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SITES INCLUDING THOSE OF ORIGIN AND TERMINATION OF REPLICATION ARE NOT FREELY AVAILABLE TO SINGLE-CUT RESTRICTION ENDONUCLEASES IN THE SUPERCOMPACT FORM OF SIMIAN VIRUS 40 MINICHROMOSOME*

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Summary — Simian virus 40 minichromosomes, isolated from both virions (MV) and infected cells (MI), have highly compact structures in buffer containing 0.15 M NaCl and sediment with S values of about 90–100 and 115–130, respectively. Under the electron microscope, also, MI appear the more compact of the two. Only 30–35% of the sites of origin and termination of replication in MV are freely available to the restriction endonucleases Bgl I and Bam HI. MV are similarly resistant to Eco RI and Hpa II. In contrast, almost no sites in MI are available to any of the above single-cut endonucleases. In 0.6 M NaCl, MV and MI change to relaxed structures of 45–55 S and 50–60 S, respectively, containing 20–24 nucleosomes per genome, and become more sensitive to Bgl I, Bam HI, Eco RI, and Hpa II.

In nuclei of lytically infected cells, SV40‡ DNA is associated with cellular histones in a chromatin-like structure called a minichromosome (1–4). The SV40 minichromosome inside the host cell is replicated and transcribed by use of the cellular enzymatic machinery (5–7). The nucleoprotein complexes, isolated from both mature virions and infected cells, have a subunit organization very similar to that of the cellular chromatin (1, 2). The SV40 chromatin contains about 20–24 nucleosomes (visualized when relaxed) and all five cellular histones, including histone H1 (3, 8). Under physiological salt conditions, it is possible to isolate a very compact form of the minichromosome that could be accommodated inside the viral capsid (4, 9). This compact form may closely resemble the form of the minichromosome inside the host cell nuclei.

The specificity of the nucleosomal arrangement and the availability of "active" regions in the SV40 minichromosome have been studied with a number of restriction endo-

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‡Abbreviations used: SV, simian virus; MV, minichromosomes from mature virions; MI, minichromosomes from infected cells; p.i., post infection; Tris, tris (hydroxymethyl) aminomethane; PMSF, phenylmethylsulfonylfluoride; EDTA, ethylenediaminetetraacetate.

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nucleases which cleave the SV40 genome at specific sites. However, the data available (10–14) are contradictory and do not permit definitive conclusions. We believe that the differences in results are partly due to the lack of similarity in the SV40 chromatin preparations, particularly the compactness of their structures.

Here, we describe conditions for the isolation of compact forms of the SV40 minichromosome from both virions and infected cells. MV are partially resistant to Bgl I and Bam HI, suggesting that the sites of origin and termination of replication are not freely available; they are also partially resistant to Hpa II and Eco RI. MI, which are more compact than MV, as judged by sedimentation and electron microscopy, are totally resistant to all four restriction endonucleases. However, both MV and MI at high (0.6 M NaCl) salt concentration undergo transition to lower-sedimenting, more relaxed structures containing 20–24 nucleosomes per genome and are more susceptible to the restriction endonucleases.

MATERIALS AND METHODS

Growth and labeling of cells and virions. African green monkey kidney cells (BSC-1; obtained as a gift from Dr. T. J. Kelly, Jr., Johns Hopkins University) were grown in roller bottles, each containing 100 ml of Eagle's minimum essential medium (Grand Island Biological Co.) supplemented with 10% fetal bovine serum (Grand Island Biological Co.), 2 mM L-glutamine (Schwarz/Mann), 100 units penicillin G/ml (Squibb), 100 μg streptomycin sulfate/ml (Lilly), 50 μg gentamicin sulfate/ml (Sigma), and 2.5 mM $NaHCO_3$ (complete growth medium). When cell monolayers became 80% confluent, the medium was removed from each bottle and replaced with 21 ml of the above medium except having 2% fetal bovine serum (virus growth medium); the cells were then infected with SV40 (a gift from Dr. T. J. Kelly, Jr.) suspended in 4 ml of complete growth medium at a multiplicity of 20 pfu/cell. After 2 hr, to each bottle were added 75 ml of virus growth medium (final volume, 100 ml). The cells were labeled with [methyl-^{14}C]thymidine (50 mCi/mM, Schwarz/Mann) to a final radioactivity of 0.2 μCi/ml at 24–26 hr p.i. At 36–48 hr p.i., the cells were harvested for the isolation of viral chromatin. When purification of mature virions was desired, the cells were labeled with [^{3}H]thymidine (14 Ci/mM, Schwarz/Mann) to a final radioactivity of 1–2 μCi/ml at 24–26 hr p.i. and harvested at 72–80 hr p.i. The virions were purified and collected by 2–3 CsCl gradients (25–40%) containing 10 mM Tris-HCl (pH 7.4).

