and *Hpa*II, each of which cleaves SV40 chromatin at a different single site. These sites are located at fractional-length map positions 0.14 (61) and 0.735 (50) for *Bam*HI and *Hpa*II, respectively, relative to the single *EcoRI* site, which is arbitrarily assigned the zero position (12).

Separate kinetic experiments were done on the same preparation of ³²P-labeled SV40 chro-

FIG. 2. Kinetics of digestion of SV40 DNA and SV40 chromatin by *EcoRI* at 100 mM NaCl. SV40 DNA (5.5 μ g) and SV40 chromatin (8.3 μ g) were incubated separately in a volume of 120 μ l of the "standard" reaction buffer (see text) to which 2.5 U of *EcoRI* was added. At the times indicated, 10 μ l of reaction mixture was removed and diluted immediately into 30 μ l of a solution containing 10 mM Tris-hydrochloride (pH 7.5), 50 mM EDTA, and 0.1% SDS to stop the reaction, and the samples were analyzed by agarose gel electrophoresis. The digestion products were located by staining the gel with ethidium bromide at a final concentration of 2 μ g/ml. (a) SV40 DNA; (b) SV40 chromatin. The controls represent portions of SV40 DNA and SV40 chromatin removed from the reaction mixture before addition of the enzyme and incubated at 0°C (slot 1) or at 37°C (slot 2) for 60 min.

TABLE 1. *EcoRI* treatment of SV40 chromatin in 100 mM NaCl^a

Time (min)	Distribution of DNA (%) ^b		
	DNA I	DNA II	DNA III
0	91	9	0
1	80	16	4
3	71	14	15
6	68	13	19
15	66	13	21
60	62	15	23

^a Five units of the *EcoRI* were reacted with 380 ng of ³²P-labeled SV40 chromatin. The reaction was in a volume of 60 μ l of the standard buffer (see text). At the times indicated, 10 μ l was removed from the reaction mixture, and the samples were analyzed by agarose gel electrophoresis. Under these conditions, naked SV40 DNA I used as a control was quantitatively converted to SV40 DNA III within 1 min.

^b Values were calculated from densitometer tracings of exposed X-ray films placed in contact with agarose slab gels.

matin by using each of the three enzymes. Before digestion, the SV40 chromatin preparation contained 90% DNA I and 10% DNA II. The results of one such experiment are summarized in Table 2. Only 19 to 22% of the DNA in SV40 chromatin was converted to DNA III by each of the enzymes under the conditions of this reaction (100 mM NaCl, 5 mM MgCl₂, 0.025% Triton X-100, 10 mM Tris, pH 7.5, at 37°C). The salient feature of this experiment is that all three enzymes gave approximately the same degree of double-stranded cleavage, even though the respective sensitive sites are located at considerable distances apart on the genome. Therefore, these results indicate that there is no preferential distribution of nucleosomes within any one region of the genome. Instead, it appears that the nucleosomes are arranged along the DNA independent of nucleotide sequence.

The value of 19 to 22% for the fraction susceptible to double-stranded cleavage agrees well with the findings of Cremisi et al. (11), who digested SV40 chromatin with *Eco*RI and examined the distribution of linear and circular molecules in the electron microscope. These workers interpreted the cleavage as representing digestion in the naked DNA "bridges" or "spacers" extending between nucleosomes, since a similar sensitive region is found upon digestion of cellular chromatin (1, 10, 39, 52, 55, 57).

Effect of salt concentration on endonuclease cleavage of SV40 chromatin. In an electron microscopic study, Griffith (21) found that the SV40 chromatin went from a highly condensed circular structure with a contour length of about 210 nm to an extended beaded form with a contour length of about 514 nm when the salt concentration was lowered from

0.15 to 0.015 M NaCl. On the basis of this observation, it might be predicted that such a configurational change would cause the SV40 chromatin to be more susceptible to endonuclease cleavage.

Figure 3 shows the kinetics of cleavage of SV40 chromatin upon digestion with *Eco*RI (Fig. 3a) or *Bam*HI (Fig. 3b) in the presence of 50 mM NaCl–5 mM MgCl₂. Naked SV40 DNA I is completely converted to DNA III in 1 min (as indicated by the arrow in Fig. 3), whereas the reaction of enzyme with chromatin is not

FIG. 3. Kinetics of SV40 chromatin digestion by *Eco*RI and *Bam*HI at 50 mM NaCl. ³²P-labeled SV40 DNA and ³²P-labeled SV40 chromatin, each containing 55,000 cpm (1.52 × 10⁵ cpm/μg of DNA), were mixed separately with either: (a) 8 U of *Eco*RI or (b) 4 U of *Bam*HI in reaction mixtures containing 50 mM NaCl, 10 mM Tris-hydrochloride (pH 7.5), 5 mM MgCl₂, 0.025% Triton X-100, and 2 mM β-mercaptoethanol. Reaction mixtures were incubated at 37°C and, at the times indicated, samples were withdrawn and the reactions were terminated. SV40 naked DNA I (arrow) was completely converted to DNA III within 1 min under these conditions. Samples were analyzed by electrophoresis in 1.4% agarose gels, and the digestion products were revealed by exposure of the slab gels to X-ray films. The percentages of the various forms of SV40 DNA were calculated from the areas under the peaks obtained from densitometric tracings of the films. Curves are drawn in this figure only for DNA from SV40 chromatin. Symbols: ■, DNA I; △, DNA II; ○, DNA III.

TABLE 2. Comparison of *Eco*RI, *Hpa*II, and *Bam*HI treatment of SV40 chromatin in 100 mM NaCl^a

Restriction endo-nuclease	Distribution of DNA (%) ^b		
	DNA I	DNA II	DNA III
<i>Eco</i> RI	65	16	19
<i>Hpa</i> II	70	14	16
<i>Bam</i> HI	66	12	22

^a ³²P-labeled SV40 chromatin was incubated in separate reaction mixtures with either 4 U of *Eco*RI, 2 U of *Hpa*II, or 2 U of *Bam*HI in the standard reaction buffer. As a control, ³²P-labeled naked SV40 DNA was incubated at the same ratio of DNA to enzyme. Under these conditions, the control DNA was converted quantitatively to DNA III within 3 min.

