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Specific Association of Simian Virus 40 Tumor Antigen with Simian Virus 40 Chromatin

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Simian virus 40 tumor antigen (SV40 T antigen) was bound to both replicating and fully replicated SV40 chromatin extracted with a low-salt buffer from the nuclei of infected cells, and at least a part of the association was tight and specific. T antigen cosedimented on sucrose gradients with SV40 chromatin, and T antigen-chromatin complexes could be precipitated from the nuclear extract specifically with anti-T serum. From 10 to 20% of viral DNA labeled to steady state with [^{3}H]thymidine for 12 h late in infection or 40 to 50% of replicating viral DNA pulse-labeled for 5 min was associated with T antigen in such immunoprecipitates. After reaction with antibody, most of the T antigen-chromatin complex was stable to washing with 0.5 M NaCl, but only about 20% of the DNA label remained in the precipitate after washing with 0.5 M NaCl-0.4% Sarkosyl. This tightly bound class of T antigen was associated preferentially with a subfraction of pulse-labeled replicating DNA which comigrated with an SV40 form I marker. A tight binding site for T antigen was identified tentatively by removing the histones with dextran sulfate and heparin from immunoprecipitated chromatin labeled with [^{32}P]phosphate to steady state and then digesting the DNA with restriction endonucleases *Hin*fI and *Hpa*II. The site was within the fragment spanning the origin of replication, 0.641 to 0.725 on the SV40 map.

Large-T antigen, an early protein of simian virus 40 (SV40), is found in the nuclei of infected or transformed cells (2, 5, 12, 15, 29, 38, 42, 48, 49). It is required for initiating viral DNA synthesis (6, 28, 54) and for stimulating host cell DNA synthesis during lytic infection (7, 20, 34, 59). Large-T antigen regulates its own synthesis (57) by controlling the transcription of early SV40 RNA (3, 27, 41) and is also involved in initiating late SV40 transcription (11) in a way that is not yet clear (35). Large-T antigen binds specifically to SV40 DNA and to SV40 chromatin in vitro (25, 37, 40, 58) and is bound to nucleoprotein complexes isolated from infected cells (33). In vivo, T antigen must interact with SV40 chromatin in different ways in order to participate in functions as different as initiation of replication, repression of early transcription, and initiation of late transcription. For example, there may be more than one binding site for T antigen on chromatin, or chemically different forms of T antigen may be involved. Different forms may result from post-translational modification of large-T antigen or may reflect the presence of more than one protein of the approximate size of large-T antigen. To learn more about the detailed interactions in vivo, we have isolated SV40 T antigen-chromatin complexes from infected cells and partially characterized the DNA species involved, and we have determined a probable position for a tight binding site for T antigen.

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MATERIALS AND METHODS

Cells and virus. The CV-1 line of African monkey kidney cells was grown in a CO_2 incubator in Lux plastic plates (100 by 15 mm) in Eagle medium as modified by Dulbecco (GIBCO) with 5% fetal calf serum (Microbiological Associates). H65-90B, an SV40-transformed hamster cell line (14, 15), and TRK-54, an SV40-transformed line of rabbit kidney cells (10), were kindly provided by S. Tevethia and P. H. Black, respectively. Wild-type SV40 strain VA45-54 (55) was grown and purified as described by Estes et al. (16).

^{32}P-labeled SV40 DNA. The procedure of Sambrook et al. (43) was used for ^{32}P-labeled SV40 DNA. Cells were lysed by the method of Hirt (24), and the supercoiled viral DNA was purified by density gradient centrifugation in the presence of CsCl and ethidium bromide (47). The DNA was dialyzed against 10 mM Tris-hydrochloride (pH 7.5)-0.1 M NaCl-1 mM EDTA and precipitated with 2 volumes of ethanol. It was purified further by centrifugation on 10 to 30% neutral sucrose gradients containing 50 mM Tris-hydrochloride, pH 7.5, and 1 mM EDTA at 40,000 rpm for 5.5 h at 15°C in a Beckman SW41 rotor. SV40

DNA was digested with restriction endonucleases *HinfI* and *HpaII* under the conditions suggested by the suppliers (New England Biolabs and Bethesda Research Laboratories).

Gel electrophoresis. Restriction fragments were separated by electrophoresis at room temperature in 5 or 10% composite polyacrylamide-agarose slab gels (23 by 14 by 0.15 cm) containing Tris-acetate buffer, pH 7.8 (32). The gels were dried and autoradiographed with Kodak XR-5 X-ray film and Du Pont Cronex Lightning Plus XL intensifying screens (30). Electrophoresis of SV40 DNA in 1% (wt/vol) agarose gels was in a buffer containing 40 mM Tris, 36 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7 (22). Tube gels (0.5 by 9 cm) were run for 6 h at 10 V/tube. The gels were cut into 2-mm slices which were dissolved in 50% formamide at 100°C for 10 min and then counted with a toluene-Triton scintillation fluid.

