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Distribution of DNase I-Sensitive Sites in Simian Virus 40 Nucleoprotein Complexes from Disrupted Virus Particles

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Nucleoprotein complexes (core particles) released from simian virus 40 (SV40) virions were compared with similar complexes (SV40 chromatin) extracted from nuclei of infected cells. Core particles were sensitive to cleavage by DNase I at about the same enzyme concentration required to cleave SV40 chromatin. DNase I preferentially cleaved SV40 chromatin adjacent to the viral origin of replication; however, cleavage of core particles occurred with much less selectivity. The difference between these nucleoproteins was not due to a structural alteration induced by the virion disruption procedure, since SV40 chromatin retained its pattern of DNase I-sensitive sites when subjected to this treatment. On the other hand, core particles did not acquire the nuclease-sensitive feature typical of SV40 chromatin when they were exposed to infected cell nuclei and the Triton X-100–EDTA extraction procedure. Hence, the nuclease-sensitive feature was lost or altered during the normal process of virion assembly and maturation.

Simian virus 40 (SV40) DNA occurs in combination with cellular histones in vivo, and nucleoprotein complexes which contain repeating units typical of nucleosomes in cellular chromatin have been isolated from virus particles (2, 8, 15, 20, 22, 27) as well as from infected cells (3, 10, 16, 22, 23, 35, 38). There appears to be little constraint on the location of nucleosomes with respect to viral DNA sequence (2, 10, 11, 21, 25, 27, 28, 36); however, some evidence for nonrandom arrangement has been reported (21, 24, 25, 28, 32, 37). In contrast to this flexibility in overall viral chromatin structure, a short segment near the origin of DNA replication contains a highly sequence specific nucleoprotein configuration, at least in a substantial subpopulation of SV40 chromatin molecules. This is detected as a nuclease-sensitive region of the viral genome (29, 31, 32, 37, 39–42) and as a gap in the nucleosome pattern when observed with the electron microscope (18, 29).

Since virus particles represent a distinct physiological state, it is of interest to determine the distribution of nuclease-sensitive sites in nucleoprotein recovered from virions. In this report we show that virion core particles obtained by the disruption procedure of Brady et al. (5, 6) are cleaved by DNase I at about the same rate as is SV40 chromatin from infected cells; however, cleavage occurs with little preference for the region adjacent to the origin of replication. It appears, therefore, that the unique nucleoprotein configuration exists in intracellular viral chromatin and is altered during assembly of virus particles.

MATERIALS AND METHODS

Cells and virus. BSC-1 cells were propagated in minimal essential medium (Earle salts) supplemented with 10% fetal calf serum (GIBCO Laboratories or KC Biologicals) at 37°C in an atmosphere of 5% CO_2. Confluent cultures were infected with wild-type SV40 (strain 776) at a multiplicity of 3 to 4 and incubated in the same medium containing 2% fetal calf serum.

Isolation of virus particles. Infected cultures were labeled from days 1 to 7 after infection with [^{32}P]phosphate (100 μCi/75-cm^2 tissue culture flask; carrier-free isotope; ICN) in 7 ml of phosphate-free medium supplemented with 2% fetal calf serum. Alternatively, infected cultures were labeled for the same period with [^{3}H]thymidine (100 μCi/75-cm^2 flask; 6.7 Ci/mmol; New England Nuclear Corp.) in 7 ml of medium containing phosphate and 2% fetal calf serum.

Virus particles were isolated from the cell lysate by chloroform extraction, centrifugation onto a cushion of CsCl, and centrifugation to equilibrium in CsCl as previously described (30). Purified virus was dialyzed against TN buffer (1 mM Tris-hydrochloride, pH 7.4, and 50 mM NaCl) and stored at 4°C until used.