^b Values were calculated from densitometer tracings of the 20-min reaction products, at which time the reactions were 90% complete.

complete until sometime between 5 and 10 min. It is clear that there is a significant increase in the susceptibility of SV40 chromatin upon lowering the salt concentration. Whereas 19 to 22% of the molecules were converted to DNA III at 100 mM NaCl (Table 2), a decrease in concentration to 50 mM increased yields of DNA III to 47 to 50% (Fig. 3). There was no significant increase in yields of DNA III when the NaCl concentration was lowered even further to 10 mM (data not presented).

Examination of the kinetic data in Fig. 3 reveals that digestion is a two-phase process. During the first minute, 25% of the chromatin DNA is digested, whereas the remaining 25% is cleaved during the subsequent 10-min period. The first fraction, presumably corresponding to the internucleosomal fraction, appears to be quite accessible to the enzyme, since control naked DNA is also completely digested during the first minute. This is consistent with the electron microscopic data of Griffith (21), which showed naked internucleosomal DNA regions exposed at the lower salt concentrations. The additional 25% which is cleaved upon prolonged incubation suggests that regions presumably near, or perhaps within the nucleosome, are less accessible for cleavage by the enzyme. Importantly, the total susceptible fraction of 50% corresponds well with the estimate of coverage as determined by digestion with nonrestricted endonucleases (8, 9, 32, 41).

Resistance of SV40 chromatin containing DNA II to cleavage by single-site endonucleases. In most of the experiments described above, upon purification from cell extracts, chromatin preparations contained about 90% DNA I and about 10% DNA II. Since the fraction of DNA II molecules remained approximately constant or increased upon endonuclease treatment (Fig. 2b and 3; Tables 1 and 2), it appeared that the chromatin containing DNA II was resistant to double-stranded cuts at all of the salt concentrations tested.

To determine whether this resistance was perhaps a property of DNA II chromatin derived from an *in vivo* mechanism (14) or, instead, was a general characteristic of DNA II chromatin generated by random single-strand cuts of DNA I chromatin, the following experiment was done. SV40 chromatin labeled with ^{32}P was stored for several weeks at 4°C , during which time random single-strand nicks were introduced by ^{32}P decay. This chromatin and naked SV40 DNA prepared from the chromatin were used as substrates in separate reactions with *Bam*HI and *Eco*RI. Figure 4 shows results of an experiment in which about 25% of the chromatin employed as a substrate for the enzyme *Bam*HI contained

FIG. 4. Resistance of SV40 chromatin DNA II to *Bam*HI. ^{32}P -labeled DNA and chromatin were stored at 4°C for 4 weeks, during which time about 25% of the DNA was found to be in the linear DNA III form, presumably converted to this form by decay of the ^{32}P . SV40 chromatin and DNA were reacted with *Bam*HI under conditions similar to those described in the legend to Fig. 3, except that the NaCl concentration was 10 mM instead of 50 mM.

DNA II. In contrast to naked DNA, in which DNA I and DNA II are both rapidly converted to DNA III, the fraction initially present as DNA II remained essentially unchanged. This SV40 chromatin preparation represents only one of a number of different SV40 chromatin preparations in which the amount of SV40 DNA II did not decrease significantly upon incubation with the single-site restriction enzymes.

Digestion of SV40 DNA and SV40 chromatin with multiple-site restriction endonucleases. The apparent resistance of the nicked-circular form of SV40 chromatin to cleavage suggested that, upon relaxation of the closed-circular DNA, a conformational change may have occurred, causing the DNA to become resistant to digestion by restriction endonucleases. Thus it would follow that, regardless of the number of potential restriction enzyme cleavage sites, the remaining sites might be rendered inaccessible after receiving the first nick or chop. To test this hypothesis, ^{32}P -labeled SV40 chromatin and ^{32}P -labeled naked SV40 DNA were reacted separately with the multiple-site enzymes *Hind*II + III for various time periods, and the resultant fragments were separated on 4% acrylamide slab gels. The location of cleavage sites in SV40 DNA for *Hind*II + III and the other enzymes used in this study are shown in Fig. 1. The digest patterns are shown in Fig. 5. SV40 DNA yielded 11 fragments within 15 min after exposure to the enzymes. Not shown are two very small fragments, 30 and 20 base pairs, respectively, which run off the gels under conditions required for resolution of the other fragments (66). The naked SV40 DNA used in this experiment was obtained by the removal of protein from intracellular SV40 chromatin, but it

FIG. 5. *Hind*II + III digestion of SV40 naked DNA and SV40 chromatin. Naked ^{32}P -labeled SV40 DNA (1.3 μg) and ^{32}P -labeled SV40 chromatin (2.5 μg) were incubated separately at 37°C with *Hind*II + III (5 U) in a volume of 250 μl of a buffer containing 50 mM NaCl, 6 mM Tris, 6 mM MgCl_2 , 0.6 mM β -mercaptoethanol, 0.6% glycerol, and 0.01% Triton X-100 (pH 7.5). At the times indicated, 50- μl samples were removed from each tube, the reactions were terminated, and the samples were electrophoresed on 4% polyacrylamide slab gels (40 by 0.3 cm) for 21.5 h at 100 V, 53 mA. To locate the DNA bands, gels were placed in contact with X-ray films for 72 h.

gave the same digestion pattern as DNA from purified virus.

In contrast to the 11 fragments of the naked SV40 DNA digest pattern, the majority of the DNA in chromatin was resistant to multiple cleavage by *Hind*II + III (Fig. 5). Even 4 h after incubation, most of the SV40 DNA in chromatin had received only one cut and migrated to a position characteristic of linear full-length DNA. Furthermore, a significant fraction was not susceptible to cleavage and, instead, failed to mi-

grate in the gel. Only about 10% of the molecules received more than one chop, as indicated by the mixture of incomplete digest products, whereas less than 1% corresponded to any of the 11 limit digest products—in this case, only to fragment A. If all the cleavage sites were equally susceptible, on the average, the SV40 chromatin molecules should have received six to seven cuts under these reaction conditions. The fact that most of the cleavage sites in SV40 chromatin were protected from digestion after the initial cut provided additional evidence that a rearrangement or a configurational change or both took place such that potentially susceptible sites became covered by histones. To what extent these configurational changes could be influenced by the reaction conditions was next examined.

