Salt-Resistant Association of Simian Virus 40 T Antigen with Simian Virus 40 DNA in Nucleoprotein Complexes

KAREN RUDOLPH AND KRISTINE MANN*

Biology Department, University of Alaska, Anchorage, Alaska 99508

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Treatment of nucleoprotein complexes (NPCs) from simian virus 40 (SV40)-infected TC7 cells with NaCl (1 or 2 M) or guanidine-hydrochloride (1 or 2 M) resulted in a significant fraction of T antigen still associated with SV40 (I) DNA. Immunoprecipitation of the salt-treated NPCs with SV40 anti-T serum indicated that T antigen is preferentially associated with SV40 (I) DNA rather than with SV40 (II) DNA. Treatment of the NPCs with 4 M guanidine-hydrochloride, however, resulted in a substantial decrease in the amount of SV40 (I) and (II) DNA associated with T antigen. As the temperature was increased to 37°C during incubation of NPCs with NaCl or guanidine-hydrochloride, there was a decrease in the amount of SV40 (I) and (II) DNA immunoprecipitated with SV40 anti-T serum. In the absence of salt, temperature had no effect on the association of T antigen with the SV40 DNA in the NPCs. Treatment of NPCs from SV40 wild-type or *tsA58*-infected cells grown at the permissive temperature with 1 or 2 M NaCl indicated that *tsA* T antigen has the same sensitivities as wild-type T antigen to high salt treatment when bound to DNA in NPCs. Characterization of the proteins associated with SV40 (I) DNA after high salt treatment revealed that, in addition to T antigen, a certain amount of viral capsid proteins VP1 and VP3 remained associated with the DNA. Complexes containing SV40 (I) DNA had a sedimentation value of 53S after 1 M NaCl treatment and 43S after 2 M NaCl treatment.

Simian virus 40 (SV40) large tumor antigen (T antigen), encoded by the viral A gene, is a multifunctional protein whose functions include initiation of SV40 DNA replication (3, 44), initiation of late-strand SV40 transcription (5), auto-regulation of its own early-strand mRNA transcription (36), stimulation of host cell DNA synthesis during lytic infection (4, 10, 29, 50), and the initiation (19, 44) and maintenance (2, 26, 33, 45) of transformation. The binding of purified SV40 T antigen to SV40 DNA in vitro has been extensively characterized. Purified T antigen from different sources binds to SV40 DNA at three distinct, but closely spaced recognition sites (I, II, and III) which span the origin of replication of the SV40 DNA molecule (41, 48, 49). Binding studies with SV40 T antigen from infected permissive cells indicate that the most stable binding site of T antigen on the SV40 DNA molecule encompasses all of site II, extending to sequences on either side (46). The effect of mutations in site I or site II suggests that binding of T antigen to site II is essential for SV40 DNA replication in vivo (6, 30, 42). The replication defects of these *ori* mutants were suppressed by site II mutations mapping between 0.43 and 0.50 map units on the SV40 genome (42). SV40 T antigen has been shown to

have both specific and nonspecific affinities for DNA (7, 9, 32, 34), with newly synthesized T antigen binding more efficiently and tightly to origin-containing sequences (32). The region of the T antigen molecule necessary for specific DNA binding has been localized to the coding sequence between 0.49 and 0.51 SV40 map units (28, 34, 35, 42).

The replication of SV40 DNA in infected cells occurs in the form of viral nucleoprotein complexes (NPCs) (11, 14, 43, 51). The proteins in these complexes consist of host cell histones, along with small amounts of the viral capsid proteins VP1 and VP3 (12, 15, 27). In addition, SV40 large T antigen has been found in SV40 NPCs extracted from infected cells (24, 37, 40), associated with both SV40 (I) and replicating (RI) DNA-containing complexes (24, 37, 40). It is specifically bound to sequences spanning the origin of SV40 DNA replication (37). The association of T antigen with the NPCs is temperature sensitive in cells infected with the SV40 *tsA58* mutant, with T antigen no longer present in the NPCs at the nonpermissive temperature (24). However, once bound to the SV40 DNA, *tsA* T antigen remains associated with the NPCs until elongation of the SV40 DNA is completed, even after shift-up to the nonpermissive temperature (40).

Treatment of SV40 NPCs with 1 M NaCl results in dissociation of the bulk of protein from the SV40 DNA molecule, releasing a high salt-stable complex in which protein is bound to the SV40 (I) DNA at 0.7 ± 0.05 map units (13). In addition, treatment of SV40 virions with sodium dodecyl sulfate (SDS) in the presence of EDTA and dithiothreitol results in a protein-DNA complex in which protein is covalently attached to the end of nicked SV40 (II) DNA at 0.67 map units (16, 17). The protein attached to the SV40 DNA has not been identified in either case, but our present knowledge of the multifunctions of T antigen, particularly with respect to initiation of viral DNA replication and its binding to the SV40 DNA, suggests that this salt-stable protein-DNA complex could involve T antigen.

