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Simian Virus 40 T Antigen Binds to DNA

(sedimentation/multiple species/dissociation/denatured DNA/native DNA)

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ABSTRACT **The T antigen of simian virus 40, which may play a role in the control of viral DNA replication, is recovered from nuclei of cells transformed by simian virus 40 in several forms sedimenting at different rates. The large molecular weight forms are converted to the smallest (5 S) form by high salt, suggesting that they differ in the degree of aggregation. All the forms of the antigen bind efficiently to double-stranded DNA–cellulose columns at pH 6.2 and low salt, and elute in two fractions: one at pH 8.0 and low salt, the other at pH 8.0 and high salt. The antigen has little affinity for single-stranded DNA.**

Simian virus 40 (SV40) T antigen is detected by complement fixation or immunofluorescence in the nuclei of cells transformed (1) or lytically infected (2) by SV40. In lytic infection it is detectable before the onset of viral DNA synthesis, and its appearance is not inhibited if DNA synthesis is stopped by FdU and arabinosylcytosine (3, 4). Hence, its appearance is controlled by the "early" region of the viral genome. The appearance of a normal T antigen requires the activity of the viral *A* gene of both SV40 and of the related polyoma virus. In fact, Osborn and Weber have reported an alteration in the sedimentation pattern of the SV40 T antigen in cells infected with a *tsA* mutant at the nonpermissive temperature (abstract in Cold Spring Harbor virus meeting 1973), and in cells infected or transformed by the *tsA* mutant of polyoma virus, the T antigen is not detectable by immunofluorescence at the nonpermissive temperature (5). These findings show that the T antigen may be the product of the *A* gene, or related to it. This relationship makes the antigen especially interesting, because the *A*-gene function has been shown to be required for viral DNA replication in both SV40 (6) and polyoma virus (7) infection and, with polyoma virus, for initiation of transformation (8).

Not much is known about the properties of the T antigen. SV40 T antigen has been reported to sediment as multiple species with molecular weights of about 300,000, 120,000, and 70,000 by Potter *et al.* (9), who have suggested that the multiple forms are tetramer, dimer, and monomer of the same protein, but have not shown the interconversion. Del Villano and Defendi have reported that the 70,000 molecular weight form is not further dissociable (10).

In this report we show that the larger molecular weight forms can be dissociated to yield the smallest form, and that all forms bind strongly to double-stranded DNA.

MATERIALS AND METHODS

Cell Cultures. Cells of the SV40-transformed mouse line SV3T3 (11) were grown in rotating 2.5-liter bottles in 200 ml of Dulbecco's modified Eagle's medium with 10% calf serum at 37°. Before they were sealed, the bottles were gassed for 15 seconds with 20% CO_2–80% air. The cells were grown to confluency and then harvested by detaching them with trypsin–EDTA.

Cells of the SV40-transformed hamster cell line SV28 (12), derived from BHK cells, and of the SV40-transformed BSC-1 line were grown and harvested by the above procedure.

Preparation of T Antigen. Nuclei were prepared by homogenization of fresh SV3T3 cells (about 12 g wet cells) with a 40-ml Dounce homogenizer in 30 ml of 0.25 M sucrose, 3 mM $CaCl_2$. The nuclei were pelleted by centrifugation for 5 min at 16,000 × *g* in the HB-4 rotor of the Sorval preparative centrifuge. The homogenization and centrifugation were repeated in 0.25 M sucrose, 5 mM potassium phosphate (pH 6.2), 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol. The nuclear pellet was stored at −20°. Crude nuclear lysate was made by freezing and thawing 1.5 g of nuclei in 4 ml of 10 mM tris-(hydroxymethyl)aminomethane (Tris)·HCl (pH 7.1), 1 mM dithiothreitol, 0.14 M NaCl, and then using the supernatant of a centrifugation for 20 min at 16,000 × *g* in the Sorval centrifuge at 4°.