Preparation of SV40 chromatin. For the preparation of SV40 chromatin, the method of Su and DePamphilis (50) was used with minor modifications. CV-1 cells infected with 20 to 100 PFU of virus per cell were labeled with 100 μCi of [^{3}H]thymidine per plate for 12 h, starting 24 h after infection. Alternatively, the cells were pulse-labeled with 100 μCi of [^{3}H]thymidine per plate for 5 min at 36 h after infection. The cells were scraped off and lysed with five strokes of a Dounce homogenizer in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid) (pH 7.3)-5 mM KCl-0.5 mM $MgCl_2$-0.5 mM dithiothreitol. The nuclei were spun down, suspended in 0.2 ml of Dounce buffer per plate, and incubated for 2 h at 4°C with occasional shaking. After this extraction, the nuclei were removed at 8,000 rpm for 10 min. As much as 30% of the SV40 chromatin was lost during isolation of the nuclei; extraction with low-salt buffer liberated another 30%, which was used for the experiments described, and further extraction with 0.2 ml of 10 mM triethanolamine-hydrochloride (pH 7.8)-0.15 M NaCl-10 mM EDTA-0.25% Triton X-100 per plate (60) for 2 h at 4°C yielded most of the remaining chromatin. Chromatin prepared from ^{32}P-labeled virus as described by Christiansen et al. (9) or Brady et al. (4) was purified by sucrose gradient centrifugation (8).

Antisera. Hamster anti-T serum was obtained from hamsters bearing tumors induced by H65-90B cells. Rabbit anti-T serum was induced by injecting TRK-54 cells into rabbits (41a). Antisera and T-antigen preparations were titrated with the protein A assay (13), and specificities of the sera were assessed by immunoprecipitating extracts of infected CV-1 cells or by the protein transfer procedure of Renart et al. (41a). Both the hamster and the rabbit anti-T sera react primarily with SV40 large-T and small-t antigens. *Staphylococcus aureus* protein A (Pharmacia Fine Chemicals, Inc.) was labeled with ^{125}I to specific activities of 5 to 15 μCi/μg by a modification of the procedure of Syvanen et al. (52).

Immunoprecipitation of SV40 chromatin. Nuclear extracts or sucrose gradient fractions were mixed with an equal volume of NET-BSA-PEG (150 mM NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride [pH 7.4]-2 mg bovine serum albumin per ml-4% [wt/vol] polyethylene glycol 6000). Varying amounts of hamster anti-T serum or normal hamster serum were used. Similar results were obtained with two different batches of hamster anti-T serum and four different batches of rabbit anti-T serum, as well as with purified hamster or rabbit gamma globulins. After overnight incubation at 4°C, 5 or 10 μl of Formalin-fixed *S. aureus* Cowan I cells was added (26). After 15 min on ice, the bacteria were pelleted and washed twice with NET containing 0.5 M NaCl or 0.5 M NaCl-0.4% (wt/vol) Sarkosyl. SV40 DNA was eluted from the bacteria with 10 mM Tris-hydrochloride (pH 7.4)-10 mM EDTA-0.6% (wt/vol) sodium dodecyl sulfate (SDS). Alternatively, immune complexes were trapped on GF/C filters (5-mm disks; Whatman) held in 1-ml disposable plastic syringe barrels. The filters were wetted with NET containing 0.25% (wt/vol) gelatin and 0.05% (wt/vol) Nonidet P-40 (NP-40) before loading the samples and were washed with 1 ml of the same buffer. SV40 DNA was eluted as described above.

RESULTS

Cosedimentation of T antigen with SV40 chromatin. Cells were treated with [^{3}H]thymidine for 12 h at 24 to 36 h after infection to label fully replicated SV40 chromatin or for 5 min at 36 h after infection to label replicating viral chromatin. Nuclear extracts, prepared by extraction with a low-salt buffer (50), were fixed with 1% formaldehyde (8) and sedimented as shown in Fig. 1. As expected, pulse-labeled replicating chromatin (Fig. 1b) sedimented more rapidly than fully replicated chromatin (Fig. 1a). Extraction procedures similar to the one that we have used have been reported to disrupt intracellular virions (17, 18, 45). Fully replicated nuclear chromatin may therefore contain some labeled chromatin derived from virions, but the pulse-labeled replicating chromatin will not. Also shown in Fig. 1 is the sedimentation pattern of T antigen, determined with anti-T serum and the protein A-binding assay of Crawford and Lane (13). No antigen was detected with preimmune serum. One peak of T antigen sedimented near fully replicated SV40 chromatin (Fig. 1a), with a constant shoulder toward replicating SV40 chromatin (Fig. 1b). The simplest explanation for cosedimentation is that T antigen and SV40 chromatin are associated. Cosedimentation was observed with formaldehyde-fixed and with unfixed chromatin, but the results with fixed material were more reproducible. A second peak of T antigen not associated with DNA could be seen near the tops of the gradients. The amount of antigen in this peak was 5 to 10 times larger than the amount cosedimenting with DNA. Note that the protein A-binding assay is not linear, with peak heights related to the log of the amount of antigen (13).