The present study shows that treatment of the NPCs extracted from SV40-infected cells with various concentrations of NaCl and guanidine-hydrochloride leaves a fraction of the SV40 T antigen still bound to the SV40 DNA, even at high salt concentrations. In addition, there is no difference in the ability of wild-type and *tsA* T antigen to remain bound to SV40 DNA in the NPCs at different salt concentrations.

MATERIALS AND METHODS

Viruses and cells. SV40 stocks were prepared in TC7 cells grown in Dulbecco modified Eagle medium (DMEM) plus 10% fetal calf serum. The titer of the wild-type SV40 stock was 7×10^8 PFU/ml, whereas the *tsA58* stock had a titer of 2×10^7 PFU/ml. The TC7 cells were maintained in DMEM plus 10% calf serum.

Infection and labeling of cells. Confluent monolayer cultures were infected with SV40 wild-type stock at a 1:10 dilution (multiplicity of 10 PFU/cell) or SV40 *tsA58* stock at a 1:5 dilution (multiplicity of 1 PFU/cell). Approximately 21 h before extraction of the NPCs, infected cells were labeled for DNA with [3 H]thymidine (specific activity, 67 Ci/mmol; ICN) at a final concentration of 10 μ Ci/ml. Infected cells were labeled for protein in DMEM minus methionine with 5% dialyzed calf serum and 200 μ Ci of [3 S]methionine (specific activity, greater than 1,000 Ci/mmol; Amersham Corp.) per plate in 2 ml for 4 h at 37°C or for 6 h at 32°C before extraction of the NPCs.

Extraction and sedimentation analysis of SV40 NPCs. SV40 NPCs were prepared from SV40-infected TC7 cells at approximately 36 h postinfection by the method of Su and DePamphilis (43), as described previously by Mann and Hunter (24). SV40 NPCs were sedimented on linear 5 to 30% neutral sucrose gradients in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.8)–5 mM KCl–0.5 mM $MgCl_2$ at 49,000 rpm for 50 min in a Beckman SW50.1 rotor at 4°C. Portions of each gradient fraction were counted in Triton X-toluene-PPO (2,5-diphenyloxazole). Samples of each fraction were diluted with an equal volume of 2 $\times$ protein sample buffer, and 10- μ l samples were applied per slot in an analytical 12.5% SDS-polyacrylamide gel (20, 23) and electrophoresed at a constant current of 15 mA. Radioactive proteins

were detected by fluorography after treatment of gels with PPO-dimethyl sulfoxide (22).

When SV40 NPCs were prepared from infected TC7 cells by the method of Green et al. (11), 1.0 ml of lysing solution (10 mM Tris-hydrochloride [pH 7.9], 10 mM EDTA, 0.25% Triton X-100) was added per plate and incubated at 23°C for 10 min. At the end of the lysing period, sodium chloride was added to a final concentration of 0.2 M. The resulting lysate was scraped into a centrifuge tube and left at 4°C for 20 min. The lysate was centrifuged at 10,000 rpm in a Beckman JS-13 rotor for 30 min at 4°C. The crude NPCs were layered onto linear 5 to 20% sucrose gradients in 10 mM Tris-hydrochloride (pH 7.9)–100 mM NaCl–1 mM EDTA–0.25% Triton X-100 and spun at 49,000 rpm for 70 min in a Beckman SW50.1 rotor at 4°C. Portions of each gradient fraction were counted in Triton X-toluene-PPO or diluted with an equal volume of 2 $\times$ protein sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis.

DNase treatment of SV40 NPCs. Before sucrose gradient purification, SV40 NPCs were incubated with DNase I at a final concentration of 0.16 mg/ml in 10 mM HEPES (pH 7.8)–5 mM $MgCl_2$ –0.5 mM dithiothreitol for 30 min at 23°C. A control sample was incubated in buffer alone under the same conditions. The samples were layered onto linear 5 to 30% neutral sucrose gradients and centrifuged at 49,000 rpm for 50 min in a Beckman SW50.1 rotor at 4°C. Samples of each gradient fraction were analyzed by SDS-polyacrylamide gel electrophoresis.