Isolation of core nucleoprotein from virus particles. Virus particles were disrupted by the method of Brady et al. (5, 6) (incubation conditions: 10 mM Tris-hydrochloride, pH 8.6, 150 mM NaCl, 1 mM disodium ethyleneglycol-bis-*N,N'*-tetraacetic acid [EGTA], and 3 mM dithiothreitol). Except where indicated, incubation was 15 min at 27°C. After incubation, the mixture was cooled in ice and fractionated by centrifugation on a 5 to 20% sucrose gradient in TENT buffer (10 mM Tris-hydrochloride, pH 7.4, 0.5 mM disodium EDTA, 50 mM NaCl, and 0.17% Triton X-100). The gradient was centrifuged in an SW41 Spinco rotor for 120 min at 35,000 rpm (4°C), and the single peak of radioactivity was pooled and dialyzed against an appropriate buffer. Alternatively, the nucleoprotein

complex was concentrated by centrifugation through a layer of 15% sucrose onto a 60% sucrose cushion (in TENT buffer) (42). The resulting nucleoprotein complex is designated CH_V.

Isolation of viral nucleoproteins from infected cell nuclei. Nucleoproteins designated CH_T were extracted from infected BSC-1 cell nuclei by the Triton X-100–EDTA procedure as previously described (31) and purified by centrifugation into a 5 to 20% sucrose gradient in TENT buffer (same centrifugation conditions as described for CH_V). Where indicated, CH_T was further purified by centrifugation through a layer of 15% sucrose onto a 60% sucrose cushion (in TENT buffer) as described above.

Enzymatic digestion of viral nucleoproteins and analysis of resulting DNA fragments. Cleavage of viral nucleoproteins by bovine pancreatic DNase I (2,812 U/mg; type D; Worthington Biochemicals Corp.), isolation of full-length linear viral DNA by preparative gel electrophoresis on 1.4% agarose, and recovery of DNA from gel slices have been previously described (31). Restriction enzymes *Eco*RI and *Bam*HI were obtained from Miles Laboratories; *Taq*I and *Bgl*I were obtained from New England Biolabs. Digestion conditions were those recommended by the supplier. DNA fragments were analyzed by electrophoresis on 1.4% agarose (7).

Determination of SV40 DNA concentrations in solutions containing viral nucleoproteins. Samples of extract or fractions from sucrose gradients were adjusted to 1% sodium dodecyl sulfate and incubated for 10 min at 50°C. Bovine pancreatic RNase A (RAF; Worthington; heated for 20 min at 80°C) was added (10 μg/ml), and incubation was continued at 37°C for 30 min. Then autodigested (DNase-free) proteinase K (EM Biochemicals) was added to 60 μg/ml. Incubation was continued at 37°C for 60 min. A portion of the resulting mixture was fractionated by electrophoresis on 1.4% agarose, stained with ethidium bromide solution (34), illuminated at 302 nm (UV Products, Inc.), and photographed with a Polaroid M3 Land camera through a Wratten gelatin filter (no. 25), using Polaroid type 55 film.

SV40 DNA was quantitated by scanning the Polaroid negative with a Joyce-Loebl microdensitometer and comparing the result with a standard curve of form I SV40 DNA run on the same gel. This method provides a linear measurement of form I SV40 DNA in the range of 6 to greater than 60 ng. Form II SV40 DNA (which was always a minor species) was included in the quantitation, although any sample which contained a substantial amount of form II or form III viral DNA would be overestimated by this procedure.

RESULTS

Nucleoprotein released by disruption of SV40 virions. Incubation of virus particles with EGTA and dithiothreitol (5, 6) released core nucleoprotein (CH_V) which sedimented as a homogeneous species (Fig. 1a). CH_V isolated in this manner was approximately as sensitive to DNase I as was nucleoprotein from infected cell nuclei (CH_T; Fig. 2); however, the distribution of initial cleavage sites introduced by DNase I was different. Digestion conditions were selected which gave maximal yield of full-length linear DNA. The resulting linear molecules were isolated and subjected to cleavage by single-cut restriction enzymes (Fig. 3) followed by electrophoresis on 1.4% agarose (Fig. 4).

