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Nuclease-Sensitive Sites in the Two Major Intracellular Simian Virus 40 Nucleoproteins

JOHN P. HARTMANN AND WALTER A. SCOTT*

Department of Biochemistry, University of Miami School of Medicine, Miami, Florida 33101

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Simian virus 40 nucleoprotein isolated from the nuclei of infected cells contains a nuclease-sensitive site adjacent to the viral origin of replication (between 0.66 and 0.73 map unit). Nuclear extracts were subfractionated by sucrose gradient centrifugation to yield provirions (200S) and simian virus 40 chromatin (80S). The 80S fraction was cleaved either by DNase I or by an endonuclease endogenous to BSC-1 cells with high preference for the 0.66 to 0.73 region. The 200S fraction was treated to release core particles that were sensitive to nuclease cleavage; however, DNase I showed little or no preference for the 0.66 to 0.73 region of the provirion core nucleoprotein.

Simian virus 40 (SV40) chromatin contains a region which is not associated with typical nucleosome structures (12, 17). This region (ca. 400 base pairs located on the late side of the viral origin of replication) is preferentially cleaved by endonucleases (18, 22, 23). Recently, isolation procedures have been described (1, 2, 8, 9, 11, 13) that yield nucleoprotein complexes sedimenting at about 200S (provirions or intracellular viruses) and 80S (SV40 chromatin or minichromosomes) (Fig. 1). We investigated each of these subfractions and concluded that the nuclease-sensitive site is a prominent feature of the 80S subfraction but is not observed in the 200S subfraction.

To prepare nucleoprotein fractions, BSC-1 cells (propagated in minimal essential medium supplemented with 10% newborn calf serum [Biocell] at 37°C in an atmosphere of 5% CO_2) were infected with wild-type SV40 (strain 776; multiplicity of infection, 3 to 4) and incubated in the same medium containing 2% newborn calf serum. Viral DNA was labeled with [^{32}P]phosphate (ICN) in phosphate-free medium from 24 to 42 h after infection. Nucleoproteins were extracted from nuclei isolated 42 h after infection by the isotonic method of Fernandez-Munoz et al. (8).

80S nucleoprotein (Fig. 1) was incubated with DNase I, staphylococcal nuclease, or endogenous endonuclease (added as a crude Triton-EDTA extract of nuclei from uninfected cells). Full-length linear DNA was isolated, and the distribution of nuclease cleavage sites was determined by redigestion with *Bam*HI or *Eco*RI (Fig. 2). DNase I and endogenous nuclease cleaved preferentially in the 0.66 to 0.73 region. Endogenous nuclease was more selective for this region than was DNase I (18). Staphylococcal nuclease did not cleave selectively within the 0.66 to 0.73 region of chromatin—in fact, there is some indication that the region was preferentially avoided by this enzyme. This finding is in contrast to some previous reports (6, 21) but is in agreement with others (15, 19). It is possible that differences in incubation conditions could account for this discrepancy. We did not attempt to determine whether this enzyme would become selective under other incubation conditions; however, the fact that staphylococcal nuclease and DNase I gave strikingly different cleavage patterns, even when we carried out the reactions under identical conditions (data not shown), may suggest that the 0.66 to 0.73 region is not simply a stretch of naked DNA but, rather, consists of nucleoprotein which is more accessible to DNase I than to staphylococcal nuclease. The nature of proteins located in this region is of considerable interest.

When the 80S nucleoprotein recovered in these experiments was compared with a similar subfraction prepared by a procedure using Triton X-100 and EDTA under hypotonic conditions (18), it was evident that the degree of specific cleavage by either DNase I or endogenous nuclease was greater for chromatin isolated by the isotonic method. Endogenous nuclease cleaved within the 0.66 to 0.73 region ca. 43% of the time (densitometric analysis of the data shown in Fig. 2), as compared with 26% specific cleavage when 80S nucleoprotein was recovered from parallel cultures by the Triton-EDTA procedure. The amount of SV40 DNA recovered in the 80S subfraction was similar by the two procedures (3 to 4 μg per 150-mm culture dish), but different subpopulations may have been rep-

