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Different Forms of Simian Virus 40 Large Tumor Antigen Varying in Their Affinities for DNA

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In various permissive monkey cell lines infected with simian virus 40 there are two major forms of large T antigen which differ in their rate of sedimentation through sucrose gradients. The lighter (5 to 7S) form sedimented slightly more rapidly than the 4S tRNA marker, whereas the heavier (16S) form sedimented slightly more slowly than the 18S rRNA marker. The small t antigen did not form complexes which sedimented as rapidly as those formed by the large T antigen. The 16S T antigen form was converted to the slowly sedimenting 5 to 7S form in the presence of 1.0 M NaCl. The majority of large T antigen synthesized in cell-free protein-synthesizing systems primed by mRNA isolated from infected cells sedimented as the 5 to 7S form even when premixed with excess quantities of cellular T antigen. The formation of the 16S form in infected cells did not require ongoing viral or cellular DNA replication because considerable quantities of this T antigen class were produced in the presence of DNA synthesis inhibitors, such as cytosine arabinoside. Both 5 to 7S and 16S forms could be isolated separately and, therefore, each could be analyzed as to its individual properties. The 5 to 7S T antigen form bound more efficiently and tightly to DNA and had specific affinity for sequences at the viral origin of replication, whereas the 16S form bound less efficiently to DNA and exhibited very little specificity for origin-containing DNA sequences. It is therefore likely that the active DNA-binding species of T antigen isolated from infected cells is the 5 to 7S form.

The early region of the genome of the DNA tumor virus simian virus 40 (SV40) encodes two polypeptides, the large T and small t antigens (for a review see reference 42). Genetic studies have shown that large T antigen is required for initiating viral DNA replication (36) and viral L-strand-specific RNA transcription (4) and for initiation (12) and maintenance (2, 16, 25, 37) of the transformed phenotype in nonpermissive cells. In addition, this gene product exerts negative feedback control over early mRNA synthesis (31, 39). Small t antigen has no apparent role in the lytic cycle of the virus but appears to play a role, by slowing tumor growth, in virus-induced malignant transformation (17). Although little is known about the properties of small t antigen, considerable information has been acquired regarding the biochemistry of the large T antigen. Studies utilizing either authentic T antigen from lytically infected or transformed cells, or the closely related D-2 protein, have shown it to be a phosphoprotein (38) exhibiting both tight (specific) (23, 34, 40) and loose (nonspecific) affinities for DNA (3, 23), as well as having ATPase activity (41). In addition, both earlier (3, 13, 15, 24, 27) and more recent (5, 6, 18, 28) papers have described the ability of the T antigen protein to form rapidly sedimenting complexes. As both biological and biochemical evidence supports the idea that T antigen is a multifunctional protein, we sought to isolate different forms of the T antigen which may have different functions. More specifically, we isolated and characterized two forms of the T antigen from infected cells which differ in their sedimentation properties and affinities for DNA.

MATERIALS AND METHODS

Cells and viruses. BSC-1 or CV-1 lines of African green monkey kidney cells were used, both for infected cell extracts and to grow SV40 virus strains 777 and 776.

Labeling and extractions of cells. Extraction and labeling of infected cells were done by previously published procedures (29). Generally, cultures of 4×10^6 cells were labeled with [^{35}S]methionine (100 μCi/ml) in methionine-free medium from 42 to 46 or 44 to 46 h postinfection with SV40. All subsequent operations were performed at 4°C. Cells were washed three times in phosphate-buffered saline and then lysed in 0.6 ml of HIP buffer (pH 8.2) containing 0.14 M NaCl, 0.02 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.001 M $MgCl_2$, and 0.5% Noni-

det P-40. Phenylmethylsulfonyl fluoride and 1-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) were added to a final concentration of 0.25 mg/ml of extract immediately before lysis. Nuclei and debris were removed by centrifugation at 2,000 rpm for 2 min.

Sucrose gradient sedimentation and immunoprecipitation. Cytoplasmic extracts (200 μl) were layered onto 5-ml gradients of 5 to 20% sucrose in HIP buffer containing 50 μg of freshly added TPCK per ml and 100 μg of phenylmethylsulfonyl fluoride per ml. Gradients were centrifuged at 4°C for 180 min at 48,000 rpm in a Beckman SW50.1 swinging bucket rotor. Ten 520-μl or twenty 260-μl fractions were collected per gradient. After removal of 15-μl samples of each gradient fraction for direct analysis by polyacrylamide gel electrophoresis (PAGE), the remaining portion was immunoprecipitated with 10 μl of anti-SV40 T serum for 1 h at 4°C. Immune complexes were precipitated by the addition of 50 μl of a 10% suspension of formaldehyde-fixed *Staphylococcus* A bacteria for 15 min. Immunoprecipitates were washed, suspended in electrophoresis sample buffer, and subjected to PAGE as previously described (29). Gels were fluorographed, dried, and exposed to Kodak XR-5 film.