Salt treatment of SV40 NPCs followed by immunoprecipitation with SV40 anti-T serum. Purified SV40 NPC peak fractions from linear 5 to 30% neutral sucrose gradients were pooled, and 90- μ l samples were incubated with either NaCl or guanidine-hydrochloride at the appropriate final salt concentration at 4, 23, or 37°C for 30 min. The samples were immunoprecipitated with SV40 anti-T serum or normal hamster serum for 2 h at 4°C, followed by fixed *Staphylococcus aureus* overnight (18). In addition to the usual wash procedure with 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1% Trasylol, 150 mM NaCl, and 10 mM sodium phosphate (pH 7) (39), the immunoprecipitates were washed with 0.1 M Tris (pH 6.8), with 0.5 M LiCl to minimize the nonspecific trapping of DNA by antigen-antibody complexes. The immunoprecipitates were eluted in 35 μ l of 1 $\times$ protein sample buffer; 10- μ l samples were applied per slot in a 12.5% analytical SDS-polyacrylamide gel for analysis of proteins present, and 15- μ l samples were applied per slot on a 0.7% horizontal agarose gel for analysis of SV40 DNA present. DNA gels were run at a constant voltage of 75 V for 16 h at room temperature in Tris-borate buffer. The agarose gels were subjected to fluorography in PPO-methanol (21). The amount of labeled SV40 DNA and T antigen in the immunoprecipitates was quantitated by densitometry with a Helena quick scan R & D densitometer and a Helena quick quant II-T.

Salt treatment and sedimentation analysis of gradient-purified SV40 NPCs. Peak NPC fractions containing SV40 (I) DNA from linear 5 to 30% neutral sucrose gradients or linear 5 to 20% sucrose plus Triton X-100 gradients were pooled and dialyzed against TE (10 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA) or 10 mM Tris-hydrochloride (pH 7.9), 1 mM EDTA, 100 mM

FIG. 1. Sedimentation analysis of [^{35}S]methionine-labeled NPCs before and after DNase treatment. Cells infected with wild-type SV40 were labeled with [^{35}S]methionine for 4 h before harvest. NPCs were extracted as described in the text. Crude NPCs were treated with DNase I (0.16 mg/ml) for 30 min at 23°C and sedimented on 5 to 30% neutral sucrose gradients. A portion of each fraction was analyzed directly by SDS-polyacrylamide gel electrophoresis. Gradient fractions 7 to 27 are shown, with the lower numbers referring to the bottom of the gradient. Fractions 13 to 14 in (A) correspond to the peak of SV40 NPCs containing the [^3H]thymidine-labeled SV40 (I) DNA. The fluorograms were exposed for 18 h. (A) NPCs before DNase treatment. (B) NPCs after DNase treatment.

NaCl, and 0.25% Triton X-100, respectively, at 4°C for 3 h. The samples were then concentrated with an Amicon ultrafiltration apparatus and spun at 2,500 rpm until approximately 0.1 of the initial volume remained. Samples were incubated with NaCl adjusted with water to a final concentration of 1 or 2 M NaCl for 30 min at 23°C. Salt-treated samples were layered onto linear 20 to 30% neutral sucrose gradients and centri-

fuged at 49,000 rpm for 2 h 10 min in a Beckman SW 50.1 rotor at 4°C. For NPCs prepared by the method of Green et al. (11), salt-treated samples were layered onto linear 20 to 30% sucrose plus Triton X-100 gradients and centrifuged at 49,000 rpm for 2 h in a Beckman SW50.1 rotor at 4°C. Portions of each gradient fraction were counted in Triton X-toluene-PPO; [^{35}S]methionine-labeled proteins were analyzed by

SDS-polyacrylamide gel electrophoresis, and [^3H]thymidine-labeled DNA was analyzed by agarose gel electrophoresis.

RESULTS

DNase treatment of SV40 NPCs before sucrose gradient purification. SV40 NPCs labeled with both [^3H]thymidine and [^{35}S]methionine were extracted from SV40-infected TC7 cells by the low salt method of Su and DePamphilis (43). With this method, SV40 T antigen has previously been detected in association with the NPCs (24). It was of interest to determine whether SV40 T antigen was specifically associated with the SV40 NPCs or whether it was sedimenting independently to the NPC region of the gradient. If T antigen was specifically associated with the NPCs, it should have dissociated as a result of DNase treatment and no longer be associated with the NPCs after sucrose gradient centrifugation. Crude NPCs were incubated with 0.16 mg of DNase I per ml for 30 min at 23°C, and a control sample was incubated with buffer under

FIG. 2. Immunoprecipitation of SV40 NPCs with SV40 hamster anti-T serum versus normal hamster serum. SV40 NPCs labeled with [^3H]thymidine and [^{35}S]methionine were extracted as described in the text and sedimented on 5 to 30% neutral sucrose gradients. The NPC peak fractions were immunoprecipitated with SV40 anti-T serum or normal hamster serum. A 10- μl portion of each immunoprecipitate was analyzed by SDS-polyacrylamide gel electrophoresis. The positions of T antigen, VP1, VP3, and the histones are indicated. NHS, Normal hamster serum; HaT, hamster anti-T serum. The fluorogram was exposed for 6 days.