Enzymes. The restriction endonucleases Bgl I, Eco RI, Bam HI, and Hpa II were purchased from New England Biolabs.

Isolation of SV40 chromatin. (i) From infected cells: Cells in each roller bottle were washed with 50 ml of 1 mM Tris-HCl (pH 6.8) containing 0.14 M NaCl, harvested in 50 ml of 10 mM Tris-HCl (pH 7.4) containing 0.5 mM $MgCl_2$, and centrifuged immediately (5000 rpm for 5 min in a Sorvall centrifuge). They were resuspended in 10 ml of the same medium for another 15 min for swelling, then homogenized in a tight-fitting glass homogenizer (10–12 strokes). An equal volume of 10 mM Tris-HCl (pH 7.4), containing 0.5 M sucrose, 0.3 M NaCl, and 0.1 mM PMSF, was immediately added to the homogenate to prevent disruption of the nuclei. Nuclei were collected by low-speed centrifugation

(6000 rpm for 5 min) and were disrupted by gentle stirring for 1 hr in 10 mM Tris-HCl (pH 7.4) containing 0.25% Triton, 0.15 M NaCl, 1 mM EDTA, and 0.1 mM PMSF. The cellular chromatin was first removed by centrifugation at 15,000 rpm for 15 min. The viral chromatin in the supernatant was immediately purified by sucrose (5–20%) gradient centrifugation in an SW41 rotor for 1 hr at 39,000 rpm in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1 mM PMSF, 0.25% Triton, and either 0.15 or 0.6 M NaCl. All operations were performed at 0–4°C.

(ii) From mature virions: Purified virions (0.4 ml in 10 mM Tris-HCl, pH 7.4) were suspended in 0.6 ml of the following buffer for 2 hr at 4°C for complete disruption: 0.15 M Tris-HCl (pH 9.3) containing 20 mM dithiothreitol, 10 mM EDTA, 1 mM PMSF, and 0.10 M NaCl. The viral chromatin was then purified by sucrose density gradient centrifugation as described above, except that no Triton was present.

Digestion of minichromosomes with restriction endonucleases. The fractions from the sucrose gradients containing the viral chromatin were used either directly or after dialysis for 12 hr against 10 mM Tris-HCl (pH 7.4). In either case, the final composition of the digestion buffer was 10 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA, 8 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.1 mM PMSF. Approximately 20-fold more restriction endonuclease was used than was required to completely digest an equal quantity of purified SV40 DNA under identical conditions. Digestions were carried out at 37°C for 2 hr. The reaction was stopped by the addition of EDTA (pH 7.5) to a final concentration of 15 mM. The percentage of the minichromosomes cleaved by the endonucleases was estimated by scoring for linear DNA molecules under the electron microscope after the dissociation of the histones in 2 M NaCl.

Electron microscopy. Electron microscopy of the chromatin samples was done following the method of Dubochet et al. (15) as modified by de Murcia et al. (16). Spreading of the DNA was done as described by Inman and Schnös (17).

RESULTS AND DISCUSSION

Characterization of the minichromosomes. Fig. 1 shows the sedimentation behavior in buffer containing 0.15 M NaCl (solid lines) and 0.6 M NaCl (dashed lines) of the minichromosomes isolated from mature virions and from infected cells. The slowly sedimenting material at the top of the gradient for MI (possibly derived from degraded cellular material) is absent in the case of MV. In buffer containing 0.15 M NaCl, the sedimentation coefficients for MV and MI are 90–100 S and 115–130 S, respectively, with T7 DNA (a gift of Dr. Warren Masker, Biology Division, ORNL) as marker (S = 31.5). All five histones, including histone H1, were present in both samples, as determined by sodium dodecylsulfate–gel electrophoresis (data not shown).

When visualized under the electron microscope (Fig. 2), both MV (Fig. 2a) and MI (Fig. 2b) in 0.15 M NaCl appear as compact structures of globular shape. MV often appear as clusters. The MI are more compact, which is consistent with the respective S values. Relaxed structures of 45–55 S from MV and 50–60 S from MI (dashed lines in