It is known that rearrangement and exchange of histones can occur at certain salt and divalent ion concentrations (8). Thus, one of the factors studied was the effect of magnesium concentration on the susceptibility of SV40 chromatin to *Hind*II + III. In the experiment just discussed (Fig. 5), the concentration of magnesium chloride was 6 mM. Shown in Fig. 6 are the digestion products of SV40 chromatin DNA at 0, 1, 20, and 100 mM magnesium concentrations. At very low concentrations of magnesium (less than 1 mM), control naked SV40 DNA was only partially converted to limit digest products, so these reactions with SV40 DNA and chromatin are not shown (also, the reactions had to be incubated for long periods at 37°C, conditions which could cause degradation and denaturation of chromatin).

At all of the magnesium concentrations tested, SV40 DNA in chromatin was either resistant or was converted mostly to linear DNA III, indicating that the majority of the molecules had one cut or less. An interesting feature of this experiment was that SV40 chromatin DNA was digested to a greater extent at the lower magnesium concentration (1 mM), whereas the opposite was true for the control naked SV40 DNA (e.g., Fig. 6, slots 3 and 4 compared with slots 7 and 8). These reactions were carried out at 37°C for 4.5 h, so it is difficult to conclude that this effect was solely the result of a magnesium-dependent configurational change, since it may have been caused by partial denaturation or degradation of proteins. However, it is the opposite effect that would be predicted for exchange or removal of histones, since these phenomena appear to increase with increasing concentration of divalent cation (8). It should be noted that at 100 mM magnesium concentration, both the SV40 DNA and chromatin were resistant, probably due to the aggregation of DNA,

FIG. 6. Effect of magnesium concentration on the digestion of SV40 DNA and chromatin by HindIII + III. Naked ^{32}P -labeled SV40 DNA or ^{32}P -labeled SV40 chromatin were incubated at 37°C for 4.5 h with HindIII + III (2 U) in a volume of 72 μl of a buffer containing 80 mM NaCl, 8 mM Tris, 1 mM β -mercaptoethanol, 0.02% Triton X-100 (pH 7.5), and various concentrations of MgCl_2 . Reactions were terminated, and 50- μl samples were electrophoresed on a 4% polyacrylamide slab gel for 19.5 h at 120 V, 48 mA. Each slot represents a different reaction mixture. Slots 1 through 6, SV40 DNA; slots 7 through 9, SV40 chromatin; slot 10, SV40 DNA plus EcoRI control. Slot 1, 0 M MgCl_2 , no enzymes; slot 2, 0 M MgCl_2 , enzymes; slots 3 and 7, 1 mM MgCl_2 ; slots 4 and 8, 20 mM MgCl_2 ; slots 5 and 9, 100 mM MgCl_2 ; slot 10, 5 mM MgCl_2 .

chromatin, enzyme, or a combination of these.

Further attempts were made to detect the removal or exchange of histones under the stan-

dard enzyme reaction conditions by sedimentation analysis. ^3H -labeled SV40 chromatin was mixed with ^{32}P -labeled SV40 naked DNA at a weight ratio of chromatin to DNA of 16 to 1, incubated for 1 h in 50 mM NaCl–6 mM MgCl_2 –1 mM β -mercaptoethanol–0.01 M Tris (pH 7.5)–0.01% Triton X-100, and sedimented in a sucrose gradient containing the same buffer. Except for a small shoulder on the leading edge of the DNA peak, there was no evidence of a change in the sedimentation pattern of the DNA or chromatin (Fig. 7). Thus, the conclusion from the two aforementioned experiments is that protein exchange is not a determining factor in the acquisition of resistance of SV40 chromatin upon the initial cleavage of SV40 chromatin.

Reactivity of restriction enzymes with SV40 chromatin. Another possible explanation for the inability to generate limit digest fragments by multiple-site restriction enzymes is that the chromatin or some other component in the reaction mixture may cause a drastic reduction or change in the enzyme activity so that only sufficient activity remains to generate a

FIG. 7. Sedimentation of mixtures of SV40 chromatin with SV40 DNA at high ratios of chromatin to DNA. A 2- μg amount of SV40 [^3H]chromatin (20,000 cpm/ μg) was mixed with 0.125 μg of ^{32}P -labeled SV40 DNA (122,000 cpm/ μg) in a volume of 50 μl and incubated as described in the text. Samples were layered onto 5 to 20% sucrose gradients and sedimented for 2 h at 36,000 rpm in a Spinco SW 50.1 rotor at 4°C . Radioactivity represents trichloroacetic acid-insoluble products. (a) DNA alone; (b) DNA plus chromatin. Sedimentation was from right to left.

single cleavage in most of the molecules. Consequently, SV40 chromatin and naked SV40 DNA were incubated together in various combinations with *Hind*III to determine the extent of enzyme activity during the course of the reaction. Such an experiment is shown in Fig. 8 which shows the reaction of ^3H -labeled SV40 chromatin and SV40 [^{32}P]DNA with *Hind*III endonuclease. *Hind*III cleaves SV40 DNA into six fragments (12, 66). Incubation of *Hind*III with SV40 chromatin for 2 h (slot 2) did not generate limit digest fragments. Instead, the major fraction was cleaved only once to yield full-length linear DNA (position of RI marker), whereas the remainder of the DNA either did not migrate into the gel (this fraction was a mixture of closed-circular and nicked-circular DNA) or was converted into partial digests (8%). The addition of fresh enzyme after 2 h and incubation for an additional 2 h did not significantly change the digest pattern (slot 3, Fig. 8). To demonstrate that the enzyme was indeed in excess and still active after 2 h of incubation, [^{32}P]DNA added to the SV40 chromatin reaction mixture at this time and incubated for 2 h more was completely digested (slots 4 and 4B). When SV40 DNA and chromatin were mixed before the addition of enzyme, the composite result was the same as that obtained when DNA and chromatin were incubated separately (slots 7 and 7B). These experiments show that the resistance of the SV40 chromatin to limit digestion by *Hind*III does not result from a change or reduction in enzyme activity.