FIG. 3. Effect of NaCl and guanidine-hydrochloride on the association of T antigen with the SV40 DNA. The [^3H]thymidine-labeled DNA from the NPC region of 5 to 30% neutral sucrose gradients was treated with increasing concentrations of NaCl (0, 1, and 2 M) or guanidine-hydrochloride (0, 1, 2, and 4 M) for 30 min at the various temperatures indicated (4, 23, and 37°C). Samples treated with NaCl were then immunoprecipitated with SV40 anti-T serum; samples treated with guanidine-hydrochloride were immunoprecipitated with SV40 anti-T serum or normal hamster serum. Portions of each sample were analyzed by agarose gel electrophoresis. The positions of SV40 (I) and (II) DNA are indicated. SV40 (III) DNA can be seen migrating between SV40 (I) and (II) DNA in the untreated (0 M salt) NPC samples. The fluorogram in (A) was exposed for 30 days, whereas that in (B) was exposed for 28 days. (A) NPCs treated with NaCl. (B) NPCs treated with guanidine-hydrochloride. NHS, Normal hamster serum; HaT, hamster anti-T serum.

the same conditions. Both samples were analyzed by sedimentation on 5 to 30% neutral sucrose gradients. A portion of each fraction of the gradients was subjected to SDS-polyacrylamide electrophoresis and fluorography to analyze the [^{35}S]methionine-labeled proteins present. In the control gradient (Fig. 1A), fractions 13 to 15 correspond to the peak of SV40 NPCs which contains the [^3H]thymidine-labeled SV40 (I) DNA. In these fractions, T antigen, VP1, VP3, and cellular histones are detectable. Treatment of the crude NPCs with DNase (Fig. 1B) resulted in the complete disappearance of T antigen and the cellular histones from the NPC region of the gradient containing SV40 (I) DNA. There was also a partial reduction in the amount

FIG. 4. Effect of NaCl on the NPC-associated proteins immunoprecipitated with SV40 anti-T serum. The NPCs used in the experiment shown in Fig. 3A were labeled with [^{35}S]methionine as well as [^3H]thymidine. After the NPCs were treated with NaCl and immunoprecipitated with SV40 anti-T serum as described in the legend to Fig. 3, portions of each sample were analyzed by SDS-polyacrylamide gel electrophoresis. The positions of T antigen, VP1, and the histones are indicated. The fluorogram was exposed for 22 days. Lg T, Large T antigen.

of VP1 associated with this region after DNase treatment (Fig. 1B). Gradient fractions before DNase treatment showed the typical sedimentation pattern when analyzed by agarose gel electrophoresis, with SV40 (I) DNA peaking in the region of the gradient containing the peak of [^{35}S]methionine-labeled T antigen. After DNase treatment, no DNA was detected, indicating that the DNase treatment was effective. These results suggest that there is a specific association of T antigen with the SV40 DNA in the SV40 NPCs, although it is still possible that T antigen is associated with the NPCs as a result of binding to another protein, which in turn is specifically bound to the SV40 DNA.

Salt treatment of SV40 NPCs followed by immunoprecipitation with SV40 anti-T serum. Immunoprecipitation of sucrose gradient-purified SV40 NPCs with SV40 hamster anti-T serum versus normal hamster serum showed a specific recognition of T antigen by the hamster anti-T serum (Fig. 2). In addition, there was an increase in the amount of VP1 and histones immunoprecipitated by hamster anti-T serum compared with control serum, presumably due to an association of these proteins and T antigen with the SV40 DNA in the NPCs (24). Sucrose gradi-

ent-purified SV40 NPCs, labeled with [^{35}S]methionine and [^3H]thymidine, were treated with increasing concentrations of NaCl (0.4 to 1.7 M) for 30 min at 4°C, followed by immunoprecipitation with SV40 anti-T serum to determine whether T antigen remained associated with the SV40 DNA after high salt treatment. Both SV40 (I) and (II) DNA immunoprecipitated with SV40 anti-T serum, even after treatment with 1.7 M NaCl (data not shown), which is sufficient to remove the cellular histones (8, 31). To extend these studies further, we looked at the effect of temperature (4, 23, and 37°C) during incubation with 1 and 2 M NaCl on the association of T antigen with the SV40 DNA in the NPCs (Fig. 3A). Sucrose gradient-purified NPCs were treated with 1 and 2 M NaCl and incubated at their respective temperatures for 30 min. The samples were subsequently immunoprecipitated with SV40 anti-T serum, and portions of the immunoprecipitates were examined by SDS-polyacrylamide gel electrophoresis for analysis of protein and by agarose gel electrophoresis for analysis of DNA. The amount of T antigen present in the immunoprecipitates before and after salt treatment remained about the same (Fig. 4). However, there was a marked decrease in the amount of histone present in the immunoprecipitates as the salt concentration was increased (Fig. 4). Densitometer tracings of the agarose gels indicate a considerable decrease in the amount of SV40 (II) DNA immunoprecipitated with SV40 anti-T serum after incubation in high salt (Table 1). In contrast, as much as 75% of the SV40 (I) DNA which immunoprecipitated before treatment was still precipitable after incubation with 2 M NaCl at 23°C (Table 1). The decrease in the amount of both SV40 (I) and (II) DNA immunoprecipitated after salt treatment at 37°C appears to be real. Repeated experiments have given similar results, although less of a decrease was evident.