The more purified material used for the 1 M NaCl dissociation was made by a modification of the procedure of Lazarus *et al.* (13) by twice freezing and thawing 6–9 grams of nuclei in 5 mM potassium phosphate (pH 6.2), 0.1 M NaCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol, and centrifuging for 20 min at 16,000 × *g* at 4° in the Sorval centrifuge. The precipitate was extracted twice with 15 ml of 20 mM Tris·HCl (pH 8.0), 0.8 M NaCl, 20 mM EDTA, 1 mM dithiothreitol and centrifuged as before. The two pH 8.0 extracts were pooled, and the fraction precipitating between 18 and 45% saturation with ammonium sulfate was prepared. The precipitate was resuspended in 2.5 ml of 10 mM Tris·HCl (pH 8.0), 1.0 M NaCl, 1 mM dithiothreitol and then applied directly to the sucrose gradient.

It has been reported (14) that many antisera with T-specificity have also U-specificity. Since the T antigen is heat labile, losing its antigenicity after incubation at 50° for 30 min, whereas the U antigen is stable under these conditions, we have tested the heat stability of all of the sedimenting species and DNA–cellulose column fractions reported in this paper. All were found to be heat labile, and, therefore, contained T antigen.

Preparation of Antiserum. SV28 cells were harvested, washed twice with medium, and resuspended in Dulbecco's modified Eagle's medium at a concentration of 2 × 10^6 cells per ml. Four-month-old golden hamsters were injected subcutaneously with 10^6 cells each. When the tumors were about

Abbreviation: SV40, simian virus 40.

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3 cm in diameter, the hamsters were bled. The serum, separated out on storage at 4°, was heated at 55° for 30 min to inactivate complement, and was then stored at −20°. The antiserum reacted with SV3T3 nuclei but not with Balb/c 3T3 nuclei in the immunofluorescence test.

In a number of experiments, we used antiserum purchased from Flow laboratories. This, and antiserum prepared by us, gave us indistinguishable results.

Complement Fixation. The assay was carried out as described by Levine (15) except that a volume of 0.6 ml was used. A dilution of 1/400 of the antibody was used. Guinea pig complement, rabbit hemolytic serum, and sheep erythrocytes were obtained from Wellcome Reagents, Ltd. In assaying sucrose gradient and column eluate fractions, 5-μl aliquots of each fraction were added to a final volume of 0.6 ml of the complement fixation assay mixture. The results were expressed as the percentage of inhibition of hemolysis by means of the absorbance of the supernatant at 413 nm.

Sucrose Gradient Band Centrifugation. Linear sucrose gradients of 5–20% sucrose (with a 60% sucrose pad) were poured with the aid of a mixing device. The 4.4-ml analytical gradients (with 0.4-ml 60% sucrose pad) contained: 10 mM Tris·HCl (pH 7.4), 1 mM dithiothreitol, 0.14 M NaCl. Over this gradient was layered 0.2 ml of sample; then the gradient was centrifuged for 18 hr at 23,000 rpm (about 50,000 × *g*) in the 6 × 5 swing-out rotor in the MSE preparative ultracentrifuge. The 60-ml preparative gradients (with 4-ml 60% sucrose pads) contained: 10 mM Tris·HCl (pH 8.0), 1 mM dithiothreitol and 1 M NaCl in addition to sucrose. The tubes were pierced by a needle and 6-drop (0.14–0.16 ml) fractions were collected from the analytical gradients; 2-ml fractions were collected from the preparative gradients.

DNA–Cellulose Column Chromatography. Double-stranded DNA–cellulose was made by a modification of the method of Alberts *et al.* (16) by drying 100 ml [2 mg/ml in 10 mM Tris·HCl (pH 7.1)] Sigma calf thymus DNA with 30 g of Machery, Nagel & Co. cellulose powder. Single-stranded DNA–cellulose was made with the same DNA that had been denatured by titration to pH 12 with NaOH, allowed to stand 15 min, and then adjusted to pH 7.1 with HCl. The single-stranded DNA was then dialyzed against 10 mM Tris·HCl (pH 7.1).