As previously reported, CH_T is preferentially

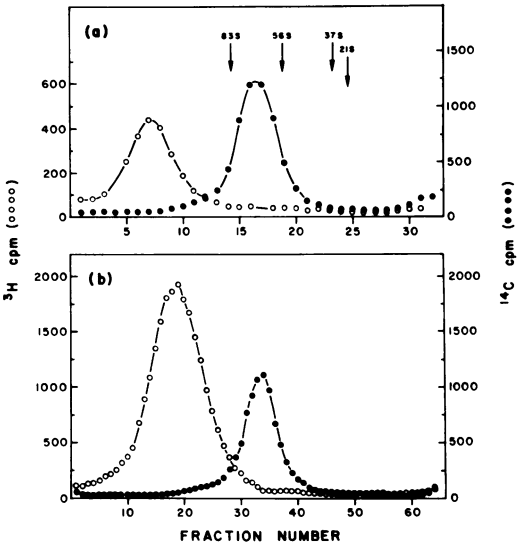

FIG. 1. *Sedimentation analysis of SV40 nucleoproteins CH_T, CH_V, and CH_V after treatment by the Triton X-100–EDTA extraction procedure. (a) Infected BSC-1 cells were labeled with [^{14}C]thymidine (20 μCi/150-mm dish in 15 ml of medium; specific activity, 40.8 Ci/mmol; New England Nuclear Corp.) 24 to 42 h postinfection. Nuclear extract (prepared as described in Materials and Methods) was fractionated by centrifugation on a 15 to 30% sucrose gradient in TENT buffer (150 min, 35,000 rpm, SW50.1 Spinco rotor, 4°C), yielding ^{14}C-labeled CH_T. ^{3}H-labeled CH_V (prepared by disruption of ^{3}H-labeled virions for 15 min at 27°C) was dialyzed into TENT buffer and fractionated on a separate gradient. Total radioactivity in each fraction was determined by liquid scintillation counting. (b) A portion of the ^{3}H-labeled CH_V preparation was dialyzed into 11 mM Tris-hydrochloride (pH 7.4), 0.56 mM disodium EDTA, and 0.25% Triton X-100 and added to a nuclear pellet from infected BSC-1 cells (labeled as described above) as the first step in the extraction procedure. A portion of nuclear extract was centrifuged as described above. Fractions were collected, and trichloroacetic acid-precipitable radioactivity was determined. Sedimentation standards include monosomes (83S) and ribosomal subunits (56S and 37S [44]) from BSC-1 cells and form I SV40 DNA (21S) analyzed on parallel gradients. Monosomes and subunits were analyzed on 15 to 30% sucrose gradients in 50 mM Tris-hydrochloride (pH 7.4), 200 mM KCl or 400 mM KCl, and 5 mM $MgCl_2$ (4).*