Initial cleavage sites of *Hpa*I in SV40 DNA chromatin. Since the majority of the SV40 chromatin molecules could only be cut once by the multiple-site enzymes, it was of interest to ascertain whether or not the initial cleavage occurred at a single site or at preferred sites on the SV40 genome. The position of the initial cleavage site was determined by restriction enzyme mapping. To simplify the analysis, the multiple-site restriction endonuclease *Hpa*I was used to generate linear DNA III, since it chops SV40 DNA at only four positions along the genome (12, 50, 66). Two of these sites are situated very close together, being only 0.004 map units apart (66), and for the purposes of mapping, they can be treated as a single site.

A time course of digestion of control naked SV40 DNA by *Hpa*I is shown in Fig. 9. Limit digestion generated by cleavage at all sites, i.e., 0.755 (actually, 0.753 and 0.757), 0.375, and 0.175 produces three major fragments: (A) 41% fragment; (B) 38% fragment; and (C) 20% fragment (the percentages denote the fractional length of the DNA). Incomplete digestion caused by

FIG. 8. *Hind*III digestion of mixtures of SV40 chromatin and naked SV40 DNA. SV40 chromatin and ^{32}P -labeled naked SV40 DNA were either incubated separately or mixed together in approximately equal amounts (2 μg) and digested with *Hind*III (2 U) in 100 μl of a buffer containing 85 mM NaCl, 8.5 mM Tris, and 15 mM MgCl_2 (pH 7.5). Reactions were terminated after 4 h. Samples were electrophoresed on a 4% polyacrylamide slab gel (0.15 by 10 cm) at 60 V and 70 mA for 9 h. Slot 1, chromatin, no enzyme; slot 2, chromatin with *Hind*III; slot 3, chromatin, *Hind*III (2 U), 2 h, additional *Hind*III (2 U) added and incubated for 2 more h; slot 4, chromatin, *Hind*III 2 h, DNA added and incubated for 2 additional h; slot 5, DNA, *Hind*III; slot 6, DNA, no enzyme; slot 7, chromatin, DNA, no enzyme, 2 h, *Hind*III added and incubated for 2 additional h. Slots 1 through 7 show ethidium bromide-stained gels. Slots 4b through 7b represent autoradiograms of slots 4 to 7.

cleavage at only two sites yields the following "partial" digest fragments: an 80% fragment (0.175 and 0.375); a 62% fragment (0.755 and 0.375); and a 58% fragment (0.755 and 0.175). In Fig. 9, the 58% and 62% fragments are not clearly resolved.

Figure 10 shows a time course of SV40 chromatin digestion by *Hpa*I. Unlike the SV40 DNA, which is cleaved to the limit digest fragments within 1 h, digestion of SV40 chromatin results in less than 3% of any limit digest fragment, even after prolonged incubation in a vast excess of enzyme for 6 h. Instead, the major digest product was SV40 DNA III. This is consistent with the previous data obtained after digestion with *Hind*III and *Hind*II + III.

FIG. 9. Kinetics of SV40 DNA digestion by *HpaI*. ^{32}P -labeled SV40 DNA (2 μg) was incubated with *HpaI* (2U) in a volume of 50 μl of a buffer containing 50 mM NaCl, 6 mM KCl, 15 mM Tris, 5 mM MgCl_2 , 1 mM β -mercaptoethanol, and 1% glycerol (pH 7.5) and incubated at 25°C. At the times indicated, 5 μl was removed and the reactions were terminated. Samples were electrophoresed at 100 V, 48 mA on a 1.4% agarose slab gel (0.3 by 30 cm) at 48 mA, 100 V for 7 h. DNA fragments were detected by autoradiography. Fragments A, B, and C represent the limit digest fragments for *HpaI* digestion of SV40 DNA, whereas 80% and 58% represent partial digest fragments produced by cutting the genome at only 2 sites. There is also a 62% fragment which is not resolved from the 58% fragment on this gel.

About 12% of the chromatin DNA appeared as partial digest fragments—a 62% fragment and a 58% fragment. These partial digest fragments migrate very close to the position of DNA I and are not resolved in these gels. However, no significant amount of DNA could be detected at the position of the 80% partial digest fragment. Since cleavage at 0.755 is required to produce the 62 and 58% fragments, it appeared that 0.755 was more probable as a first (or second) cleavage site.

To determine the site(s) of the first cleavage, linear DNA III was eluted from the gels after digestion of SV40 chromatin by *HpaI*, and the DNA III was redigested with restriction enzymes which cleave SV40 DNA at only one site. Since the cleavage position for each single-site enzyme is known, the position of the first site cleaved by *HpaI* can be calculated from the size of the

FIG. 10. *HpaI* digestion of SV40 chromatin. ^{32}P -labeled SV40 chromatin (3 μg) was incubated at 37°C with *HpaI* (1.5 U) in 175 μl of a buffer containing 75 mM NaCl, 3 mM KCl, 0.7 mM β -mercaptoethanol, 3 mM MgCl_2 , 13 mM Tris (pH 7.5), 0.02% Triton X-100, and 0.02 mM EDTA. At various times between 1 and 120 min, 25- μl samples were removed and the reactions were terminated. After 120 min, 1.0 U of *HpaI* enzyme was added, and the remainder of the reaction mixture was incubated for an additional 4 h. As a control, naked ^{32}P -labeled SV40 DNA was incubated under similar reaction conditions for 120 min. Samples were applied to a 1.4% agarose slab gel (22 by 0.3 cm) at 45 mA, 100 V for 6 h. DNA digestion products were detected by exposure of the slab gels to X-ray films.

resulting DNA fragments. As a control, naked SV40 DNA was partially digested with *HpaI* to the stage where the DNA had only a single chop.

HpaI-derived DNA III from naked SV40 DNA and SV40 chromatin were redigested with *Bam*HI, *Eco*RI, and *Hpa*II (Fig. 11). Inspection of the right panel of Fig. 11 shows that the initial cleavage of the SV40 chromatin by *HpaI* was not at a unique site, for had this been the case, redigestion with *Bam*HI should have yielded only two distinct fragments. Instead, redigestion yielded six fragments, five shown in Fig. 11 (right panel, slot c) and one very small fragment which can only be resolved on a higher-percentage

acrylamide gel (not shown in Fig. 11). Indeed, comparison of the naked SV40 DNA III and SV40 chromatin DNA III digestion patterns by *Bam*HI shows that these patterns are quite similar (Fig. 11, middle and right panels, slot c).