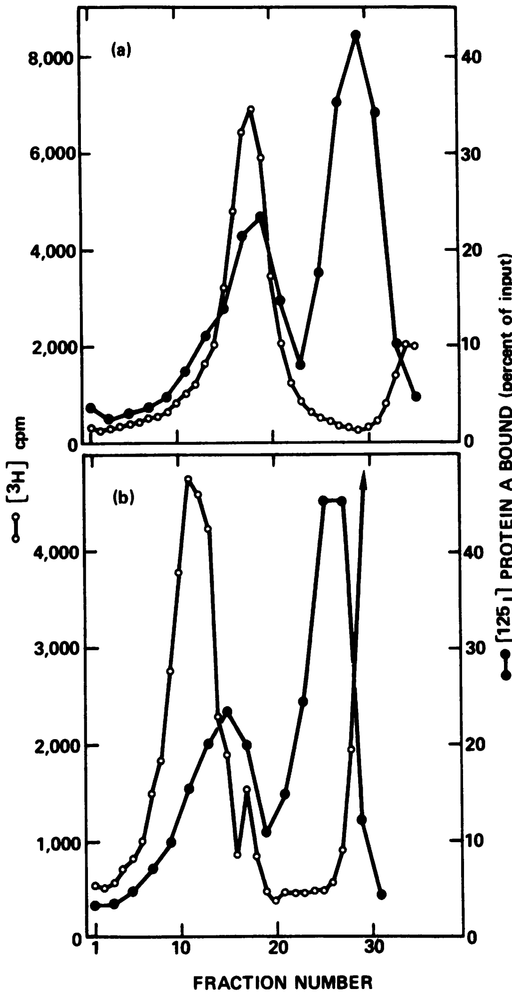

FIG. 1. *Sedimentation analysis of SV40 chromatin and SV40 T antigen. Infected cells from five 100-mm dishes were labeled with 100 μCi of [³H]thymidine per dish for 12 h (a) or for 5 min (b), and nuclear extracts were prepared. The extracts were fixed with 1% neutralized formaldehyde, and 0.5-ml portions were centrifuged through a 5 to 30% neutral sucrose gradient with a Beckman SW41 rotor at 40,000 rpm for 100 min at 4°C. The buffer was 10 mM HEPES (pH 7.3)-5 mM KCl-0.5 mM $MgCl_2$-0.5 mM dithiothreitol. Fractions of 0.35 ml were collected, and 70-μl portions were dried on glass fiber filters and counted. Portions of 75 μl from alternate fractions were immunoprecipitated with 5 μl of anti-T serum and 50 μl of NET-BSA-PEG. After incubation overnight at room temperature, the immune complexes were reacted with ¹²⁵I-labeled protein A from S. aureus as described by Crawford and Lane (13). The tops of the gradients are to the right.*

Immunoprecipitation of SV40 chromatin with anti-T serum. SV40 chromatin labeled with [³H]thymidine for 5 min or 12 h was fixed with formaldehyde and purified as described in the legend to Fig. 1. Constant amounts of the pooled peak fractions were treated with increasing amounts of anti-T or preimmune serum and then precipitated with fixed *S. aureus* cells to determine the maximum fraction of labeled chromatin associated with T antigen. As shown in Fig. 2, the fraction of labeled chromatin precipitated with anti-T serum depended on the length of the labeling period. A maximum of 40 to 50% of pulse-labeled chromatin or 15 to 20% of the chromatin labeled for 12 h precipitated with excess serum. In each case, five to seven times less chromatin precipitated with preimmune serum. Since SV40 chromatin tends to be trapped nonspecifically during immunoprecipitation, to obtain low backgrounds the complexes must be washed repeatedly with buffer containing high salt.

Unfixed nuclear extracts from cells labeled for 12 h were immunoprecipitated without centrifugation (Table 1). The fraction of labeled SV40 chromatin associated with T antigen was similar to the fraction obtained in the experiment of Fig. 2, with or without fixation. Therefore, fixation did not destroy the ability of T antigen to react with anti-T serum and was not necessary for the