Sucrose gradient-purified NPCs were also treated with increasing concentrations of guanidine-hydrochloride (1, 2, and 4 M) over the same temperature range (4, 23, and 37°C) for 30 min. After the incubation period, samples were diluted to a final salt concentration of 1.0 M or lower before immunoprecipitation with SV40 anti-T serum to prevent variability in immunoprecipitation efficiency as a result of high salt. Approximately 22% of the SV40 (I) DNA which immunoprecipitated with hamster anti-T serum in the absence of salt pretreatment was still precipitable after 2 M guanidine-hydrochloride treatment at room temperature, as opposed to only 3% of SV40 (I) DNA immunoprecipitated after 4 M guanidine-hydrochloride treatment (Fig. 3B and Table 1). Comparison of the amount of SV40 (I) DNA immunoprecipitated by hamster anti-T se-

rum versus normal hamster serum indicates that the amount of SV40 (I) DNA precipitated was significantly above background, and as the salt concentration increased, there was a considerable decrease in the amount of nonspecific trapping of the DNA (Fig. 3B). Similar results were obtained when NPCs treated with NaCl were immunoprecipitated with normal hamster serum (data not shown). As in the case of NaCl treatment, the temperature at which the NPCs are treated with guanidine-hydrochloride affects the amount of SV40 DNA which remains associated with SV40 T antigen (Table 1). The marked effect of 4 M guanidine-hydrochloride on the amount of SV40 (I) and (II) DNA immunoprecipitated after treatment may be due to denaturation of T antigen by this particular salt, resulting in a decrease in recognition of T antigen by the polyvalent antiserum. When the proteins in the immunoprecipitates were examined by SDS-polyacrylamide gel electrophoresis, there was a significant decrease in the amount of T antigen immunoprecipitated after 4 M guanidine-hydrochloride treatment compared with that seen after 1 or 2 M treatment with the same salt (data not shown).

Characterization of the proteins associated with SV40 (I) DNA after high salt treatment of NPCs. SV40 NPCs labeled with [^3H]thymidine and [^{35}S]methionine were extracted from SV40-infected TC7 cells and sedimented on 5 to 30% neutral sucrose gradients. The peak NPC gradient fractions containing SV40 (I) DNA were pooled, dialyzed against TE buffer, and concentrated to approximately 0.1 of the initial volume. After incubation with 1 or 2 M NaCl for 30 min at 23°C, the NPCs were centrifuged on 20 to 30% neutral sucrose gradients. For markers, [^3H]thymidine-labeled SV40 (I) DNA and a sample of cytoplasmic extract containing the ribosomal subunits were centrifuged on parallel gradients. Liquid scintillation counting and agarose gel electrophoresis indicated that the SV40 NPCs containing SV40 (I) DNA sedimented as a 96S peak in the untreated sample, whereas the NPCs containing SV40 (I) DNA after 1 and 2 M NaCl treatment sedimented at 53S and 43S, respectively (data not shown). The SV40 (II) DNA which had been associated with the NPCs was found to sediment as a 17S peak after both 1 and 2 M NaCl treatment relative to markers run in parallel gradients.

Portions of each fraction of the gradients were further examined by SDS-polyacrylamide gel electrophoresis and fluorography to analyze the [^{35}S]methionine-labeled proteins present in the SV40 NPCs after salt treatment. In Fig. 5A, before salt treatment, fractions 10 to 12 correspond to the peak of SV40 NPCs containing [^3H]thymidine-labeled SV40 (I) DNA. It is clear

TABLE 1. Immunoprecipitation of SV40 (I) and (II) DNA from NPCs after treatment with NaCl and guanidine-hydrochloride^a

Treatment and temp (°C)	Salt concn (M)	% DNA immuno- precipitated	
		SV40 (I)	SV40 (II)
NaCl			
4	1	69	11
	2	63	11
23	1	79	15
	2	76	17
37	1	38	4
	2	32	1
HCl			
23	1	49	25
	2	22	6
	4	3	<1
37	1	27	14
	2	14	6
	4	1	<1

^a Numbers are expressed as percentages of SV40 (I) or (II) DNA immunoprecipitated after salt treatment, with the amount of DNA immunoprecipitated from untreated (0 M salt) controls taken as 100%. Densitometer tracings were done on the gels shown in Fig. 3. Repeated experiments showed some variability in the percentage of DNA immunoprecipitated, but the overall pattern reported here was consistent.

that there is a peak of [^{35}S]methionine-labeled proteins in this region which contains the viral capsid proteins VP1 and VP3, T antigen, and the histones. After 1 M NaCl treatment, fractions 16 to 18 correspond to the peak containing [^3H]thymidine-labeled SV40 (I) DNA (Fig. 5B); after 2 M NaCl treatment, fractions 17 to 19 contain the SV40 (I) DNA peak (Fig. 5C). In both cases, T antigen, VP1, and VP3 can be detected in these fractions but in decreased amounts from those found in the untreated NPC peak. These proteins do not appear as a discrete peak as in the untreated NPCs, but rather appear more diffuse in several fractions surrounding the SV40 (I) DNA peak. After salt treatment, there was an increase in the [^{35}S]methionine-labeled proteins found at the top of the gradients, particularly VP1 and the histones and less so T antigen (Fig. 5).