Quantitative analysis of the *Hpa*I redigestion of the *Hpa*I-derived DNA III revealed that the initial cut in the naked DNA as well as in the chromatin is more likely to occur at the 0.755 position than at the other two susceptible sites (Table 3). For the DNA, initial cuts occur about 1.5 times more frequently at this site than at the other sites, whereas in the chromatin, initial cuts are made about 2.5 times more frequently than at the other sites (Table 3). The 0.755 position contains two very closely spaced *Hpa*I cleavage sites, so that it is reasonable that the initial cuts should be more frequent in this region. For instance, if it were assumed that cleavage occurs with equal probability at each of the four *Hpa*I sites, there should have been twice as many DNA fragments generated by cleavage at the 0.755 sites relative to each of the other cleavage sites. However (Table 3), the frequency of cleavage at the 0.755 site is significantly higher for cleavage of SV40 chromatin and significantly

less frequent for naked SV40 DNA. These data indicate that the probability of cleavage is different for each site and is different in naked DNA compared with that in chromatin. Unfortunately, these experiments were done with a single preparation of SV40 chromatin, and further work would be needed to determine the significance of these differences.

DISCUSSION

In this study, the susceptibility of SV40 chromatin to restriction endonucleases was found to be dependent on the ionic environment and on the topological configuration of the chromatin DNA. The SV40 chromatin employed in these experiments was devoid of H1 histones, so the observed dependence was presumably due to conformational changes between the nucleosomal histones and the SV40 DNA.

Recent biochemical data indicate that there are about 200 DNA base pairs associated with each SV40 or polyoma nucleosome and a stretch of about 50 DNA base pairs connecting adjacent nucleosomes (4, 6, 32, 43). Consistent with this model is our finding that only about 20% of the SV40 chromatin DNA was attacked by the re-

FIG. 11. Cleavage of *Hpa*I-derived DNA III by single-site enzymes. 32 P-labeled SV40 DNA III was removed from preparative agarose slab gels after limit digestion of SV40 chromatin or after partial digestion of naked SV40 DNA. The DNA III was then digested by either *Eco*RI, *Hpa*II, or *Bam*HI in a buffer containing 70 mM NaCl, 4 mM KCl, 14 mM Tris (pH 7.5), 3.5 mM $MgCl_2$, and 0.7 mM β -mercaptoethanol. DNA fragments were separated on 1.4% agarose slab gels. Left panel, SV40 DNA digest products as markers: (a) untreated DNA; (b, c, and d) partial digestion by *Hpa*I; (e) *Hpa*II digestion; (f) *Hpa*I + *Hpa*II. Middle panel, *Hpa*I-derived DNA III from partial digestion of naked DNA: (a) untreated DNA; (b) *Eco*RI digestion; (c) *Bam*HI digestion; (d) *Hpa*I digestion. Right panel, *Hpa*I-derived DNA III from limit digestion of chromatin: (a) *Hpa*II digestion (very low activity); (b) *Hpa*I and *Hpa*II (*Hpa*II, very low activity); (c) *Bam*HI digestion.

TABLE 3. *Bam*HI digestion of *Hpa*I-derived SV40 DNA III^a

Fragment size ^b	Map position	Digestion					
		Predicted ^c		DNA		Chromatin	
		Wt (%)	No. of molecules (%)	Wt (%)	No. of molecules (%)	Wt (%)	No. of molecules (%)
96.5	0.175	24.4	14.3	24.1	14.5	22.2	12.9
76.5		19.3	14.3	26.2	19.9	16.9	12.4
23.5	0.375	5.9	14.3	6.2	15.3	5.2	12.4
62.5		31.5	28.6	27.6	25.6	34.7	31.0
37.5	0.755	18.9	28.6	15.9	24.6	21.0	31.3

^a Data were derived from densitometric tracings of gel patterns. Values represent averages for duplicate runs.

^b Fragment size represents percentage of the length of SV40 DNA. For each initial cut made by *Hpa*I, redigestion by *Bam*HI will generate two fragments. Thus, the 76.5/23.5 pair is generated by *Bam*HI digestion of DNA III terminated at the 0.375 map position, etc. A 3.5% fragment runs off this gel and is not included in this analysis; thus, the 96.5 fragment is lacking its complement.

^c Predictions were based on the assumption that each of the four *Hpa*I sites is cleaved with equal probability. Since 0.755 represents a double site, cleavage at the site should be at twice the frequency of cleavage at the other two sites.

striction enzymes at 100 mM NaCl, suggesting that nucleosome-associated DNA was entirely resistant to the enzymes at this salt concentration. However, our kinetic experiments indicate that the susceptible DNA, presumably in the "spacer" regions between nucleosomes, did not exist in a state analogous to free SV40 DNA. For example, in the short time required to convert control naked SV40 DNA to its limit digest products, only about 4% of the chromatin DNA was cleaved (Table 1). Complete digestion of the susceptible DNA required incubation times 6 to 15 times longer.

At lower salt concentrations, however, about 50% of the SV40 chromatin DNA was digested by the restriction enzymes. This value is in agreement with values found in studies in which chromatin was digested with nonrestricted endonucleases (8, 9, 32, 41). Of this susceptible fraction, about half of the DNA was cleaved rapidly, i.e., during a time period comparable to that required for limit digestion of equivalent amounts of control naked SV40 DNA (Fig. 3). From these experiments, we propose that the internucleosomal DNA has very little association with the histones at low salt concentrations. In contrast, an incubation period at least 10 times greater was required to digest the remaining susceptible chromatin DNA. This fraction of the chromatin DNA may represent sites within nucleosomes which are only exposed at the lower salt concentrations but nevertheless remain partially associated with histones. The fraction totally resistant to digestion at the lower salt concentration probably contains sites within the

140-base-pair nucleosome core (1, 2, 10, 52, 53, 55, 57).