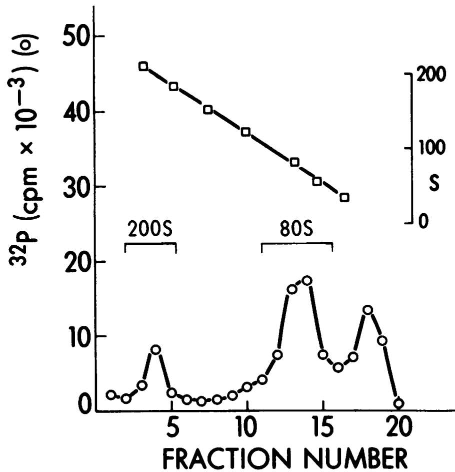

FIG. 1. Sedimentation of viral nucleoproteins extracted from nuclei of infected cells. Nuclear extracts were prepared from infected BSC-1 cells (labeled from 24 to 42 h postinfection with 50 μCi of [^{32}P]phosphate per 150-mm culture dish) by the isotonic method of Fernandez-Munoz et al. (8). Cells from each culture dish were scraped into 3 ml of TD buffer (136 mM NaCl, 7 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris-hydrochloride, [pH 7.4]) and lysed by the addition of an equal volume of TD buffer containing 0.5% NonidetP-40 (Bethesda Research Laboratories). The suspension was gently mixed; nuclei were collected by brief centrifugation at 4°C and suspended in TD buffer (1×10^8 to 1.5×10^8 nuclei per ml). The suspension was homogenized at 0°C (30 strokes, Thomas tissue grinder with a Teflon pestle), and nuclei were removed by centrifugation (Sorvall SA600 rotor, 7,000 rpm, 15 min, 4°C). The supernatant solution was fractionated by centrifugation into a 15 to 30% sucrose gradient in TD buffer (120 min, 35,000 rpm, 4°C, SW41 Spinco rotor). Radioactivity was determined by Cerenkov radiation counting. Sedimentation standards included ribosomal subunits (56S and 37S), monosomes (83S), disomes (123S), trisomes (154S), tetrasomes (183S), and pentasomes (211S) fractionated in parallel 15 to 30% sucrose gradients in 50 mM Tris-hydrochloride (pH 7.4)–200 mM KCl or 400 mM KCl–5 mM $MgCl_2$ (3).

resented. For example, it has been reported (8, 9, 20) that provirions and virus particles are disrupted during some extraction procedures to give rise to nucleoproteins which sediment at about 80S; however, it is not clear that such breakdown occurred during either of our extraction procedures since a stable 200S component was recovered in each case. It is also possible that some of the 80S nucleoprotein was damaged during the Triton-EDTA extraction. Whatever the reason for the difference in preferential nuclease cleavage between material prepared by these two methods, the isotonic procedure yields nucleoproteins that are more suitable for study. Extracts prepared by this method contained less nonviral material (as judged by gel electrophoresis and ethidium bromide staining), and DNA in these extracts showed less evidence of endonucleolytic damage than in Triton-EDTA extracts.

The 200S fraction was not susceptible to cleavage by even high concentrations of DNase I. To determine the pattern of nuclease-sensitive sites in this fraction, core nucleoprotein was released by the method of Brady et al. (5) (Fig. 3). Purified virions yielded 110S core particles within 5 min of treatment. The sedimentation coefficient decreased slightly (to about 90S) with extended treatment. Intracellular 200S nucleoprotein was disrupted more slowly than purified virions present in the same mixture, and the early disruption products had heterogeneous sedimentation properties; however, after 60 min of treatment, most of the radioactivity sedimented as a distinct peak of 100S.