The gradient fractions containing the SV40 (I) DNA peak from each of the three gradients (untreated and treated with 1 or 2 M NaCl) were also analyzed by immunoprecipitation of a portion of each fraction with SV40 anti-T serum. In addition to T antigen, SV40 (I) and (II) DNA were still immunoprecipitated from the resedimented complexes in the 1 and 2 M salt-treated samples as well as in the untreated control sample (data not shown).

Since Griffith et al. (13) observed by means of

electron microscopy a protein associated with SV40 (I) DNA at the origin of SV40 DNA replication after 1 M NaCl treatment, we decided to characterize the proteins associated with the SV40 DNA in the NPCs after high salt treatment using their extraction procedure. With this system, which utilizes the detergent Triton X-100, we observed a decrease in the sedimentation rate of the [3 H]thymidine-labeled SV40 NPCs containing SV40 (I) DNA from 78S in the untreated sample to 46S in the 1 M NaCl-treated sample and 32S in the 2 M NaCl-treated sample (data not shown). When gradient fractions were subjected to SDS-polyacrylamide gel electrophoresis, it was noted that T antigen, VP1 and VP3 were still associated with the SV40 (I) DNA after 1 and 2 M NaCl treatment. The histones, on the other hand, were still detectable after 1 M NaCl treatment in the SV40 (I) DNA-containing region of the gradient but were no longer associated with the complexes after 2 M NaCl treatment (data not shown).

Comparison of the effect of high salt on the association of T antigen with SV40 DNA in NPCs from SV40 wild-type versus *tsA58*-infected cells. The association of T antigen with the SV40 NPCs is temperature sensitive in cells infected by SV40 *tsA58*, with T antigen no longer present in the NPCs at the nonpermissive temperature (24). It was of interest to determine whether T antigen from *tsA58*-infected cells was more sensitive to high salt treatment than was wild-type T antigen in its association with the SV40 DNA in the NPCs. Gradient-purified NPCs extracted from *tsA58*- and wild-type-infected cells grown at 32°C were treated with 1 and 2 M NaCl at the permissive (32°C) and nonpermissive (41°C) temperatures *in vitro* and immunoprecipitated with SV40 anti-T serum. Portions of the immunoprecipitates were analyzed by both agarose (Fig. 6) and SDS-polyacrylamide gel electrophoresis (data not shown). The overall pattern of the immunoprecipitates indicates that similar amounts of SV40 (I) and (II) DNA are precipitated with SV40 anti-T serum from *tsA58* versus wild-type NPCs (Fig. 6). Densitometer tracings of the agarose gels show 60% of SV40 (I) DNA immunoprecipitated after 1 M NaCl treatment and 50% immunoprecipitated after 2 M NaCl

FIG. 6. Effect of NaCl on the association of T antigen with SV40 DNA in NPCs from wild-type (wt) versus *tsA58*-infected cells grown at 32°C. [3 H]thymidine- and [35 S]methionine-labeled NPCs were extracted from wild-type and *tsA58*-infected cells at 32°C and centrifuged on 5 to 30% neutral sucrose gradients. The gradient-purified NPCs were treated with 1 and 2 M NaCl for 30 min at 32 or 41°C and then immunoprecipitated with SV40 anti-T serum. Portions of each sample were analyzed by agarose gel electrophoresis. The fluorogram was exposed for 2 weeks. Since there was less [3 H]thymidine-labeled SV40 DNA in the NPC peak from the *tsA58*-infected cells than from the wild-type-infected cells, each immunoprecipitate should be compared with its respective untreated (0 M salt) control.

treatment in both wild-type and *tsA58* NPCs incubated at 32°C. After incubation at 41°C, approximately 40% of SV40 (I) DNA was immunoprecipitated after 1 M NaCl treatment and 35% after 2 M NaCl treatment of both wild-type and *tsA58* NPCs. In general, there was a decrease in SV40 DNA immunoprecipitated from both wild-type and *tsA58* NPCs after treatment with NaCl at 41°C as opposed to 32°C (Fig. 6). The same sensitivities of wild-type and *tsA58* T antigen to high salt treatment suggest that *tsA* T antigen, once bound to the SV40 DNA in the NPCs at the permissive temperature, remains as tightly associated with the SV40 DNA as wild-type T antigen when shifted up to the nonpermissive temperature.