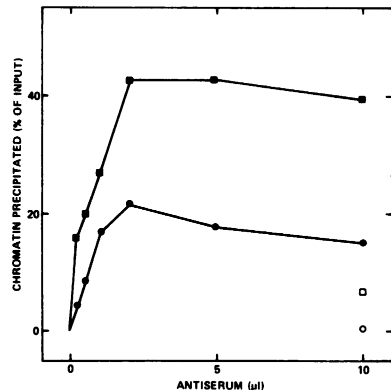

FIG. 2. *Immunoprecipitation with anti-T serum of formaldehyde-fixed SV40 chromatin. The chromatin was prepared, fixed with formaldehyde, and centrifuged through neutral sucrose gradients as described in the legend to Fig. 1. Portions of the pooled peaks (100 μl) were immunoprecipitated by adding 100 μl of NET-BSA-PEG and varying amounts of anti-T or normal serum. After 28 h at 4°C, 5 μl of a 10% suspension of fixed S. aureus cells was added, and the samples were incubated for 15 min at 0°C. The bacteria were pelleted and washed twice with 100 μl of NET containing 0.5 M NaCl-0.4% Sarkosyl. Symbols: ●, chromatin labeled for 12 h, anti-T serum; ■, chromatin pulse-labeled for 5 min, anti-T serum; ○, chromatin labeled for 12 h, normal serum; □, chromatin pulse-labeled for 5 min, normal serum. Backgrounds of 2 or 7% were subtracted from the samples containing chromatin labeled for 12 h or pulse-labeled chromatin, respectively.*

immunoprecipitation of chromatin which had not been centrifuged through sucrose gradients. Washing the unfixed immunoprecipitate-*S. aureus* complex with 0.5 M NaCl-0.4% Sarkosyl caused a large reduction in the amount of DNA bound (Table 1), indicating that most, but not all, of the association between T antigen and chromatin was sensitive to this solvent. Note that 0.4% Sarkosyl removes most of the histones from DNA (21). The Sarkosyl-resistant fraction of bound T antigen is characterized in more detail below.

As an alternative to the use of fixed *S. aureus* cells, large immune complexes can be trapped on GF/C filters (13). Results obtained with this method (Table 2) were in good agreement with those obtained with *S. aureus* cells (Fig. 2 and Table 1). With fixed fractions from sucrose gradients, the formation of immune complexes large enough to be trapped on GF/C filters was much slower than direct absorption to *S. aureus* cells, which took only a few minutes.

As a test for the association of chromatin with unbound T antigen during the preparation of the nuclear extracts, ^{32}P-labeled chromatin from virions, initially free of T antigen, was mixed with ^{3}H-labeled nuclear extracts before precipitation. As shown in Table 3, significant association did occur with the nuclear extracts, but when purified fixed chromatin derived from cells and chromatin derived from virions were mixed, there was no coprecipitation.

TABLE 1. *Immunoprecipitation of T antigen-SV40 chromatin complexes from nuclear extracts, 12-h label*[a]

Antiserum	Fixation with formaldehyde	Wash solution	% of labeled DNA precipitated
Anti-T	Yes	0.5 M NaCl	12
Normal			3
None			1
Anti-T	No	0.5 M NaCl	11
Normal			2
None			2
Anti-T	Yes	0.5 M NaCl-0.4% Sarkosyl	15
Normal			1
None			2
Anti-T	No	0.5 M NaCl-0.4% Sarkosyl	3
Normal			0.5
None			0.3

[a] The fixed samples were centrifuged before precipitation as described in the legend to Fig. 1, and the unfixed samples were not. Portions of 100 µl from pooled gradient fractions or 25-µl portions of the nuclear extracts were mixed with 100 µl of NET-BSA-PEG and 5 µl of serum and incubated for 20 h at 4°C. Five microliters of a 10% suspension of fixed *S. aureus* cells was added, the suspensions were kept on ice for 15 min, and the *S. aureus* cells were pelleted and washed twice with 100-µl portions of NET containing 0.5 M NaCl, with or without 0.4% Sarkosyl.

TABLE 2. *Immunoprecipitation of T antigen-SV40 chromatin complexes, with collection on GF/C filters*[a]

SV40 chromatin	Antiserum	% of labeled DNA precipitated
Fixed, 12-h label	Anti-T	12
	Normal	3
	None	5
Unfixed, 12-h label	Anti-T	13
	Normal	7
	None	6
Unfixed, 5-min label	Anti-T	51
	Normal	6
	None	11

[a] The fixed samples were centrifuged as described in the legend to Fig. 1 before precipitation, and the unfixed samples were not. Pooled gradient fractions (75-µl portions) or 30-µl portions of the nuclear extracts were mixed with 100 µl of NET-BSA-PEG and 5 µl of serum, incubated for 39 h at 4°C, mixed with 0.5 ml of NET-gelatin-NP-40, and filtered through GF/C filters. The filters were washed twice with 0.5 ml of NET-gelatin-NP-40, and the DNA was eluted with 200 µl of 10 mM Tris-hydrochloride (pH 7.4)-10 mM EDTA-0.6% SDS.

Analysis of DNA from immunoprecipitated SV40 chromatin. Nuclear extracts from cells pulse-labeled for 5 min were treated with SDS and phenol, and the DNA was fractionated by electrophoresis in 1% agarose gels along with ^{32}P-labeled SV40 form I and form II DNA standards (Fig. 3a). The same extracts were treated with anti-T serum, and the immunoprecipitates were trapped on GF/C filters and washed as described in Table 2, footnote *a*. The DNA was eluted with SDS and analyzed by electrophoresis (Fig. 3b). Alternatively, immunoprecipitates adsorbed to *S. aureus* cells were washed stringently with 0.5 M NaCl-0.4% Sarkosyl (Table 1) before analysis of the DNA (Fig. 3c). A portion of the supernatant solution from the immunoprecipitate of Fig. 3b was also analyzed (Fig. 3d). A quantitative comparison of the DNA in immunoprecipitates washed with 0.5 M NaCl or 0.5 M NaCl-0.4% Sarkosyl with the DNA in the starting nuclear extract is given in Table 4. See Fig. 3a for a definition of zones A through D.