SV40 chromatin containing DNA in a topologically relaxed configuration, i.e., with DNA having either a single- or a double-stranded break, was highly resistant to further cleavage by restriction enzymes. The resistance of SV40 chromatin containing nicked DNA was revealed in experiments with single-site enzymes, in which it was found that DNA II initially present in chromatin failed to be converted to DNA III (Fig. 3 and 4). At the higher salt concentration (100 mM NaCl) and at low enzyme-to-chromatin ratios, the quantity of DNA II actually increased during the course of the reactions (Fig. 2 and Table 1). In these experiments, the SV40 chromatin used as a substrate for the enzymes consisted initially of mixtures of chromatin with DNA I and II present, so that the possibility that DNA II was an intermediate being formed and then converted to DNA III could not be ruled out. Nonetheless, kinetic experiments such as those shown in Fig. 3 and 4 in which the fraction of DNA II remained essentially unchanged throughout prolonged incubation while DNA I was quantitatively converted to DNA III are not consistent with such a mechanism.

The conclusion that a single double-stranded break can render SV40 chromatin resistant to further digestion by multiple-site enzymes was based on experiments such as the one shown in Fig. 5 in which most of the DNA was converted to DNA III and only 1% of the DNA could be detected at positions corresponding to limit digest fragments. The small amount of limit digest

products was not due to insufficient enzyme activity, since naked SV40 DNA added to reaction mixtures before or during the reactions was rapidly digested to limit digest fragments (Fig. 8). In contrast to these results, Persico-DiLauro et al. (41) found that 20 to 25% of the chromatin DNA was digested to limit digest fragments after exposure to *Hind*III. The discrepancy may be related to differences in the purification of the chromatin and differences in the specific activity and source of enzymes.

Upon observing that most of the DNA in SV40 chromatin received only a single cleavage after digestion with multiple-site enzymes, we attempted to determine whether this initial cleavage occurred at some preferred site(s) on the chromatin. Consequently, the initial cleavage sites were mapped after digestion with *Hpa*I. Subsequent experiments revealed that, in naked SV40 DNA as well as in chromatin, all potential sites were cleaved, but not all sites were attacked with equal probability (Table 3). For instance, it was shown that certain sites, specifically those sites which map at position 0.755 on the SV40 genome, were cut at a much higher frequency than the other two sites. However, the frequency was not high enough to justify a conclusion that these sites were devoid of histones. Had this been the case, the ratio of cuts at these sites to those at the other sites should have been even greater, no DNA I ought to have remained undigested, and limit digest fragments A and B should have been generated at higher levels and in equal amounts (see Fig. 10 and Table 3). Nonetheless, the results of our experiment with *Hpa*I demonstrate that the distribution of histones in SV40 chromatin is not random. This conclusion is in agreement with the data found for the distribution of histones in polyoma chromatin (44). It also raises the possibility that more extensive analysis of chromatin by restriction enzymes might reveal certain sites which preclude the binding of histones.

One type of conformational change likely to render internucleosomal DNA resistant to digestion after relaxation of the SV40 DNA I in chromatin is one which causes the exposed DNA to associate with nucleosomes. Before relaxation, SV40 DNA I in chromatin is constrained in a high-energy state (64, 65), but upon relaxation the DNA can become free to rotate. In a model suggested by Worcel and Benyajati (63), successive right-hand rotations of nucleosomes can cause internucleosomal DNA to coil bringing adjacent nucleosomes together. In this model, the spacer DNA forms a coil in the groove created by the contiguous nucleosomes. Alternatively, it is conceivable that grooves exist or

are formed upon rearrangement of the core structure. The extent of coiling and spacing of nucleosomes would of course depend on other factors, such as salt and divalent ion concentration. An association between spacer DNA and nucleosomes similar to that proposed in this model can be concluded from recent endonuclease studies which reveal that certain repeating elements found in nucleosomes extend into the spacer region (31, 44). Indeed, others have suggested that native chromatin ordinarily exists in a compact structure with nucleosomes almost in contact (15, 21, 59).

The two proposed models of chromatin, i.e., the compact form and the beaded-string configuration appear to represent a conversion of one form to the other, depending on the conditions of observations. With regard to SV40 chromatin, Griffith (21) observed the compact form under physiological salt conditions and the beaded form at low salt conditions. In subsequent experiments, Christiansen and Griffith (6) showed that magnesium ions can condense SV40 chromatin even at low salt concentrations into a form which resembles compact chromatin. This would suggest that, under the conditions employed in our experiments (i.e., 1 to 10 mM $MgCl_2$), the SV40 chromatin became condensed when the DNA was relaxed, whereas, had the magnesium concentrations been reduced further, the internucleosomal DNA may have still remained partially extended. In agreement with this prediction, we noted above (see Results) that SV40 chromatin was digested to a greater extent at the lower concentrations of magnesium (Fig. 6).

Additional data relate to the mechanism of action of the restriction enzymes. Ruben and co-workers (47) and Modrich and Zabel (36) found that the reaction of *Eco*RI with DNA takes place in two distinct steps; a single-stranded break is made rapidly in one strand, followed by a slower reaction which generates a break in the complementary strand. When *Eco*RI was reacted with SV40 DNA at a low concentration of enzyme relative to DNA, a nicked-circular DNA II intermediate was generated before conversion to DNA III (Fig. 2a). In contrast, when *Eco*RI was reacted with SV40 chromatin under the same conditions, a significant amount of DNA II was generated, but it failed to convert to DNA III. In agreement with this observation, Persico-DiLauro et al. (41) observed a 15 to 25% increase in DNA II during digestion with *Eco*RI. In our work, however, this increase in DNA II did not occur at high concentrations of enzymes (Fig. 3). If we are correct in assuming that a nick in chromatin DNA can cause structural changes

which sequester cleavage sites, a possible explanation for the failure to convert DNA II to DNA III at low enzyme concentrations would be that these changes occur before the enzyme can cleave the complementary strand. We intend to further investigate this possibility in more detailed studies.