mRNA preparation and translation. Cytoplasmic polyadenylic acid-containing RNA was purified from SV40-infected cells and translated in the nuclease-free reticulocyte lysate system according to previously published procedures (30). mRNA (20 μg) was added per 500 μl of translation reaction mixture containing 400 μCi of [^{35}S]methionine. Translation products were layered onto 5 to 20% sucrose gradients, centrifuged through sucrose gradients, and immunoprecipitated as above.

DNA-binding experiments. Binding of T antigen in sucrose gradient fractions to DNA-cellulose columns was carried out by procedures described by Oren et al. (23). Samples (200 μl) of [^{35}S]methionine-labeled infected cell extracts were centrifuged through five sucrose gradients run in parallel, separately fractionated, and stored. One set of gradient fractions was immunoprecipitated with anti-T serum, subjected to PAGE, and then autoradiographed to identify peak tubes of T-antigen-sedimenting forms, which were then pooled separately from the other individual gradients. Samples to be bound to DNA were adjusted to pH 7.0 with 2-μl increments of 0.1 N acetic acid and loaded onto a 1-ml calf thymus DNA-cellulose column or F-161 SV40 multiorigin variant DNA-cellulose column (23) which had been preequilibrated with 10 mM potassium phosphate–0.1 M NaCl–10% (vol/vol) glycerine–0.5% Nonidet P-40, pH 7.0. After allowing 30 min for the sample to bind, the nonbound fraction was collected, and the column was washed with equilibration buffer. Bound proteins were eluted stepwise with buffers containing 0.4 M and 1.0 M NaCl. Nonbound and bound eluted fractions were then adjusted to 0.15 M NaCl, immunoprecipitated with anti-T serum, and subjected to sodium dodecyl sulfate-PAGE and autoradiography as above.

DNA binding in solution was performed by recently described procedures (A. Scheller, L. Covey-Nichols, B. Barnet, C. Prives, submitted for publication). Sucrose gradient fractions were adjusted to pH 6.5 with 2-μl samples of 0.1 N acetic acid. Five nanograms of *Bst*N1-digested SV40 DNA labeled with ^{32}P by nick-translation (32), as well as 5 μg of sheared salmon sperm DNA, was added to each fraction for 1 h followed by immunoprecipitation of the T antigen DNA complexes with 10 μl of anti-T serum and *Staphylococcus* A bacteria as above. After the complexes had been washed three times, DNA fragments were released from the immune complexes with 50 μl of buffer containing 0.01 M Tris, 0.001 M EDTA, and 0.5% sodium dodecyl sulfate (pH 7.4), subjected to electrophoresis through 2.0% agarose gels in Tris-borate buffer, then dried, and autoradiographed.

Materials. [^{35}S]methionine and [^{32}P]deoxynucleoside triphosphates were purchased from the Radiochemical Centre, Amersham, England. TPCK, phenylmethylsulfonyl fluoride, hydroxyurea, 2'-deoxy-2'-azidocytidine, aphidicolin, and cytosine arabinoside were bought from Sigma Chemical Co., St. Louis, Mo., and *Bst*N1 restriction endonuclease was purchased from New England Biolabs, Beverly, Mass. *Staphylococcus* A bacteria were purchased from Calbiochem, La Jolla, Calif., as Pansorbin. Anti-SV40 T serum was obtained from the Research Resources Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md.

RESULTS

Existence of T antigen as two major sedimenting forms in infected monkey cells. To extend studies describing different oligomeric forms of T antigen, extracts of [^{35}S]methionine-labeled BSC-1 cells were subjected to sedimentation through sucrose gradients. Individual gradient fractions were collected and immunoprecipitated with anti-T serum, followed by PAGE and autoradiography. Under the conditions of infection, labeling, extraction, and centrifugation described herein, the large T antigen appears as two major sedimenting classes (Fig. 1). The lighter class cosedimenting with 5S RNA and slightly less rapidly than aldolase (molecular weight, 159,000), was termed the 5 to 7S form. The heavier class, sedimenting less rapidly than 18S rRNA or thyroglobulin (molecular weight, 670,000), was termed the 16S form. It was not feasible to assign definitive sedimentation coefficients or molecular weights for these forms, isolated as they are from the crude extracts of infected cells. In many experiments a 45,000-molecular-weight polypeptide was immunoprecipitated from all gradient fractions. This may well be the major capsid protein VP-1 which has this molecular weight, reacts with many anti-T sera (35), and forms rapidly sedimenting assembly complexes (26).