As shown in Fig. 3a, pulse-labeled SV40 DNA migrated with SV40 form I and form II DNA, as well as behind and between these markers, a result typical for replicating SV40 DNA (53).

TABLE 3. *Association of free T antigen with SV40 chromatin in vitro*[a]

Chromatin	Antiserum	% of labeled DNA precipitated	
		^{32}P-labeled virion chromatin	^{3}H-labeled nuclear chromatin
Virion (method A)	Anti-T	1	
	Normal	1	
Virion (method B)	Anti-T	2	
	Normal	1	
Virion (method A) plus nuclear extract	Anti-T	5	16
	Normal	1	3
Virion (method B) plus nuclear extract	Anti-T	7	18
	Normal	2	3
Virion (method B) plus fixed nuclear chromatin	Anti-T	1	14
	Normal	1	7

[a] ^{32}P-labeled chromatin was prepared from purified virions by treatment with dithiothreitol at pH 9.8 (Christiansen et al. [9], method A) or by treatment with ethylene glycol-bis-*N,N'*-tetraacetic acid (Brady et al. [4], method B). Chromatin labeled with [^{3}H]-thymidine for 12 h was prepared as described in the legend to Fig. 1. Immunoprecipitation, adsorption to fixed *S. aureus* cells, and washing with NET–0.5 M NaCl were as described in Table 1, footnote *a*.

Chromatography of pulse-labeled DNA on benzoylated naphthoylated DEAE-cellulose confirmed that this label marks replicating chromatin since about 80% of the counts were bound to the column and were eluted with 2% caffeine (31). Precipitation with anti-T serum brought down DNA similar to the total labeled pool, although some differences were evident (compare Fig. 3a with 3b and line 1 of Table 4 with line 2). When the immunoprecipitate was washed stringently with 0.5 M NaCl–0.4% Sarkosyl so that only the DNA bound most tightly to T antigen was retained, there was a substantial increase in the relative amount of SV40 form I DNA (compare Fig. 3a with 3c and line 3 of Table 4 with line 4). The possible significance of these observations is discussed below. Note that the DNA present in the supernatant solution after an immunoprecipitation was similar to the input DNA and showed no evidence of degradation (Fig. 3d).

Localization of a T antigen binding site on SV40 chromatin. In the experiment for the localization of a T antigen-binding site on SV40 chromatin, histones and other proteins were removed from a precipitate of SV40 chromatin and anti-T serum, so that the DNA became susceptible to digestion with endonucleases. The only DNA fragments retained in the precipitate after digestion should have been those which retained their association with T antigen. A mixture of dextran sulfate and heparin is known to remove histones completely from cellular chromatin (1, 23), leaving free DNA (36). As shown in Fig. 4, treatment of SV40 chromatin with dextran sulfate and heparin caused the labeled DNA to cosediment with an internal SV40 form I DNA marker on a neutral gradient. In another control experiment, the fraction of pulse-labeled chromatin retained as an immunoprecipitate on a GF/C filter was found to be the same after washing with dextran sulfate and heparin as it was before such treatment. However, only about 20% of the DNA bound to the GF/C filter was still in place after incubation for 2 h at 37°C with buffer alone. Presumably, the loss reflects a gradual dissociation of T antigen from the DNA at this high temperature in the absence of fixation.

SV40 chromatin labeled with [^{32}P]phosphate to steady state was treated with anti-T or normal serum, and the precipitates collected on GF/C filters were washed with dextran sulfate and heparin. After cleaving with endonucleases and further washing, the DNA still bound to the filter was removed with SDS and analyzed by gel electrophoresis. A control without endonuclease is shown in Fig. 5, gel 1. Note that most of the counts were near the top of the gel. Little radioactivity was present if normal serum was used as a precipitant (Fig. 5, gel 3). Digestion with restriction endonucleases *Hin*fI and *Hpa*II (46, 51), although incomplete, did lead to the appearance of several new fragments (Fig. 5, gel 2), including a very prominent one which comigrated with fragment F of the *Hin*fI-*Hpa*II marker digest (Fig. 5, gel 4). Fragment F spans the region from 0.641 to 0.725 on the SV40 map (39), as shown in Fig. 5b. Small amounts of radioactivity also comigrated with fragments C and D of the marker digest. Digestion of the immunocomplexed sample was largely incomplete, and products of partial cleavage appeared near the top of the gel, near marker fragments A and B. Other enzymes, such as *Hae*III, *Alu*I, and *Hin*dIII, were also tried, but the digestions were even more incomplete, and a clear conclusion could not be drawn from these experiments. Incomplete digestion probably reflects an inhibition of the endonucleases by residual dextran