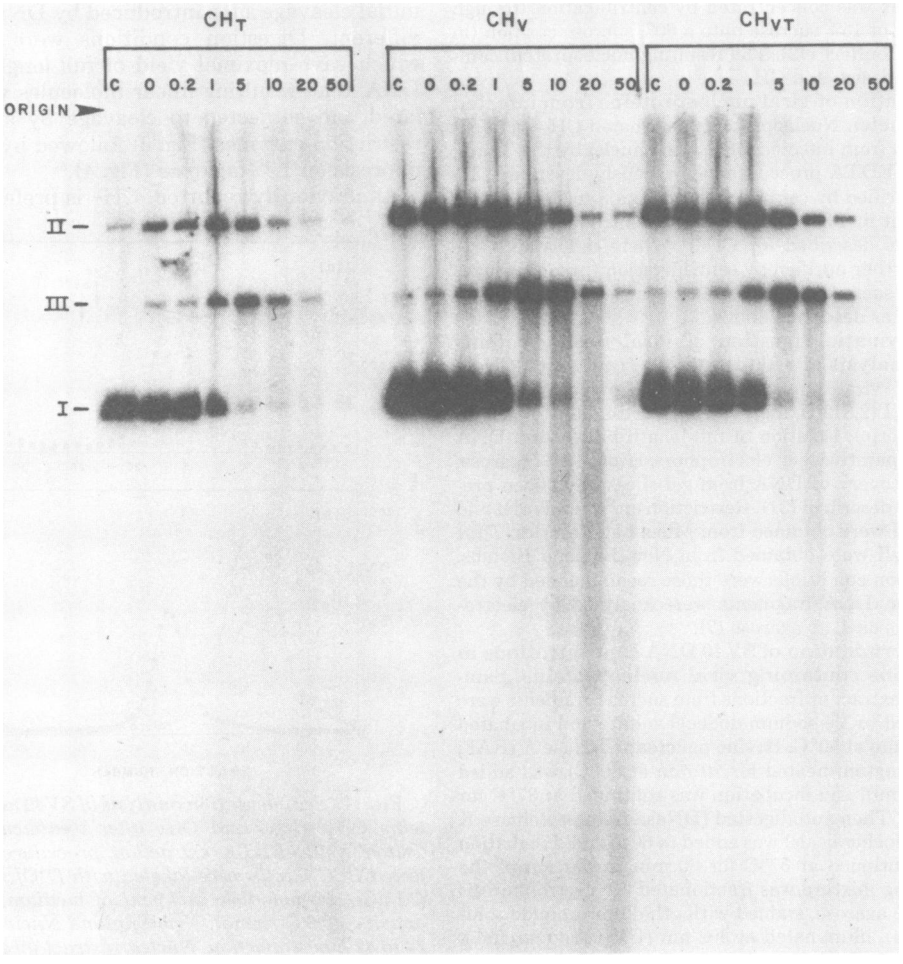

FIG. 2. *Effect of DNase I concentration on extent of cleavage of SV40 DNA in CH_V, CH_T, and CH_V after treatment by the Triton X-100–EDTA extraction procedure (designated CH_{VT}). CH_T was prepared from four 150-mm petri dishes of BSC-1 cells labeled 24 to 42 h postinfection (50 μCi of carrier-free [^{32}P]phosphate per dish in 15 ml of phosphate-free medium). CH_V was isolated by disruption of virions from five 75-cm^2 tissue culture flasks of ^{32}P-labeled infected BSC-1 cells (see Materials and Methods). A portion of CH_V was dialyzed into 11 mM Tris-hydrochloride (pH 7.4), 0.56 mM disodium EDTA, and 0.25% Triton X-100, added to a nuclear pellet from unlabeled infected BSC-1 cells, and subjected to the nuclear extraction procedure (labeled CH_{VT} in the figure). All three preparations were further purified by centrifugation through 15% sucrose onto a 60% sucrose cushion. Portions of each preparation were incubated with DNase I (concentration indicated at the top of each channel in milliunits per milliliter) in the presence of 2.0 mM $MnCl_2$ (incubation for 2.0 min at 37°C). SV40 DNA concentration was 1.3 μg/ml for CH_T, 0.8 μg/ml for CH_V, and 0.9 μg/ml for CH_{VT}. Enzyme digestion was stopped by addition of sodium dodecyl sulfate and EDTA to final concentrations of 1% and 20 mM, respectively. The mixture was heated 10 min at 50°C, and a portion from each was fractionated by electrophoresis on 1.4% agarose. DNA species were detected by autoradiography. Channel C for each series is a control without $MnCl_2$ or 37°C incubation.*

cleaved between map positions 0.66 and 0.73 (29, 31, 32, 37, 39–42). Subsequent cleavage by *Eco*RI produced clusters of DNA fragments of 3.5 to 3.8 kilobases (kb) (Fig. 4a) and 1.5 to 1.8 kb (not shown). *Taq*I gave clusters of 4.4 to 4.7 kb (Fig. 4b) and 0.5 to 0.8 kb (not shown). *Bam*HI gave clusters of 2.7 to 3.0 kb and 2.2 to 2.5 kb (Fig. 4c). *Bgl*I produced a cluster of 4.9 to 5.2 kb (Fig. 4d), with the remaining fragments smaller than 350 base pairs.