At least 75% of the radioactivity from the provirion fraction sedimented more rapidly than that from purified virions after brief ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid–dithiothreitol (EGTA-DTT) treatment (Fig. 3B), indicating that most of the intracellular 200S nucleoprotein is distinguishable from CsCl gradient-purified virions. Brady et al. (4) have also found that core nucleoprotein from provirions is distinguishable from that released from extracellular virus; however, in their experiments, the provirion core particles sedimented more slowly (75S for the nucleoprotein from provirions; 110S for that from extracellular virions). Other investigators (2, 7, 11) have reported that a variable portion of the intracellular virus is disrupted by salt concentrations in excess of 1 M. Even those provirions that survive treatment with high salt concentrations may become salt-labile when pretreated with EDTA in low salt concentrations (13). It seems likely that the intracellular 200S fraction is heterogeneous and that subpopulations of provirions may be differentially recovered by different isolation procedures.

Disruption of the 200S provirion particles has allowed analysis similar to that carried out with the 80S complex (Fig. 4). DNase I showed little or no preferential cleavage within the 0.66 to 0.73 region as has been previously observed with core particles isolated from purified virions (10). The nuclease-sensitive feature in 80S SV40 chromatin is not abolished when that fraction is subjected to the EGTA-DTT treatment (10). These results indicate that provirion core particles are structurally distinct from SV40 chromatin; however, it is not possible to conclude that the nuclease-sensitive feature is lost during provirion assembly. The nuclease-sensitive site