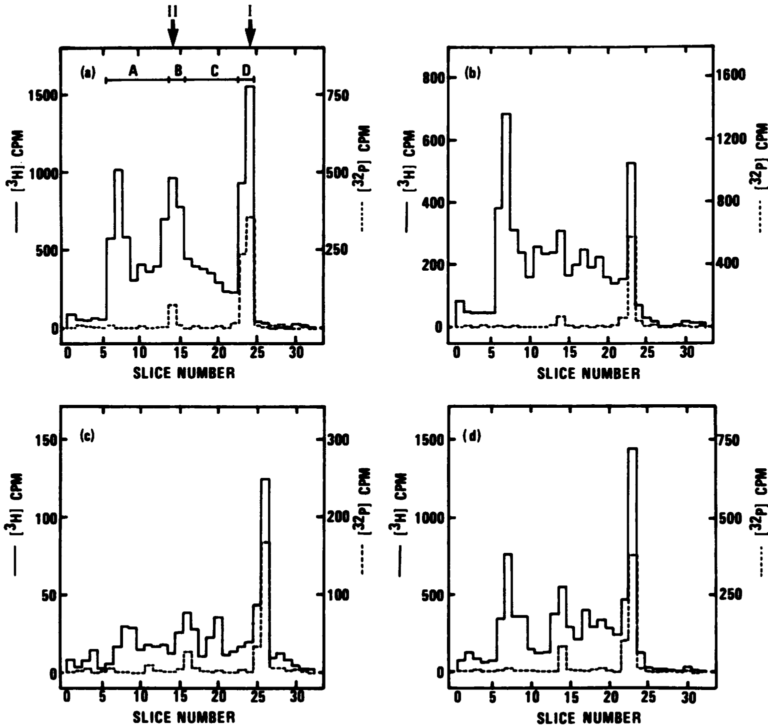

FIG. 3. *Electrophoresis in agarose gels of SV40 DNA derived from immunoprecipitated, pulse-labeled SV40 chromatin. Infected cells were labeled with [3H]thymidine for 5 min, and 50-µl portions of the nuclear extracts were immunoprecipitated for 45 h at 4°C with 150 µl of NET-BSA-PEG and 10 µl of anti-T serum. A total of 500 µl of NET-gelatin-NP-40 was added, and the immune complexes were collected on GF/C filters, which were washed twice with 500 µl of NET-gelatin-NP-40. The DNA was eluted with 200 µl of 10 mM Tris-hydrochloride (pH 7.4)-10 mM EDTA-0.6% SDS. Alternatively, the immune complexes were adsorbed to S. aureus cells and processed as described in Table 2, footnote a. Ten micrograms of yeast tRNA was added as carrier, and the samples were extracted with phenol and precipitated with ethanol. The pellets were redissolved in 1% SDS, 32P-labeled SV40 form I and form II DNA standards were added, and the samples were separated by electrophoresis in 1% agarose tube gels. (a) Nuclear extract before immunoprecipitation; (b) nuclear extract precipitated with anti-T serum, collected on a GF/C filter; (c) nuclear extract precipitated with anti-T serum, adsorbed to S. aureus cells, and washed with 0.5 M NaCl-0.4% Sarkosyl; (d) fraction from experiment (b) which was not trapped on the GF/C filter. Symbols: -----, 32P-labeled DNA; ——, 3H-labeled DNA.*

TABLE 4. *Quantitative analysis of pulse-labeled SV40 DNA in agarose gels before and after immunoprecipitation*

Sample	Wash solution	% of total counts in zones defined in Fig. 3a			
		A	B	C	D
Nuclear extract	—[a]	41	17	20	22
Immunoprecipitate (Fig. 3b)	0.5 M NaCl	47	17	22	13
Nuclear extract	—[a]	38	16	22	25
Immunoprecipitate (Fig. 3c)	0.5 M NaCl-0.4% Sarkosyl	24	21	20	35

[a] —, None.

sulfate and heparin since purified 32P-labeled SV40 DNA added to GF/C filters previously washed with the same solutions was also digested incompletely. In gels 1 and 2 of Fig. 5, the discrete bands of DNA smaller than fragment G may represent fragments protected by nucleosomes from digestion with endogenous nucleases.