By contrast, CH_V did not show a region of highly preferred cleavage. Patterns observed with restriction enzymes (Fig. 4) were approximately those expected if initial cleavage oc-

curred randomly on circular SV40 DNA molecules, bearing in mind that the DNA was uniformly labeled and the migration distance varied inversely with the logarithm of the molecular weight. Closer inspection of CH_V patterns did reveal nonrandom features. Quantitative analysis of the distribution of radioactivity in CH_V after *Eco*RI cleavage (Fig. 4a) showed a preference for radioactivity between 3.7 and 5.2 kb by comparison with the distribution expected if initial DNase I cleavage had been randomly distributed (see references 31 and 42). A similar excess of large *BglI* fragments (Fig. 4d) supported a slight preference for DNase I cleavage between map positions 0.70 and 1.00. In addition, faint clusters of fragments occurred in CH_V with approximately the same size ranges as those seen prominently in CH_T. These clusters were more pronounced in the experiment shown in Fig. 5. Even in the latter case, however, the fraction of CH_V molecules cleaved in the 0.66 to 0.73 region was small by comparison with CH_T.

Patterns produced by *TaqI* digestion (Fig. 4b), but not by other restriction enzymes, showed additional specific cleavage sites around the viral genome. These sites were the same for CH_T and CH_V except in the region between map positions 0.66 and 0.73. If DNase I introduced staggered cuts at specific sites in the nucleoprotein molecules, discrete DNA fragments might be resolved only under the conditions used for *TaqI* digestion. This phenomenon was not extensively explored since it occurred to a variable extent in different *TaqI* reactions (see, for example, Fig. 7); however it was never observed in *TaqI* digests of linear SV40 DNA produced by the action of *BglI*, indicating that the site-specific cleavage was not generated by a component of the restriction enzyme preparation. Elucidation of this phenomenon will require further experiments.

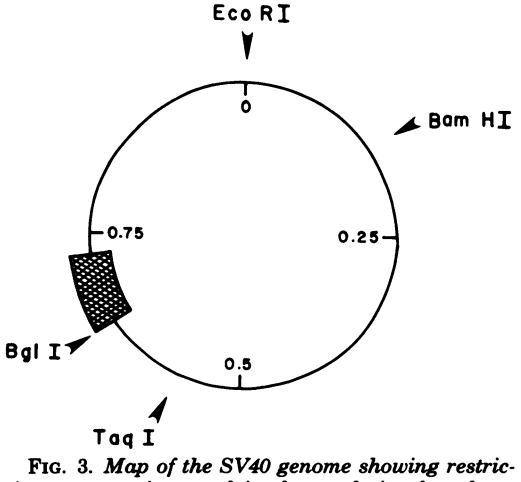

FIG. 3. *Map of the SV40 genome showing restriction enzyme sites used in the analysis of nuclease sensitivity. The region of preferential nuclease sensitivity in SV40 chromatin is indicated by the cross-hatched sector.*

Effect of more extensive disruption of virus particles. The sedimentation coefficient of CH_V depended on the time of incubation at 27°C. Incubation for 15 min gave a structure sedimenting at 120 to 130S (Fig. 1a). Incubation for 3 h gave a structure of approximately 80S. Since nuclease-sensitive features present in CH_V might be obscured in incompletely disrupted virus particles, portions of ^{32}P-labeled virus were disrupted for various time periods before sucrose