DISCUSSION

Treatment of SV40 NPCs with NaCl (1 or 2 M) or guanidine-hydrochloride (1 or 2 M) resulted in

FIG. 5. Sedimentation analysis of [3 H]thymidine and [35 S]methionine-labeled gradient-purified NPCs before and after NaCl treatment. SV40 NPCs were extracted as described in the text and sedimented on 5 to 30% neutral sucrose gradients. The NPC peak fractions were dialyzed against TE buffer, treated with 1 or 2 M NaCl, and re-centrifuged on 20 to 30% neutral sucrose gradients. A 10- μ l portion of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis. The positions of T antigen, VP1, VP3, and the histones are indicated. The fluorograms were exposed for 2 months. (A) Untreated NPCs; fractions 10 to 12 correspond to the peak of [3 H]thymidine-labeled SV40 (I) DNA. (B) 1 M NaCl-treated NPCs; fractions 16 to 18 correspond to the peak of [3 H]thymidine-labeled SV40 (I) DNA. (C) 2 M NaCl-treated NPCs; fractions 17 to 19 contain the SV40 (I) DNA peak. The difference in the amount of [35 S]methionine-labeled protein in the 1 M NaCl gradient versus the 2 M NaCl gradient is presumably due to experimental variation.

a significant fraction of SV40 (I) DNA still associated with T antigen (Fig. 3 and Table 1). The marked decrease in the amount of SV40 (II) DNA immunoprecipitated by hamster anti-T serum after salt treatment indicates that T antigen is preferentially associated with SV40 (I) DNA rather than with SV40 (II) DNA. Treatment of the NPCs with 4 M guanidine-hydrochloride, however, resulted in a substantial decrease even in the amount of SV40 (I) DNA associated with T antigen (Fig. 3B). Guanidine-hydrochloride is a powerful dissociating agent, affecting both electrostatic and hydrophobic types of interactions. It seems quite likely that the high concentration of guanidine-hydrochloride results in the denaturation of SV40 T antigen, so that it is not recognized as effectively by polyvalent hamster anti-T serum. Examination of the amount of T antigen immunoprecipitated after 4 versus 1 or 2 M guanidine-hydrochloride treatment indicates that there is a decrease in recognition of T antigen by the antiserum (data not shown). This decrease is not sufficient, however, to explain the marked decrease in immunoprecipitation of SV40 (I) DNA to 3% of that immunoprecipitated in the absence of pretreatment with guanidine-hydrochloride. If the conformation of T antigen is a critical factor in binding of the protein to the SV40 DNA, it would be reasonable to expect a marked decrease in the binding of denatured T antigen to the DNA. These data argue against a covalent bond between T antigen and the SV40 DNA, at least at the time of treatment of the NPCs with high salt concentrations. The continued association of T antigen with the SV40 DNA after 2 M NaCl or guanidine-hydrochloride treatment indicates a much tighter association of T antigen than of the cellular histones with SV40 DNA.

Studies of the *in vitro* binding of SV40 T antigen to SV40 DNA contrast with our results when sensitivity of the binding to salt is examined. Oren et al. (32) found that T antigen from SV40-infected cells bound to multiorigin SV40 DNA-cellulose *in vitro* under physiological conditions (pH 7.0; 0.15 M NaCl) and was eluted by NaCl at a concentration of 1 M or less. In addition, they found that rapidly labeled, newly synthesized T antigen bound to multiorigin SV40 DNA more tightly than older, long-term-labeled T antigen, requiring elution with 1 M NaCl as opposed to 0.4 M NaCl (32). Recently, Gidoni et al. (9) reported that the 5S to 7S form of T antigen bound more efficiently and tightly to multiorigin SV40 DNA-cellulose, requiring 1.0 M NaCl for elution from the DNA, whereas the 16S form bound less efficiently and less tightly to both calf thymus DNA and multiorigin SV40 DNA. It is not clear what factor is responsible for the increased salt resistance of T antigen

which has bound to SV40 DNA in the NPCs *in vivo* as opposed to binding to naked SV40 DNA *in vitro*. The difference may be due to the specific modification of a DNA-binding subclass of T antigen, to other proteins associated with T antigen itself or with the SV40 DNA in the near vicinity of T antigen, or to the conformation of the SV40 DNA bound to T antigen in the NPCs. Previous studies in our laboratory have shown that NPC T antigen is phosphorylated in a manner similar to the total T antigen population, with the exception of a minor phosphorylated peptide (25). It is possible, however, that there is an alternative form of modification which distinguishes the DNA-binding T antigen from the remaining T antigen molecules. By using monoclonal antibodies, Scheller et al. (38) have obtained evidence for a specific subclass of T antigen molecules which preferentially bind to the origin-containing fragment of SV40 DNA *in vitro*.