In either case, the dry DNA–cellulose powder was suspended immediately before use in pH 6.2 elution buffer: 5 mM potassium phosphate buffer (pH 6.2), 0.1 M NaCl, 1 mM dithiothreitol, and 10% (v/v) glycerol. The column was then poured and washed with 100 ml of pH 6.2 elution buffer. The pH 8 elution buffer contained 10 mM Tris·HCl (pH 8.0), 1 mM dithiothreitol, 0.10 M NaCl, and 10% glycerol.

RESULTS

Sedimentation Behavior. The three molecular weight classes of T antigen reported by others (9) have been observed repeatedly with complement fixation when nuclear extracts were centrifuged through sucrose gradients (Fig. 1*A*, peaks 1, 2, and 3). However, inclusion of 1 mM dithiothreitol as a reducing agent in the extraction buffer and in the sucrose gradient caused the disappearance of peak 1, which is the smallest of 4–5S species, sedimenting at about the same rate as 5S ribosomal RNA (Fig. 1*B*). Thus, reduced sulfhydryl groups are apparently necessary for the maintenance of T

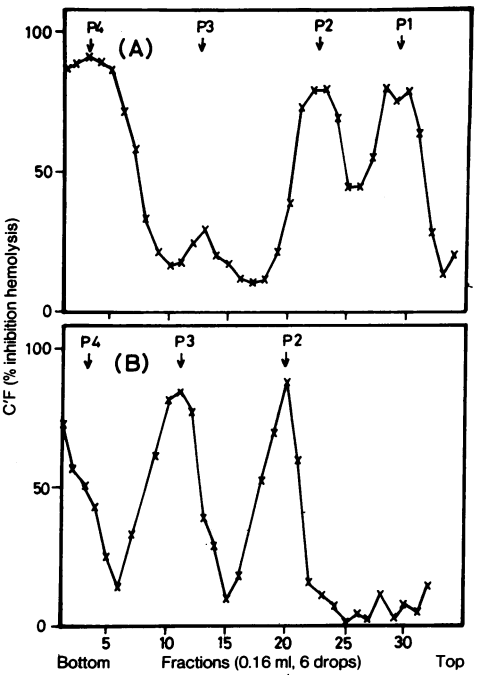

FIG. 1. Sucrose gradient band centrifugation of SV40 T antigen. (A) Extract and gradient without dithiothreitol. (B) Extract prepared in the presence of 1 mM dithiothreitol and gradient containing 1 mM dithiothreitol. The 4.4-ml gradients, each with 0.2 ml of extract, were centrifuged for 18 hr at 23,000 rpm at 4° in the MSE 6 × 5 swing-out rotor.

antigen in the higher molecular weight forms. In the absence of dithiothreitol, the amount of peak 1, relative to peaks 2 and 3, tended to increase spontaneously during storage at −20°. Even in the presence of dithiothreitol, it was increased by manipulations such as recentrifugation or purification. Given the importance of dithiothreitol, it was always present in the gradient during centrifugation.

In addition to peaks 1, 2, and 3, some T antigen apparently sedimented to the bottom of the gradient (Fig. 1*A* and *B*, position 4), but not reproducibly. On centrifugation for shorter times, this material was not detectable, probably because it was heterogeneous and, therefore, was detectable only when it accumulated on the bottom of the gradient.

Although at physiological salt concentration T antigen sedimented as the two higher molecular weight forms (Fig. 2*A*), in higher salt concentrations (0.5–1 M KCl or NaCl) it dissociated to give predominantly the 4–5S species (Fig. 2*B* and *C*). At the higher salt concentration, all forms sedimented more slowly, probably due to increased solvent density. Recentrifugation of material isolated from peak 1 (Fig. 3*A*) produced a single peak sedimenting at low salt concentration like the original peak 1 (Fig. 3*B*). No significant amount of faster sedimenting material was detected. Thus, material from peak 1 obtained by dissociation in high salt failed to reassociate when the salt concentration was again lowered to 0.14 M NaCl.