DISCUSSION

In considering the significance of our results, recall that only about 30% of the total SV40 chromatin was extracted by the low-salt procedure (see above); this fraction may not represent exactly the composition of the total chromatin

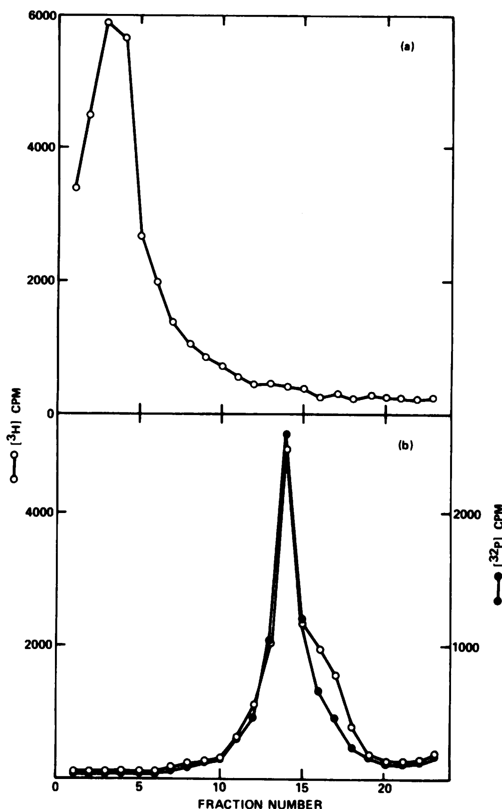

FIG. 4. *Sedimentation analysis of SV40 chromatin before and after treatment with dextran sulfate and heparin. SV40 chromatin labeled with [^{3}H]thymidine for 12 h was treated with dextran sulfate (1 mg/ml) and heparin (150 μg/ml) for 10 min at 20°C and then centrifuged through 10 to 30% neutral sucrose gradients with a cushion of 60% sucrose at 40,000 rpm for 5.5 h at 4°C in a Beckman SW41 rotor. The gradients contained 50 mM Tris-hydrochloride, pH 7.5, and 1 mM EDTA. (a) Untreated sample; (b) sample treated with dextran sulfate and heparin. Symbols: ○, ^{3}H-labeled DNA; ●, ^{32}P-labeled SV40 DNA (internal marker).*

pool. Three lines of evidence support the view that at least part of the association between T antigen and SV40 chromatin is specific. First, a portion of the antigen was bound very tightly, surviving washes with 0.5 M NaCl-0.4% Sarkosyl or dextran sulfate plus heparin. Second, tightly bound antigen was associated with a specific subclass of pulse-labeled replicating SV40 DNA, enriched for species comigrating with SV40 form I DNA in agarose gels. In addition, Mann and Hunter (33) have shown that T antigen is preferentially associated with form I DNA labeled for 21 h in chromatin isolated by the method of Su and DePamphilis (50) and sedimented in sucrose gradients in the absence of fixation. The T antigen retained in this material probably corresponds to the tightly bound class that we have observed. Third, tightly bound antigen is probably located predominantly at one binding site, at or near the origin. The binding of T antigen to the origin of SV40 DNA in vitro has been well documented previously (25, 37, 40, 58). In vivo, tightly bound T antigen may be involved in initiating DNA synthesis, perhaps through association with primer RNA. By analogy, initiated RNA polymerase binds tightly to SV40 DNA, even in the presence of Sarkosyl, presumably through RNA previously synthesized by the complex (19, 21). If tightly bound T antigen were to leave the replication complex early, after only a small fraction of the DNA had been copied, the preferential association of T antigen with pulse-labeled DNA migrating near SV40 form I DNA would result, as was observed. It is more difficult to relate the more weakly bound class of T antigen to possible functions in vivo. Most or all of the antigen in this class may be associated with SV40 chromatin nonspecifically (Table 3), or weak binding may be required for a function in replication or transcription which requires T antigen to cycle on and off SV40 chromatin readily. It may be possible to approach this question by isolating SV40 chromatin undergoing transcription and analyzing its complement of bound T antigen, or by immunoprecipitating T antigen-chromatin complexes and assaying for RNA polymerase as a function of wash procedures.

Recent evidence from several laboratories (44, 61-63) indicates that the origin of replication of SV40 chromatin is preferentially accessible to endonucleases. Varshavsky et al. (61, 62) speculate that T antigen may be involved in keeping the origin region free of nucleosomes. The observation made here that T antigen was bound to only 10 to 20% of SV40 chromatin labeled for 12 h argues against the possibility that T antigen is the only protein with such a function, since nearly all of the SV40 minichromosomes have accessible origin regions (61, 62). We must consider the possibility that our mapping data for T antigen were influenced by preferential accessibility of the origin region to restriction endonucleases, although a priori it is not easy to see how exposure of the origin can cause a DNA fragment from this region to be retained in an immunoprecipitate after digestion with endonuclease. Treatment with dextran sulfate and heparin caused SV40 chromatin to comigrate with SV40 form I DNA (Fig. 4), and one would have to postulate that the proteins involved in exposing the origin are retained after such treatment. Also, restriction endonucleases *Hae*III and *Alu*I, which cut near the origin of SV40