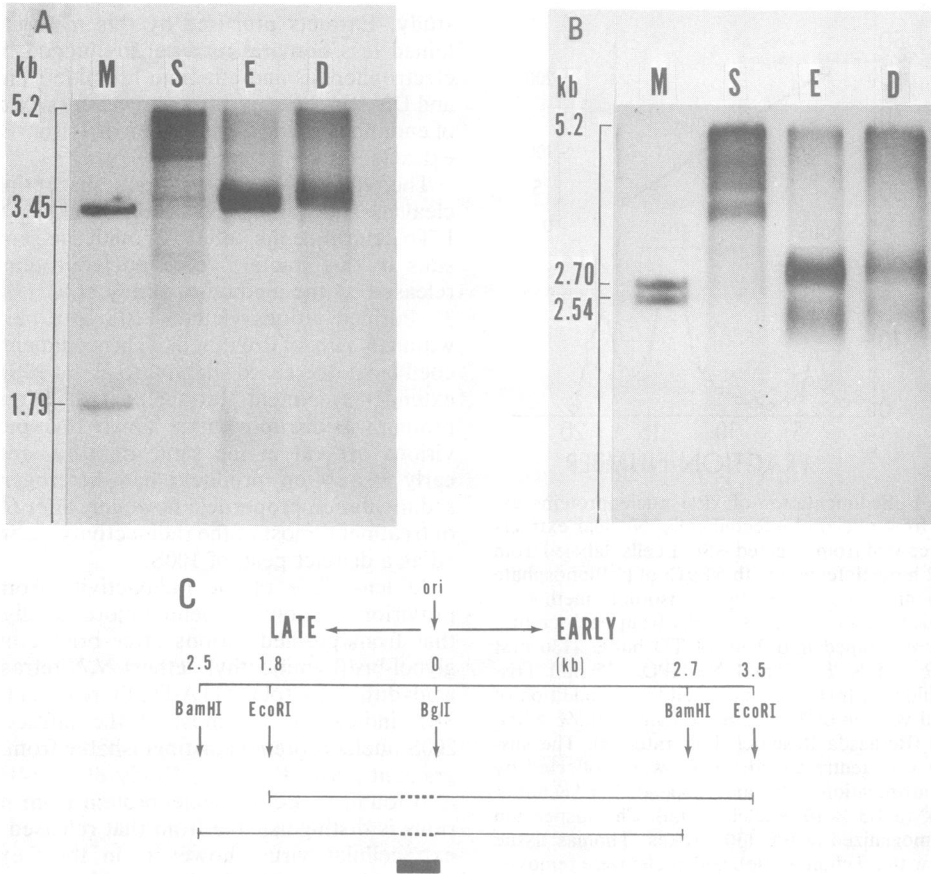

FIG. 2. Locations of sites in 80S nucleoprotein that were preferentially cleaved by nucleases. SV40-infected BSC-1 cells were labeled from 24 to 42 h postinfection with 200 μCi of [^{32}P]phosphate per 150-mm culture dish. 80S nucleoprotein was prepared as described in the legend to Fig. 1, dialyzed against TENT buffer (10 mM Tris-hydrochloride [pH 7.5], 0.5 mM disodium EDTA, 30 mM NaCl, and 0.17% Triton X-100), and incubated with nucleases under conditions which gave the maximal yield of full-length linear SV40 DNA: for DNase I (D), 0.01 U/ml for 2 min at 37°C; for staphylococcal nuclease (S), 1.0 U/ml for 10 min at 5°C. Endogenous endonuclease (E) was prepared from nuclei isolated from confluent, uninfected BSC-1 cells by Triton-EDTA extraction (18) and added to sucrose gradient-purified 80S nucleoprotein as one-third of the incubation volume. Incubation was carried out for 60 min at 37°C. Cleavage of SV40 nucleoprotein by bovine pancreatic DNase I (2,812 U/mg, type D; Worthington Biochemicals Corp.), staphylococcal nuclease (also called micrococcal nuclease, 15,000 U/mg, type NFCP; Worthington Biochemicals Corp.), and the nuclease endogenous to BSC-1 cells has been described previously (18). Full-length linear SV40 DNA was isolated (18) from each digestion mixture and redigested with *Eco*RI (A) or with *Bam*HI (B). Resulting DNA fragments were fractionated by electrophoresis on 1.4% agarose and detected by autoradiography. Standards (M) were circular SV40 DNA digested with a mixture of *Eco*RI and *Bgl*I (3.45 and 1.79 kilobases [kb]) or with a mixture of *Bam*HI and *Bgl*I (2.70 and 2.54 kb). (C) DNA fragments expected from cleavage within the region spanning 400 base pairs on the late side of the origin of replication (*ori*) (indicated by the filled bar, bottom), followed by cleavage with *Eco*RI or *Bam*HI.

may not be revealed, owing to proviron structural features which persist even after disruption with EGTA-DTT. Alternatively, the nucleoprotein structure elsewhere on the viral genome may be highly accessible to nuclease cleavage in core nucleoprotein so that we cannot differentiate between the 0.66 to 0.73 region and the rest of the viral genome by nuclease sensitivity.

Our results are in agreement with recent reports which indicate that nucleoprotein contained in virus particles and provirions is quite different from SV40 chromatin. Core nucleoprotein from these structures is substantially more accessible to nuclease digestion (4) and does not show typical nucleosome structure by electron microscope observation (14). Unlike SV40 chro-