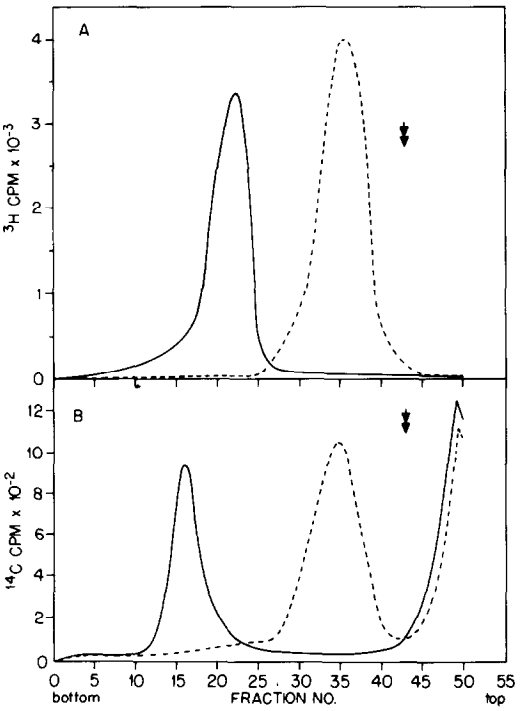

Fig. 1. Sedimentation profiles of (A) MV (labeled with [^{3}H]thymidine) and (B) MI (labeled with [^{14}C]thymidine) in a 5–20% sucrose gradient containing 0.15 M NaCl (——) or 0.6 M NaCl (-----). The marker T7 DNA (S = 31.5) is indicated by double arrows.

Fig. 1) containing 20–24 nucleosomes per genome and separated by spacers of varying length, can be obtained as follows: (a) purification of either MV (Fig. 2c) or MI (Fig. 2d) in a sucrose gradient containing 0.6 M NaCl, or (b) dialysis of compact forms against 2 mM Tris-HCl (pH 7.4) for 36–48 hr or treatment with 0.6 M NaCl (data not shown). 0.6 M NaCl is believed to remove histone H1 and nonhistone proteins (4, 18). The relaxation of the structure is in general agreement with the observations of Müller et al. (18), although different degrees of relaxation are apparent in the case of MI (Fig. 2d), for reasons that are not yet clear.

Digestion of SV40 minichromosomes with restriction endonucleases. Bgl I and Bam H1 are known (19) to cleave the SV40 DNA at map positions 0.67 and 0.16, roughly corresponding to the sites where the replication of the genome is initiated and terminated, respectively [assuming equal rates of bidirectional replication (19)]. The cleavage sites for Eco R1 and Hpa II are at positions 0.0 and 0.735 on the genetic map (19). Under the

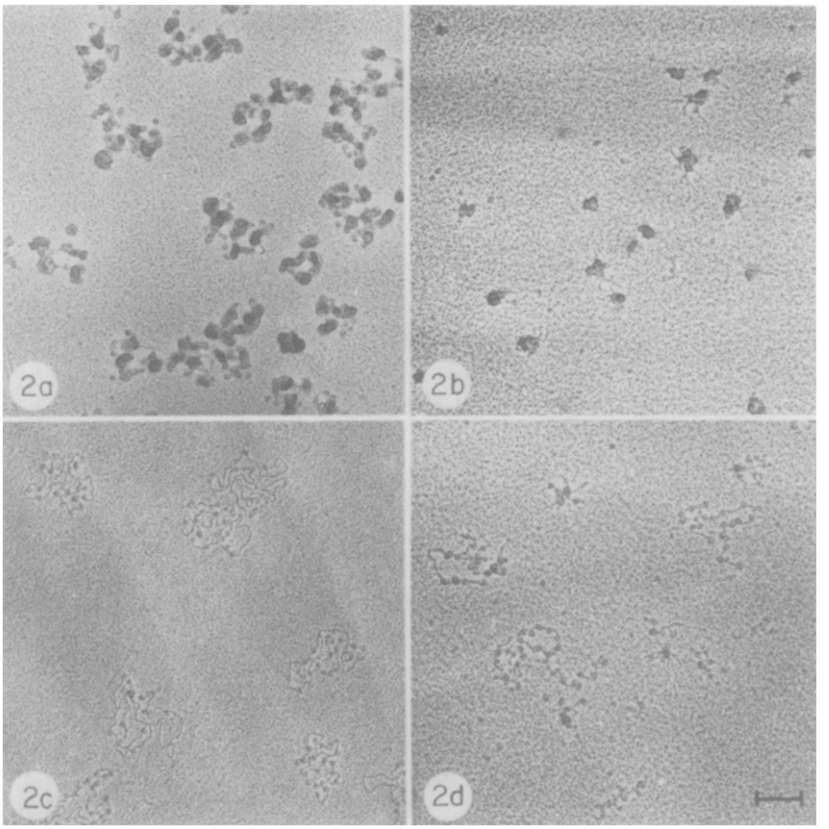

Fig. 2. Electron micrographs of minichromosomes purified in buffer containing: 0.15 M NaCl and undialyzed, (a) MV, (b) MI; or 0.6 M NaCl, then dialyzed against 10 mM Tris-HCl, pH 7.4, (c) MV, (d) MI. Electron micrographs of samples in (a) and (b), dialyzed against 10 mM Tris-HCl (pH 7.4), showed unaltered structures (data not shown). Bar is equal to 100 nm.