DNA-Binding Properties. When T antigen from nuclear extracts was applied at pH 6.2 to double-stranded DNA–cellulose, it was efficiently bound to the column. No T antigen could be eluted between pH 6.2 and 7.4. However at pH 8.0 one fraction of T antigen was eluted (Fig. 4). This first frac-

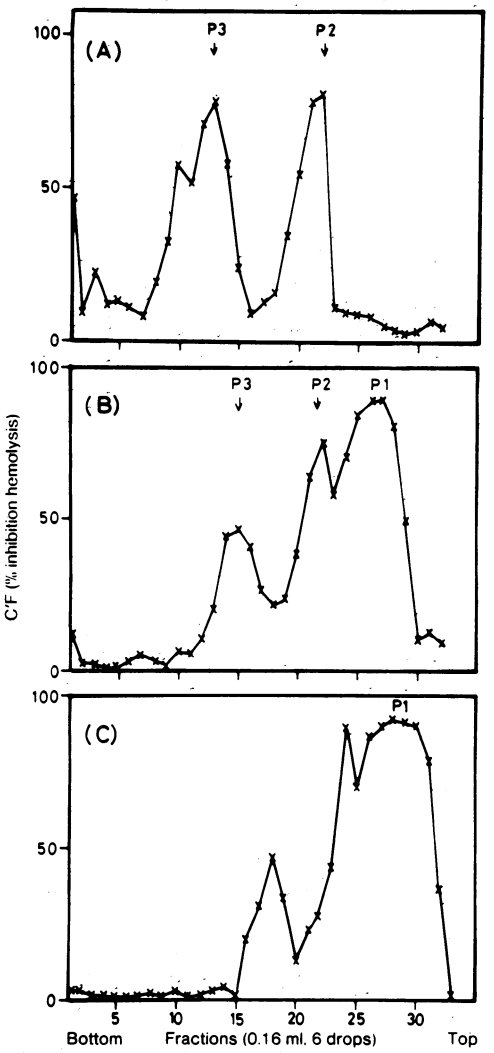

FIG. 2. The effect of NaCl concentration on the sedimentation behavior of SV40 T antigen. (A) 0.14 M NaCl; (B) 0.5 M NaCl; (C) 1.0 M NaCl. The 4.4-ml gradients, each with 0.2 ml of extract, were centrifuged for 18 hr at 23,000 rpm in the MSE 6 × 5 swing-out rotor.