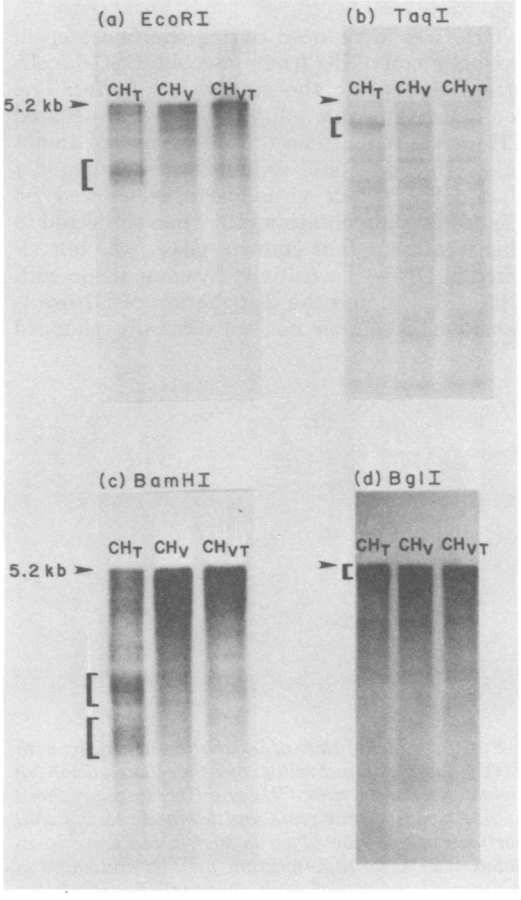

FIG. 4. *Location of sites in CH_T, CH_V, and CH_{VT} that are preferentially cleaved by DNase I. The three nucleoprotein preparations described in Fig. 2 were incubated with DNase I (5 mU/ml) as described in the legend to Fig. 2. Full-length linear SV40 DNA was isolated from each and digested with EcoRI (a), TaqI (b), BamHI (c), or BglI (d). Resulting DNA fragments were fractionated by electrophoresis on 1.4% agarose and detected by autoradiography.*

gradient isolation and DNase I cleavage. Subsequent restriction enzyme digestion gave patterns of DNA fragments which were independent of the length of the disruption period (Fig. 5). Occasionally, virion disruption complexes with lower sedimentation values were observed (e.g., approximately 50S in Fig. 6); however, the patterns of cleavage sites in such structures were not studied.

Exposure of CH_V to nuclear extraction conditions. Since CH_V and CH_T were isolated under substantially different conditions, the difference in distribution of nuclease-sensitive sites may have been due to differences in isolation procedures. To evaluate this possibility, CH_V was subjected to CH_T isolation conditions and vice versa.

CH_V was introduced during the first step in extraction of CH_T from infected BSC-1 cells after dialysis into the appropriate buffer. The sedimentation rate of CH_V (now designated CH_{VT}) was reduced as a result of this treatment (Fig. 1b), but it still sedimented more rapidly than CH_T. Intact virus particles showed no change in sedimentation rate when subjected to this treatment (not shown). CH_{VT} was not altered in DNase I sensitivity by comparison with CH_V (Fig. 2), and the distribution of DNase I-sensitive sites was not substantially changed (Fig. 4); however, a small fraction of the molecules in CH_{VT} appeared to have acquired preferential DNase I cleavage sites in the region 0.66 to 0.73 by comparison with CH_V.

Exposure of CH_T to virion disruption conditions. Nuclease sensitivity in the 0.66 to 0.73 region might be present in nucleoprotein in virus particles but be lost as a result of the disruption procedure. To evaluate this possibility, CH_T was dialyzed into the buffer used for storage of virus suspensions and was subjected to virion disruption conditions. Sedimentation properties of CH_T were not altered by this treatment (Fig. 6), although ^3H-labeled virions in the same mixture were effectively disrupted. The sedimentation coefficient of CH_T (approximately 55S in this experiment) was reduced by comparison with results shown in Fig. 1, presumably due to the

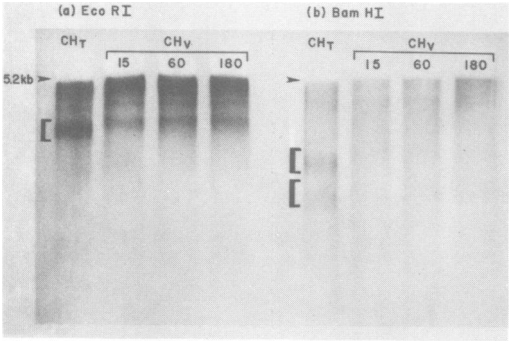

FIG. 5. *Effect of time of incubation of virions with EGTA and dithiothreitol on the distribution of DNase I-sensitive sites. CH_T and CH_V were prepared as described in Materials and Methods except that portions of ^{32}P-labeled virus suspension were incubated at 27°C for different time periods (indicated in minutes at the top of each channel). Resulting nucleoprotein sedimented approximately at 70S (CH_T), 130S (15-min CH_V), 130S (60-min CH_V), and 80S (180-min CH_V). The four nucleoprotein preparations were incubated with DNase I as described in the legend to Fig. 2; full-length linear SV40 DNA was isolated from each and digested with EcoRI (a) or BamHI (b). Resulting DNA fragments were fractionated by electrophoresis on 1.4% agarose and detected by autoradiography.*