limit-digest conditions (see Materials and Methods), the results of control reactions were as follows: (a) Practically all the naked SV40 DNA molecules are cleaved by the restriction endonucleases (Table I). (b) Incubation of the samples in digestion buffer without the restriction endonuclease does not alter the chromatin structure (data not shown). (c) The endonuclease activity associated with the SV40 chromatin (20, 21) cleaves the DNA up to a maximum of 20–25% of the molecules (Table I). The results show that in the compact form of MV, at best 30–40% of the sites are available to the restriction endonucleases. On the other hand, in MI, which are even more compact, practically no sites are available. Both the above forms, on relaxation with 0.6 M NaCl, become more sensitive to the restriction nucleases, although the extent of cleavage is somewhat variable (details to be published elsewhere).

TABLE I. Biophysical Properties of Different Forms of SV40 Chromatin and and Availability of Their Sites to Restriction Endonucleases

Chromatin extracted from	Molarity of NaCl in sucrose gradient	Further treatment	S values	% of molecules cleaved by*				
				Endogenous activity	Bgl I	Hpa II	Eco RI	Bam HI
Mature virions	0.15	none	90–100	14	46(32)	47(33)	47(33)	49(35)
	0.15	dialyzed†	same	18	47(29)	52(34)	57(39)	49(31)
	0.6	dialyzed†	45–55	25	76(51)	87(62)	80(55)	62(37)
Infected cells	0.15	none	115–130	20	26(6)	26(6)	20(0)	8(-12)
	0.15	dialyzed†	same	20	20(0)	18(-2)	8(-12)	12(-8)
	0.6	dialyzed†	50–60	25	71(46)	90(65)	65(40)	55(30)
SV40 DNA					99	98	100	97

*Numbers in parentheses were obtained by subtracting the values for the endogenous endonucleolytic activity. At least 100 molecules were scored in each case.

†Against 10 mM Tris-HCl (pH 7.5).

The structure of chromatin, both viral and cellular, depends on its method of preparation (4, 16). In the case of SV40 chromatin, S values ranging from as low as 35 to as high as 180 have been reported (e.g. 10–13, 22). Our results (Table I) are at variance with those of Varshavsky et al. (13), who suggested that the origin of replication in the minichromosome is highly exposed, as probed by Bgl 1 cleavage. We find that the MI are supercompact, as supported by our much higher S values [115–130, in contrast to 70 obtained by Varshavsky et al. (3, 13, 23)] and by our electron micrographs (Fig. 2). It has been shown by Fernandez-Munoz et al. (22) and by us (unpublished) that prolonged exposure of whole cells to Triton, as described by Varshavsky et al. (13), leads to more relaxed structures of SV40 chromatin, possibly induced by Triton-treated cellular material (22). It is possible that under these conditions, a certain percentage of the 70 S minichromosomes may be derived from mature virions present in the infected cells. In the present studies, when mature virions were disrupted under "mild" conditions in the absence of Triton, the sedimentation coefficient was about 100, as compared with 35 and 48 obtained by others (10, 24). When chromatin was extracted from infected cells, 0.25% Triton was used to disrupt isolated nuclei for a short period of time (1 hr) rather than to suspend whole cells for 3 hr (13). Compact forms of the SV40 minichromosome having sedimentation coefficients greater than 70S have also been isolated by others (22, 25).

It has been shown recently (20, 21) that the origin of replication in SV40 chromatin is recognized by an endonuclease endogenous to the chromatin and that this enzyme cleaves about 30% of the viral DNA population, in agreement with our results. This observation might be related to the reports (26, 27) that a protein is linked to SV40 DNA at the origin of DNA replication. This may also partially account for the discrepancy between our results and those of Varshavsky et al. (13, 23). Recent results of Mann and Hunter (25) showing the association of the SV40 T antigen (which is involved in viral replication and transcription) with SV40 chromatin, and those of Tjian and Robbins (28) showing that the T antigen carries both ATPase and protein kinase activities, suggest an interesting role for the T antigen in opening up the origin of replication and transcription in the supercompact form of SV40 chromatin.

Our results show that the states of chromatin in the mature virions and inside the nuclei before encapsidation may not be identical, as shown by the S values, electron micrographs, and the restriction endonuclease data.

The nonavailability of the sites (to the restriction endonucleases) in the MI suggests that the distribution of nucleosomes in the compact form may not be random. One might obtain results supporting random distribution of the nucleosomes only when the superstructure

is destroyed and nucleosomes are separated by DNA spacers along which the movement of the nucleosomes is then possible (14).

A gradual relaxation of the compact structure on exposure to high salt concentration and other perturbants leads to structures having different degrees of compactness. The structural and functional properties of such complexes will be reported elsewhere (G. C. Das et al., manuscript in preparation).

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