As the temperature was increased to 37°C during incubation of the NPCs with NaCl or guanidine-hydrochloride, there was a decrease in the amount of SV40 (I) and (II) DNA immunoprecipitated with hamster anti-T serum (Fig. 3 and Table 1). In contrast, temperature had no effect on the association of T antigen with the SV40 DNA in the absence of any pretreatment with salt (Fig. 3A). The association of T antigen with the SV40 DNA was affected by temperature only when the ionic environment surrounding the NPCs was perturbed. These results support our conclusion that T antigen is not covalently bound to the DNA in the NPCs. It is assumed that the presence of salt changes the conformation of T antigen and thereby affects the noncovalent interactions between it and the SV40 DNA so that they are more easily broken at increased temperatures.

After recentrifugation of the salt-treated NPCs, we observed a separation of the SV40 (I) and (II) DNA peaks (data not shown). Complexes containing SV40 (I) DNA had a sedimentation value of 53S after 1 M NaCl treatment and a value of 43S after 2 M NaCl treatment. After both 1 and 2 M NaCl treatment, SV40 (II) DNA sedimented at 17S, close to the sedimentation value of 16S for naked SV40 (II) DNA. It appears, therefore, that after 2 M NaCl treatment, there are no longer any proteins associated with SV40 (II) DNA, although there are still proteins bound to the SV40 (I) DNA. Our evidence indicates that, in addition to T antigen, certain amounts of VP1 and VP3 remain associated with the SV40 (I) DNA after high salt treatment. Some of the VP1 seen in this region of the gradient is thought to be an artifact, resulting from a tendency of VP1 to aggregate and smear across the gradient (Fig. 5). There

was, however, an increase in VP1 in the region of the gradient that contains SV40 (I) DNA, suggesting a specific association of VP1 with SV40 DNA, which is resistant to high salt treatment (Fig. 5). Brady et al. (1) have presented evidence for an association of VP1 with SV40 DNA, which is resistant to treatment with 0.25% Sarkosyl. The preferential association of T antigen with SV40 (I) DNA rather than with SV40 (II) DNA after high salt treatment was confirmed by the immunoprecipitation results in which SV40 (I) DNA was selectively precipitated by SV40 anti-T serum (Fig. 3 and Table 1).

When SV40 NPCs from wild-type- and *tsA58*-infected cells grown at the permissive temperature were treated with 1 and 2 M NaCl, we found that *tsA58* T antigen showed the same sensitivity as wild-type T antigen to high salt treatment at the permissive and nonpermissive temperatures (Fig. 6). This suggests that *tsA* T antigen maintains the same tight association with SV40 DNA as wild-type T antigen, once bound to the SV40 DNA in the NPCs. These results are consistent with the findings of Segawa et al. (40) that both wild-type and *tsA* T antigen remain associated with SV40 NPCs during elongation, even after shift-up to the nonpermissive temperature. In addition, Tack and DePamphilis (L. C. Tack and M. L. DePamphilis, manuscript in preparation) have found that wild-type T antigen remains associated with elongating SV40 DNA in NPCs until replication is 70% completed. Differences have been found between wild-type and *tsA* T antigen when the in vitro binding of T antigen to SV40 DNA is examined after preheating T antigen at different temperatures. Tenen et al. (47) demonstrated that *tsA* T antigen was more heat labile than wild-type T antigen in its ability to bind to SV40 in vitro. Wilson et al. (52) found that class II *tsA* mutants, including *tsA58*, produced T antigen which bound poorly in vitro to the origin of SV40 DNA replication after heat shock but exhibited wild-type binding to the origin of SV40 DNA replication if not heat shocked. We have previously shown that the association of *tsA* T antigen with the SV40 NPCs is temperature sensitive in cells infected by *tsA58*, with T antigen no longer present in the NPCs after shift-up of the *tsA*-infected cells to the nonpermissive temperature for 1 h before harvest of the NPCs (24). In keeping with the original observation of Tegtmeier (44) that SV40 T antigen is temperature sensitive for initiation of SV40 DNA replication but not for elongation, it appears that T antigen is temperature sensitive for initial binding to SV40 DNA in the NPCs but not for the ability to remain bound to SV40 DNA once associated with the NPCs. It is possible that the enzymatic function of T antigen required for initiation is not temperature sensitive

in the *tsA* mutants but is simply not expressed because *tsA* T antigen cannot bind to the DNA to perform its function. However, once bound to the DNA in the NPCs, both wild-type and *tsA* T antigen appear to be stabilized, so that they remain tightly associated with the DNA despite high salt concentrations or elevated temperatures. If T antigen expresses an enzymatic activity that is involved in elongation, it seems clear that this activity is either different from that involved in initiation, or, if it is the same as that involved in initiation, then this enzymatic activity must not be temperature sensitive in the *tsA* mutants.

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