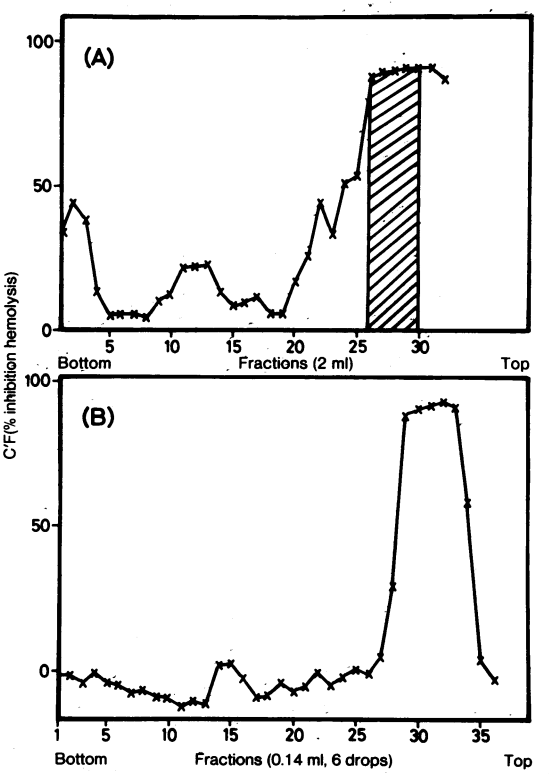

FIG. 3. Resedimentation of salt-dissociated SV40 T antigen. (A) Preparative centrifugation in 1.0 M NaCl. Partially purified T antigen (4 ml), prepared as noted in *Materials and Methods*, was centrifuged on a 60-ml 5–20% sucrose gradient with 1 M NaCl for 18 hr at 23,000 rpm at 4° in a MSE 3 × 70 swing-out rotor. (B) Recentrifugation in 0.14 M NaCl. The pooled salt-dissociated material, indicated by crosshatching in panel A, was precipitated with 45% saturated ammonium sulfate and resuspended in 1 ml of centrifugation buffer. Of this, 0.3 ml was layered over the standard 4.4-ml gradient and centrifuged 18 hr at 23,000 rpm at 4° in an MSE 6 × 5 swing-out rotor.

tion could also be eluted by adding 1.0 M NaCl to the pH 6.2 elution buffer. A second fraction of T antigen was eluted by pH 8.0 elution buffer containing 0.6 M NaCl (Fig. 4). Thus, T antigen has a strong affinity for DNA at pH 6.2. The fact that two fractions were eluted from the DNA cellulose at different salt concentrations indicates that T antigen binds to calf thymus DNA with two affinities.

When T antigen, dissociated with 1.0 M NaCl to the slowly sedimenting form, was applied to the double-stranded DNA–cellulose column in pH 6.2 elution buffer, it was found to bind completely and to elute in the same two peaks as it had prior to dissociation. Thus, T antigen, which sedimented in 0.14 M NaCl as one species, bound to double-stranded DNA with two affinities.

T antigen was found to bind poorly to single-stranded calf thymus DNA. In fact it never binds completely at pH 6.2, even with large increases in column size, and what binds has low affinity since it is completely eluted at pH 8 in low salt. The unbound material was found to be in equilibrium with the bound material, as successive passages, through successively larger columns, retained more of the previously unbound material.

DISCUSSION

The dissociation of the two faster sedimenting forms of T antigen to the slowest sedimenting, smallest molecular weight form demonstrates that there is a unique subunit; the two faster sedimenting forms may represent oligomers, as already put forward by Potter *et al.* (9). The results do not answer the question of whether the multiple forms are due to self-association, perhaps in cooperation with a small ligand, or due to binding to some other moiety, either protein or polynucleotide. The inability of the salt-dissociated, slowly sedimenting form to reassociate under the conditions used suggests that some other moiety is necessary. Lack of reassociation cannot be attributed to damage of the protein by 1 M salt because the protein so treated retained antigenic activity and binding affinity for double-stranded DNA.

The results demonstrate that T antigen binds strongly to double-stranded DNA. The occurrence of two affinities shown by both salt-dissociated and undissociated T antigen may be indicative of its function on the DNA. The two affinities may be the result of two different classes of sites on the DNA, as with RNA polymerase (17), or may be the result of cooperative binding, as shown by T4 gene-32 protein (18).