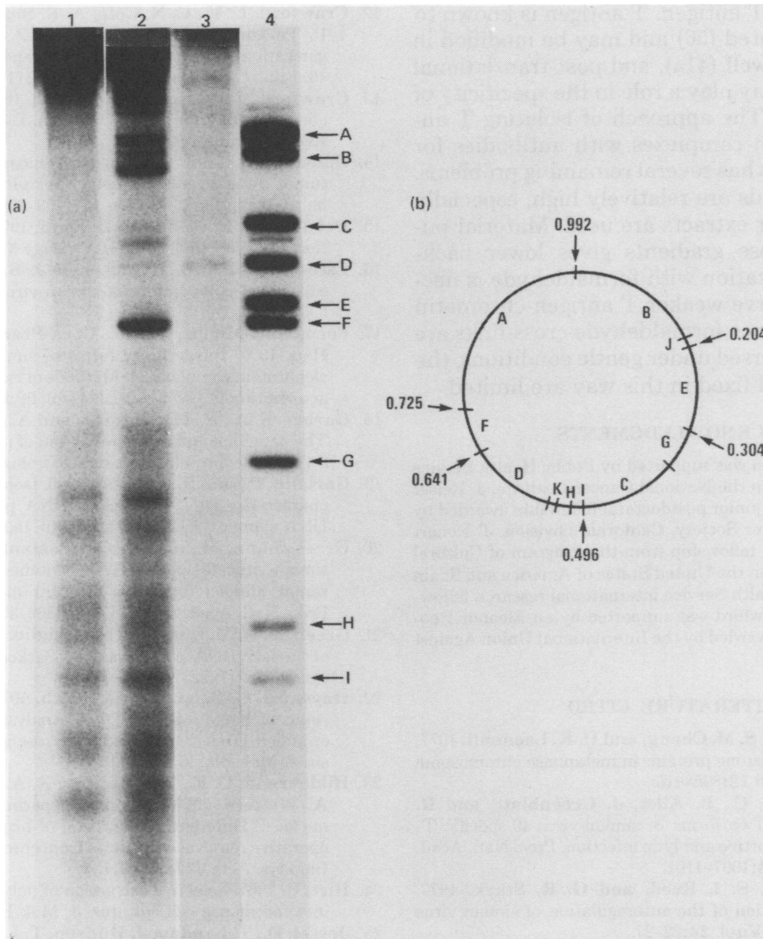

FIG. 5. *(a) Localization of a probable T antigen-binding site on SV40 chromatin. Cells were labeled with [^{32}P]phosphate (0.35 μCi/plate) in complete medium containing 2% fetal calf serum immediately after infection, and nuclear extracts were prepared 36 h later. Portions of 50 μl were immunoprecipitated for 41 h at 4°C with 10 μl of antiserum and 150 μl of NET-BSA-PEG. A total of 500 μl of NET-gelatin-NP-40 was added, and the precipitates were collected on GF/C filters. The filters were washed with 500 μl of NET containing heparin (150 μl/ml) and dextran sulfate (1 mg/ml), with 500 μl of NET-gelatin-NP-40, and then with 1 ml of the buffer used for digestion with restriction enzymes plus gelatin (100 μg/ml). To the damp filters was added 10 μl of restriction enzyme in buffer. After digestion for 2 h at 37°C, the reactions were stopped by adding 10 μl of 0.25 M EDTA. The filters were rinsed once with 1 ml of NET-gelatin-NP-40, and the DNA was eluted with 200 μl of 10 mM Tris-hydrochloride (pH 7.4)-10 mM EDTA-0.6% SDS. Carrier SV40 DNA (6 μg) was added, the samples were digested with 5 μg of proteinase K for 60 min at 37°C, extracted with phenol, and precipitated with ethanol. The DNA fragments were analyzed in 5% polyacrylamide-agarose composite gels. Gel 1, control without nucleases; gel 2, nuclease digestion and precipitation with anti-T serum; gel 3, nuclease digestion and precipitation with normal serum; gel 4, digest of SV40 DNA with HinfI-HpaII. (b) Map of HinfI and HpaII cleavage sites in SV40 DNA. The positions of the sites are those described by Reddy et al. (39).*

minichromosomes preferentially (62), gave only partial digestion in our experiments, with no indication of any preferential cleavage (data not shown). Finally, the cut made by *Hin*fI (0.641) is outside the region Varshavsky et al. (62) have shown to be free of nucleosomes and accessible to endonucleases.

A full understanding of the various roles of T antigen in replication and transcription will require an extension of the approach that we and others (33) have begun. Replication and transcription complexes will have to be isolated and characterized in terms of the forms of SV40 DNA that they contain, whether or not they contain tightly or weakly bound T antigen, and whether bound T antigen differs chemically

from unbound T antigen. T antigen is known to be phosphorylated (56) and may be modified in other ways as well (41a), and post-translational modification may play a role in the specificity of DNA binding. The approach of isolating T antigen-chromatin complexes with antibodies for further analysis has several remaining problems. The backgrounds are relatively high, especially if crude nuclear extracts are used. Material purified on sucrose gradients gives lower backgrounds, but fixation with formaldehyde is necessary to preserve weaker T antigen-chromatin interactions. Since formaldehyde cross-links are not readily reversed under gentle conditions, the uses of material fixed in this way are limited.

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