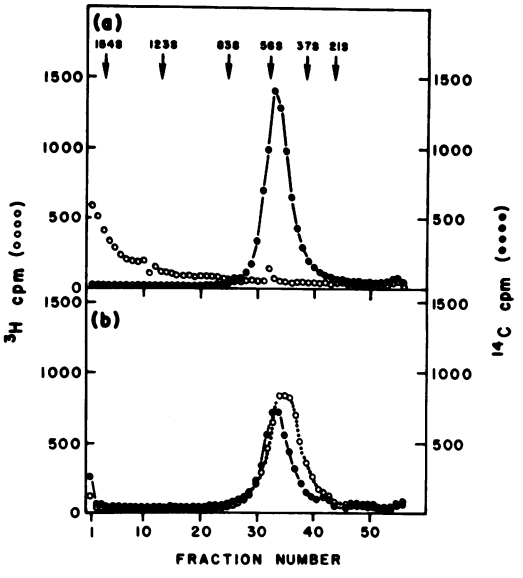

FIG. 6. *Effect of the virion disruption procedure on the sedimentation properties of CH_T. CH_T was prepared from two 150-mm petri dishes of BSC-1 cells labeled 24 to 42 h postinfection with [^{14}C]thymidine (30 μCi/dish in 15 ml of medium; specific activity, 40.8 Ci/mmol), fractionated on a 5 to 20% sucrose gradient (see Materials and Methods), and dialyzed into TN buffer. ^3H-labeled virus particles were added and the mixture was (a) fractionated without further treatment or (b) subjected to the virion disruption procedure described in Materials and Methods (incubation for 30 min at room temperature). Both samples were fractionated in 15 to 30% sucrose gradients in TENT buffer as described in the legend to Fig. 1. Fractions were collected on filter disks and precipitated with trichloroacetic acid before counting. Sedimentation standards were those described in Fig. 1 and, in addition, disomes (123S) and trisomes (154S) from BSC-1 cells (26).*

conditions of dialysis and recentrifugation. This shift has previously been reported to be a result of a number of factors (summarized in reference 14). There was no change in the extent of DNase I cleavage at a given enzyme concentration (not shown), and DNase I still cleaved preferentially in the 0.66 to 0.73 region of the genome (Fig. 7), indicating that the nuclease-sensitive feature characteristic of CH_T was not destroyed by the treatment.

DISCUSSION

The nuclease-sensitive feature near the origin of replication which is characteristic of SV40 chromatin extracted from nuclei of infected cells is much less in evidence in SV40 nucleoprotein released from virus particles. Application of the virion disruption procedure to SV40 chromatin does not alter the sedimentation rate or the overall DNase I sensitivity and does not abolish specific DNase I cleavage between map positions 0.66 and 0.73.