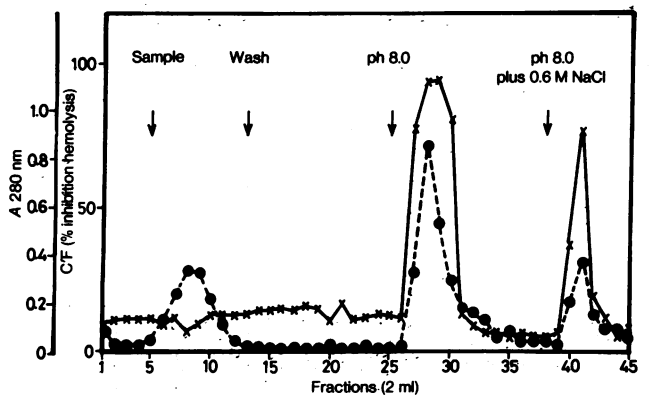

FIG. 4. Double-stranded DNA–cellulose column chromatography of SV40 T antigen. Nuclear extract was prepared as noted in *Materials and Methods* from 9 g of nuclei and applied to a 1.4 × 15-cm double-stranded DNA–cellulose (4 g) column, which was then eluted with 15 ml of pH 6.2 buffer, 25 ml of pH 8.0 buffer, and 25 ml of pH 8.0 buffer plus 0.6 M NaCl. ●, Absorbance at 280 nm; ×, C'F.

They are evidently not the result of differing affinities of the different molecular weight forms of T antigen, as dissociated T antigen also binds to double-stranded DNA, and does so with the same two affinities.

The stronger affinity of T antigen for double-stranded than for single-stranded DNA indicates that T antigen is not similar to T4 gene-32 protein, which has higher affinity for single-stranded DNA. The affinity for double-stranded DNA and the double affinity suggests that the T antigen has similarities to the λ repressor protein (19).

This similarity is consistent with the relatedness of the T antigen to the *tsA* gene, whose product is required for initiating both the replication of the viral DNA and cell transformation. It is conceivable that both functions are the result of the interaction of the T antigen with the double-stranded DNA.

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1. Black, P. H., Rowe, W. P., Turner, H. C. & Huebner, R. J. (1963) *Proc. Nat. Acad. Sci. USA* **50**, 1148–1156.
2. Rapp, F., Kitahara, T., Butel, J. S. & Melnick, J. L. (1964) *Proc. Nat. Acad. Sci. USA* **52**, 1138–1142.
3. Gilden, R. V., Carp, R. I., Taguchi, F. & Defendi, V. (1965) *Proc. Nat. Acad. Sci. USA* **53**, 684–692.
4. Rapp, F., Butel, J. S., Feldman, L. A., Kitahara, T. & Melnick, J. L. (1965) *J. Exp. Med.* **121**, 935–944.
5. Oxman, M. N., Takemoto, K. K. & Eckhart, W. (1972) *Virology* **49**, 675–682.
6. Tegtmeyer, P. (1972) *J. Virol.* **10**, 591–598.
7. Francke, B. & Eckhart, W. (1973) *Virology* **55**, 127–135.
8. Fried, M. (1965) *Proc. Nat. Acad. Sci. USA* **53**, 486–491.
9. Potter, C. W., McLaughlin, B. C. & Oxford, J. S. (1969) *J. Virol.* **4**, 574–579.
10. Del Villano, B. C. & Defendi, V. (1973) *Virology* **51**, 34–46.
11. Tonegawa, S., Walter, G., Bernardini, A. & Dulbecco, R. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 823–831.
12. Wiblin, C. N. & MacPherson, I. A. (1972) *Int. J. Cancer* **10**, 296–309.
13. Lazarus, H. M., Sporn, M. B., Smith, J. M. & Henderson, W. R. (1967) *J. Virol.* **1**, 1093–1095.
14. Lewis, A. & Rowe, W. P. (1971) *J. Virol.* **7**, 189–197.
15. Levine, L. (1967) in *Handbook of Experimental Immunology*, ed. Weir, D. M. (Blackwell, Oxford and Edinburgh), pp. 707–719.
16. Alberts, B. M., Amodio, F. J., Jenkins, M., Gutmann, E. D. & Ferris, F. L. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33**, 289–305.
17. Hinkel, D. C. & Chamberlin, M. J. (1972) *J. Mol. Biol.* **70**, 157–185.
18. Alberts, B. M. (1970) *Fed. Proc.* **29**, 1154–1163.
19. Ptashne, M. (1971) in *The Bacteriophage Lambda*, ed. Hershey, A. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), pp. 221–237.