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LITERATURE CITED

1. Axel, R. 1975. Cleavage of DNA in nuclei and chromatin with staphylococcal nuclease. *Biochemistry* 14:2921-2925.
2. Axel, R., W. Melchoir, B. Sollner-Webb, and G. Felsenfeld. 1974. Specific sites of interaction between histones and DNA in chromatin. *Proc. Natl. Acad. Sci. U.S.A.* 71:4101-4105.
3. Barban, S., and R. S. Goor. 1971. Structural proteins of simian virus 40. *J. Virol.* 7:198-203.
4. Bellard, M., P. Oudet, J. E. Germond, and P. Chambon. 1976. Subunit structure of simian virus-40 minichromosome. *Eur. J. Biochem.* 70:543-553.
5. Camerini-Otero, R. D., B. Sollner-Webb, and G. Felsenfeld. 1976. The organization of histones and DNA in chromatin: evidence for an arginine-rich histone kernel. *Cell* 8:333-347.
6. Christiansen, G., and J. Griffith. 1977. Salt and divalent cation affect the flexible nature of the natural beaded chromatin structure. *Nucleic Acids Res.* 4:1837-1851.
7. Christiansen, G., T. Landers, J. Griffith, and P. Berg. 1977. Characterization of components released by alkali disruption of simian virus 40. *J. Virol.* 21:1079-1084.
8. Clark, R. J., and G. Felsenfeld. 1971. Structure of chromatin. *Nature (London) New Biol.* 229:101-105.
9. Clark, R. J., and G. Felsenfeld. 1974. Chemical probes of chromatin structure. *Biochemistry* 13:3622-3628.
10. Compton, J. L., M. Bellard, and P. Chambon. 1976. Biochemical evidence of variability in the DNA repeat length in the chromatin of higher eukaryotes. *Proc. Natl. Acad. Sci. U.S.A.* 73:4382-4386.
11. Cremisi, C., P. F. Pignatti, O. Croissant, and M. Yaniv. 1976. Chromatin-like structures in polyoma virus and simian virus 40 lytic cycle. *J. Virol.* 17:204-211.
12. Danna, K. J., G. H. Sack, Jr., and D. Nathans. 1973. Studies of simian virus 40 DNA. VII. A cleavage map of the SV40 genome. *J. Mol. Biol.* 78:363-376.
13. Estes, M. K., E.-S. Huang, and J. S. Pagano. 1971. Structural polypeptides of simian virus 40. *J. Virol.* 7:635-641.
14. Fareed, G., M. McKerlie, and N. Salzman. 1973. Characterization of simian virus 40 DNA component II during viral DNA replication. *J. Mol. Biol.* 74:95-111.
15. Finch, J. T., M. Noll, and R. D. Kornberg. 1975. Electron microscopy of defined lengths of chromatin. *Proc. Natl. Acad. Sci. U.S.A.* 72:3320-3322.
16. Frearson, P. M., and L. V. Crawford. 1972. Polyoma virus basic proteins. *J. Gen. Virol.* 14:141-155.
17. Friedmann, T., and D. David. 1972. Structural roles of polyoma virus proteins. *J. Virol.* 10:776-782.
18. Germond, J. E., B. Hirt, P. Oudet, M. Gross-Bellard, and P. Chambon. 1975. Folding of the DNA double-helix in chromatin-like structures from simian virus 40. *Proc. Natl. Acad. Sci. U.S.A.* 72:1843-1847.
19. Gibson, W. 1974. Polyoma virus proteins: a description of the structural proteins of the virions based on polyacrylamide gel electrophoresis and peptide analysis. *Virology* 62:219-336.
20. Goldstein, D. A., M. R. Hall, and W. Meinke. 1973. Properties of nucleoprotein complexes containing replicating polyoma DNA. *J. Virol.* 12:887-900.
21. Griffith, J. D. 1975. Chromatin structure: deduced from a minichromosome. *Science* 187:1202-1203.
22. Hall, M. R., W. Meinke, and D. A. Goldstein. 1973. Nucleoprotein complexes containing replicating simian virus 40 DNA: comparison with polyoma nucleoprotein complexes. *J. Virol.* 12:901-908.
23. Hedgpeth, J., H. M. Goodman, and H. W. Boyer. 1972. DNA nucleotide sequence restricted by the RI endonuclease. *Proc. Natl. Acad. Sci. U.S.A.* 69:3448-3452.
24. Hewish, D., and L. Burgoyne. 1973. Chromatin substructure: the digestion of chromatin DNA at regularly spaced sites by nuclear deoxyribonuclease. *Biochem. Biophys. Res. Commun.* 52:504-510.
25. Howe, C. C., and K. B. Tan. 1977. Nucleoprotein complexes from simian virus 40-infected monkey cells: association with histones and the major viral structural protein. *Virology* 78:45-56.
26. Huang, E.-S., M. K. Estes, and J. S. Pagano. 1972. Structure and function of the polypeptides in simian virus 40. I. Existence of subviral deoxynucleoprotein complexes. *J. Virol.* 9:923-929.
27. Kornberg, R. D. 1974. Chromatin structure: a repeating unit of histones and DNA. *Science* 184:868-871.
28. Kornberg, R. D., and J. O. Thomas. 1974. Chromatin structure: oligomers of the histones. *Science* 184:865-868.
29. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
30. Lake, R. S., S. Barban, and N. P. Salzman. 1973. Resolution and identification of the core deoxyribonucleoproteins of the simian virus 40. *Biochem. Biophys. Res. Commun.* 54:640-647.
31. Lohr, D., K. Tatchell, and K. E. Van Holde. 1977. On the occurrence of nucleosome phasing in chromatin. *Cell* 12:829-836.
32. Louie, A. J. 1974. The organization of proteins in polyoma and cellular chromatin. *Cold Spring Harbor Symp. Quant. Biol.* 38:259-265.
33. McMillen, J., and R. A. Consigli. 1974. Characterization of polyoma DNA-protein complexes. I. Electrophoretic identification of the proteins in nucleoprotein complex isolated from polyoma-infected cells. *J. Virol.* 14:1326-1336.
34. Meinke, W., M. R. Hall, and D. A. Goldstein. 1975. Proteins in intracellular simian virus 40 nucleoprotein complexes: comparison with simian virus 40 core proteins. *J. Virol.* 15:439-448.
35. Mertz, J. E., and R. W. Davis. 1972. Cleavage of DNA by R₁ restriction endonuclease generates cohesive ends. *Proc. Natl. Acad. Sci. U.S.A.* 69:3370-3374.
36. Modrich, P., and D. Zabel. 1976. *Eco*RI endonuclease. Physical and chemical properties of the homogeneous enzyme. *J. Biol. Chem.* 251:5866-5874.
37. Mulder, C., and H. Delius. 1972. Specificity of the break produced by restricting endonuclease R₁ in simian virus 40 DNA, as revealed by partial denaturation mapping. *Proc. Natl. Acad. Sci. U.S.A.* 69:3215-3219.
38. Noll, M. 1974. Subunit structure of chromatin. *Nature*