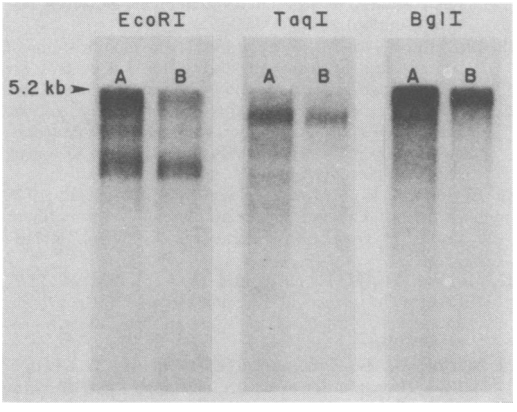

FIG. 7. *Location of DNase I-sensitive sites in CH_T and in CH_T subjected to virion disruption conditions. CH_T was isolated from 10 90-mm petri dishes of BSC-1 cells labeled 24 to 42 h postinfection (100 μCi of carrier-free [^{32}P]phosphate per dish in 5 ml of medium lacking phosphate). A portion (A) of pooled peak fractions from the 5 to 20% sucrose gradient was dialyzed into TN buffer, adjusted to conditions for virus particle disruption (see Materials and Methods), and incubated for 30 min at room temperature. A second portion (B) was diluted in TN buffer. Both preparations were further purified by centrifugation through 15% sucrose onto a 60% sucrose cushion. After dialysis against TENT buffer, DNase I (A, 1 mU/ml; B, 5 mU/ml) and $MnCl_2$ (2.0 mM) were added and the samples were incubated for 2.0 min at 37°C. Full-length linear SV40 DNA was isolated as described in Materials and Methods, digested with the restriction enzymes indicated, fractionated by electrophoresis on 1.4% agarose, and detected by autoradiography.*

It has been reported (41) that a subfraction of virion DNA is derived from molecules which are linear before encapsidation, with ends predominantly mapping between 0.69 and 0.70 map units. As much as 8% of the form III DNA observed after DNase I digestion could have preexisted in the virus preparation (Fig. 2; compare channels CH_V-C and CH_V-5). Faint clusters observed in CH_V^* (Fig. 4 and 5) which correspond to cleavage in the 0.66 to 0.73 region may be accounted for by this preexisting linear DNA. We cannot, however, exclude the possibility that the nuclease-sensitive feature persists in a small and variable portion of the virus population.

Virion-derived nucleoprotein sedimented more slowly after being subjected to the Triton X-100–EDTA treatment in the presence of infected cell nuclei. This change can be accounted for, at least partly, by the presence of a chelating agent, since extended treatment with EGTA and dithiothreitol yields species with a reduced sedimentation rate. The extent of digestion with DNase I was not substantially altered, nor was the distribution of DNase I-sensitive sites. One hope for this type of experiment is that the normal virion uncoating process might occur in a cell-free preparation, producing a nucleoprotein structure similar to that which functions in the early stages of infection. This approach might be successful if appropriate incubation conditions could be found; however, recent results by Winston et al. (43) suggest that virion-derived nucleoprotein is initially incorporated into a complex intranuclear structure.

Recently, several investigators (1, 9, 12–14, 17, 19, 33) have shown that different extraction procedures give rise to more than one nucleoprotein species from infected cells. A substantial portion has the sedimentation properties of mature virus and consists of a mixture of mature virus and late assembly intermediates (1, 9, 12, 17, 19). CH_T in our experiments is derived in part from these rapidly sedimenting forms. Experiments in this laboratory (J. P. Hartmann and W. A. Scott, manuscript in preparation) show that 210 to 220S particles obtained from infected cell nuclei by the method of Fernandez-Munoz et al. (13) can be disrupted by the EGTA–dithiothreitol treatment as are purified virions. The resulting nucleoprotein is much less sensitive to DNase I in the 0.66 to 0.73 region of the genome than is viral chromatin prepared by the Triton X-100–EDTA method. By contrast, 75S species recovered in the same experiments are sensitive to DNase I cleavage and have a stronger preference for the 0.66 to 0.73 region than does CH_T. This

latter subpopulation accounts for most of the preferential DNase I sensitivity seen in CH_T in the experiments reported here. Complexes active in replication and transcription are included with the 75S peak (1, 12–14, 17).

From these results, it seems likely that by nuclease sensitivity we have detected a unique chromatin structure which is altered in vivo during virion packaging. The disappearance of preferential nuclease sensitivity during assembly and maturation of virus particles argues against a role for this feature immediately upon release of viral nucleoprotein in an infected cell. It seems more likely that the nuclease-sensitive feature is acquired at some time after virion uncoating. Host cell proteins, virus-coded proteins, or both may be involved.

The location of the cluster of nuclease-sensitive sites and the stage of the infectious cycle when this feature has been detected suggest a role in viral late-gene transcription. The specific configuration probably persists in SV40 chromatin molecules not actively involved in transcription and is much reduced in mature virus particles.

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