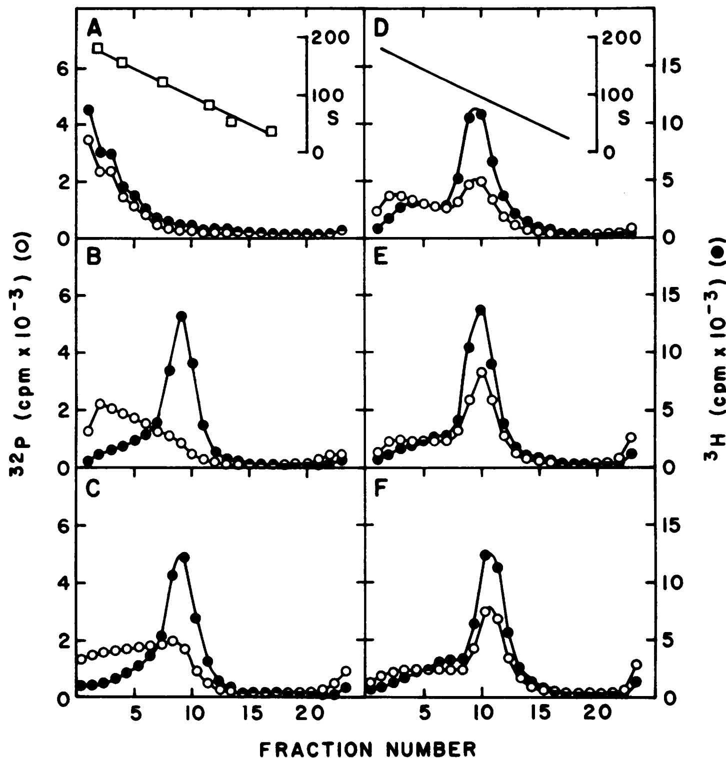

FIG. 3. Effect of time of incubation with EGTA-DTT on the sedimentation of nucleoprotein released from 200S provirions and from CsCl-purified virus particles. ^{32}P-labeled 200S nucleoprotein (○) isolated as described in the legend to Fig. 1 was mixed with ^{3}H-labeled CsCl-purified virus particles (●) and dialyzed against 1 mM Tris-hydrochloride (pH 7.4)–50 mM NaCl. A portion of this mixture was fractionated without further treatment (A). Additional portions were adjusted to conditions which release core nucleoprotein from virions (5) (incubation condition: 10 mM Tris-hydrochloride [pH 8.6], 150 mM NaCl, 1 mM disodium EGTA, and 3 mM DTT) and incubated at 27°C for 5 min (B), 15 min (C), 30 min (D), 60 min (E), or 180 min (F). At the end of these incubation periods, each mixture was fractionated by centrifugation through 15 to 30% sucrose (in TENT buffer) for 150 min at 35,000 rpm and 4°C (SW50.1 Spinco rotor). Sedimentation markers are described in the legend to Fig. 1. Core nucleoprotein from provirions (unlike core particles release from purified virions) readily adsorbed to dialysis tubing, glass surfaces, etc. To prevent this, dialysis tubing and nitrocellulose centrifuge tubes were treated with a solution of 1% bovine serum albumin and 0.02% Ficoll 400 (Pharmacia Fine Chemicals) at 45°C for 1 h before use. Glass containers were treated briefly with 1% dimethyldichlorosilane in carbon tetrachloride and rinsed before use. When these precautions were taken, recovery of DNA from the 200S fraction and that from the 80S fraction through subsequent steps was approximately equal (ca. 45%).

matin (16), virions and provirions do not show preferential reactivity in the 0.66 to 0.73 region when photolabeled with radioactive psoralen; and the extent of psoralen photoaddition is greater with SV40 DNA in virions or provirions than it is with SV40 chromatin (L. Hallick, personal communication).

The absence of the nuclease-sensitive site in core particles isolated from provirions and the enrichment for this feature in 80S nucleoprotein isolated by a procedure in which provirions are not disrupted suggest a role for this structure in the viral infection before incorporation of the genome into provirions.

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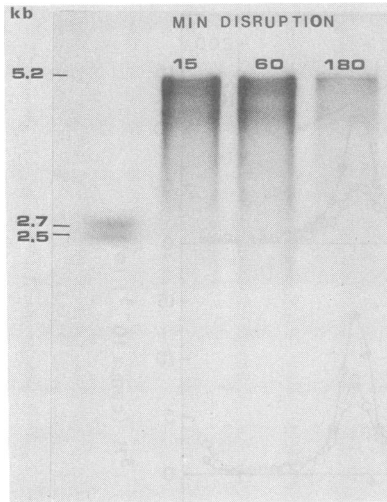

FIG. 4. Distribution of DNase I cleavage sites in 200S nucleoprotein after disruption with EGTA-DTT. ^{32}P-labeled 200S provirions were prepared from infected BSC-1 cell nuclei and treated as described in the legend to Fig. 3. Each mixture was centrifuged into a 5 to 20% sucrose gradient (in TENT buffer) for 120 min (35,000 rpm, 4°C, SW41 Spinco rotor). Pooled peak fractions were dialyzed against TENT buffer and incubated with DNAse I (0.005 U/ml) for 2 min at 37°C. Full-length linear DNA was isolated from each incubation mixture, redigested with *Bam*HI, and fractionated by electrophoresis on 1.4% agarose. Markers are the same as in Fig. 2B. kb, Kilobases.

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