- (London) **251**:249-251.
39. **Olins, A. L., and D. E. Olins.** 1974. Spherical chromatin units (*v* bodies). *Science* **183**:330-332.
 40. **Oudet, P., M. Gross-Bellard, and P. Chambon.** 1975. Electron microscopic and biochemical evidence that chromatin is a repeating unit. *Cell* **4**:281-300.
 41. **Persico-Dilauro, M., R. G. Martin, and D. M. Livingston.** 1977. Interaction of simian virus 40 chromatin with simian virus 40 T-antigen. *J. Virol.* **24**:451-460.
 42. **Polisky, B., and B. McCarthy.** 1975. Location of histones on SV40 DNA. *Proc. Natl. Acad. Sci. U.S.A.* **72**:2895-2899.
 43. **Ponder, B. A. J., F. Crew, and L. V. Crawford.** 1978. Comparison of nuclease digestion of polyoma virus nucleoprotein complex and mouse chromatin. *J. Virol.* **25**:175-186.
 44. **Ponder, B. A. J., and L. V. Crawford.** 1977. The arrangement of nucleosomes in nucleoprotein complexes from polyoma virus and SV40. *Cell* **11**:25-49.
 45. **Qureshi, A. A., and P. Bourgaux.** 1977. Distinct non-structural polypeptides in polyoma and simian virus 40 DNA-protein complexes. *Virology* **77**:418-420.
 46. **Roblin, R., E. Harle, and R. Dulbecco.** 1971. Polyoma virus proteins. I. Multiple virion components. *Virology* **45**:555-566.
 47. **Ruben, G., P. Spielman, D. T. Chen-pei, E. Jay, B. Siegel, and R. Wu.** 1977. Relaxed circular SV40 DNA as cleavage intermediate of two restriction endonucleases. *Nucleic Acids Res.* **4**:1803-1813.
 48. **Sahasrabudde, C. G., and K. E. Van Holde.** 1974. The effect of trypsin on nuclease-resistant chromatin fragments. *J. Biol. Chem.* **249**:152-156.
 49. **Seebeck, T., and R. Weil.** 1974. Polyoma viral DNA replicated as a nucleoprotein complex in close association with the host cell chromatin. *J. Virol.* **13**:567-576.
 50. **Sharp, P. A., W. Sugden, and J. Sambrook.** 1973. Detection of two restriction endonuclease activities in *Hemophilus parainfluenza* using analytical agarose-ethidium bromide electrophoresis. *Biochemistry* **12**:3055-3063.
 51. **Shaw, B. R., J. L. Corden, C. G. Sahasrabudde, and K. E. Van Holde.** 1974. Chromatographic separation of chromatin sub-units. *Biochem. Biophys. Res. Commun.* **61**:1193-1198.
 52. **Shaw, B. R., T. M. Herman, R. T. Kovacic, G. S. Beaudreau, and K. E. Van Holde.** 1976. Analysis of subunit organization in chicken erythrocyte chromatin. *Proc. Natl. Acad. Sci. U.S.A.* **73**:505-509.
 53. **Simpson, R., and J. Whitlock.** 1976. Chemical evidence that chromatin DNA exists as 160 base pair beads interspersed with 40 base pair bridges. *Nucleic Acids Res.* **3**:117-127.
 54. **Sollner-Webb, B., R. D. Camerini-Otero, and G. Felsenfeld.** 1976. Chromatin structure as probed by nucleases and proteases: evidence for the central role of histones H3 and H4. *Cell* **9**:179-193.
 55. **Sollner-Webb, B., and G. Felsenfeld.** 1975. A comparison of the digestion of nuclei and chromatin by staphylococcal nuclease. *Biochemistry* **14**:2915-2920.
 56. **Tan, K. B., and F. Sokol.** 1972. Structural proteins of simian virus 40: phosphoproteins. *J. Virol.* **10**:985-994.
 57. **Van Holde, K. E., C. G. Sahasrabudde, and B. R. Shaw.** 1974. A model for particulate structure in chromatin. *Nucleic Acids Res.* **1**:1579-1586.
 58. **Varshavsky, A. J., V. V. Bakayev, P. M. Chumackov, and G. P. Georgiev.** 1976. Minichromosome of simian virus 40: presence of histone H1. *Nucleic Acids Res.* **3**:117-127.
 59. **Varshavsky, A. J., S. A. Nedospasov, V. V. Schmatchenko, V. V. Bakayev, P. M. Chumackov, and G. P. Georgiev.** 1977. Compact form of SV40 viral minichromosome is resistant to nuclease: possible implications for chromatin structure. *Nucleic Acids Res.* **4**:3303-3325.
 60. **Walter, G., R. Roblin, and R. Dulbecco.** 1972. Protein synthesis in simian virus 40-infected monkey cells. *Proc. Natl. Acad. Sci. U.S.A.* **69**:921-924.
 61. **Wilson, G. A., and F. E. Young.** 1975. Isolation of sequence-specific endonuclease (Bam I) from *Bacillus amyloliquefaciens* H. *J. Mol. Biol.* **97**:123-125.
 62. **Woodcock, C. L. F.** 1973. Ultrastructure of inactive chromatin. *J. Cell. Biol.* **59**:368a.
 63. **Worcel, A., and C. Benyajati.** 1977. Higher order coiling of DNA in chromatin. *Cell* **12**:83-100.
 64. **Vinograd, J., and J. Lebowitz.** 1966. Physical and topological properties of circular DNA. *J. Gen. Physiol.* **49**:103-124.
 65. **Vinograd, J., J. Lebowitz, and R. Watson.** 1968. Early and late helix-coil transitions in closed circular DNA. The number of superhelical turns in polyoma DNA. *J. Mol. Biol.* **33**:173-197.
 66. **Yang, R., K. Danna, A. Van de Voorde, and W. Fiers.** 1975. Location of the small restriction fragments, *Hind*-L, *Hind*-M, and *Hpa*-E, on the simian virus 40 genome. *Virology* **68**:260-265.