The small t antigen, although sedimenting somewhat more rapidly than expected for a 17,000-molecular-weight polypeptide, was not detectable in gradient fractions containing the 16S form of large T antigen and therefore does not participate in the rapidly sedimenting large T antigen complexes. The sedimentation properties of large T and small t antigens were repro-

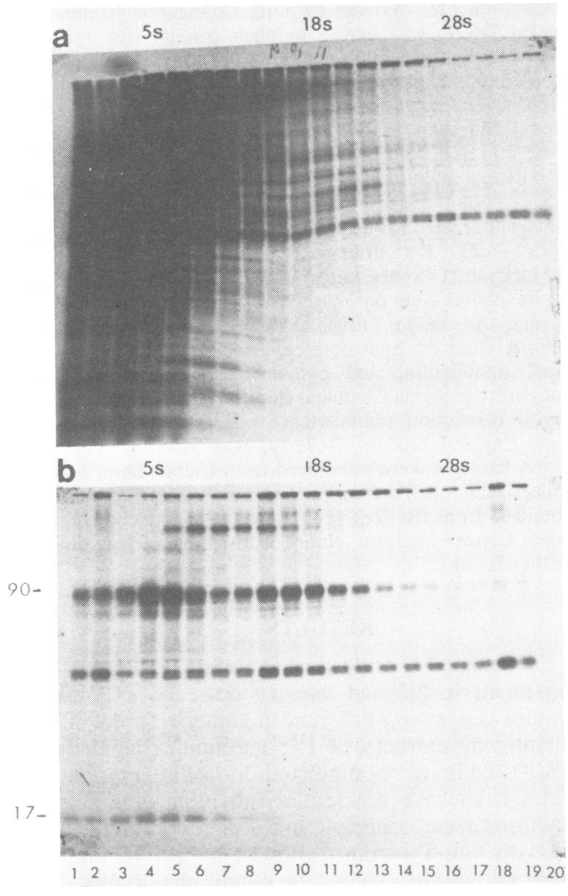

FIG. 1. Sucrose gradient sedimentation of SV40 T antigen. [^{35}S]methionine-labeled cell extracts were subjected to centrifugation through 5 to 20% sucrose gradients as described in the text. Twenty fractions were collected, and 10-μl samples of each fraction were directly analyzed by PAGE and autoradiography (a). The remainder of each sample was immunoprecipitated with anti-SV40 T serum and similarly analyzed (b). Two parallel sucrose gradients were run, containing [^{3}H]uridine-labeled rRNA markers in one and purified preparations of aldolase and thyroglobulin in the other. Peak tubes of 5S, 18S, and 28S rRNA markers were nos. 4, 11, and 17, respectively, and peak tubes of aldolase (molecular weight, 159,000) and thyroglobulin (molecular weight, 670,000) were nos. 5 and 11, respectively.

ducibly observed in several SV40-infected monkey lines, including BSC-1, CV-1, TC-7, and AGMK cells. The relative proportion of the 5 to 7S and 16S large T antigen form varied among different monkey cell lines. Generally, there was more [^{35}S]methionine-labeled 5 to 7S form than 16S form immunoprecipitated from sucrose gradient fractions. However, there was more rapidly sedimenting than slowly sedimenting T antigen when this protein was measured by the enzyme-linked immunosorbent assay, performed as recently published (11, 43), indicating that much of the accumulated T antigen exists as heavier complexes (data not shown). As the experiments in this study measured the [^{35}S]methionine-labeled T antigen, it should be kept in mind that all the data presented refer to that population of T antigen molecules synthesized during the labeling period of 2 to 4 h before extraction.

T antigen synthesized in vitro. Studies in other laboratories (5, 6, 13), as well as our own (28), have shown that *tsA* mutant T antigen does not form rapidly sedimenting complexes at the restrictive temperature, suggesting that the generation of the 16S form is related to a biological function of T antigen. To further explore this idea, we examined the sedimentation properties of T antigen synthesized in a cell-free protein-synthesizing system primed with SV40 E-strand mRNA. This T antigen would serve as an example of nonfunctional protein in that it has no opportunity to act in viral and cellular DNA replication or RNA transcription. Moreover, it may not undergo the identical post-translational modifications characteristic of T antigen isolated from infected cells.

When T antigen synthesized in the nuclease-treated reticulocyte lysate protein-synthesizing system was centrifuged through a sucrose gradient, no evidence of the rapidly sedimenting form was detected (Fig. 2). Even after co-incubation and co-sedimentation of the cell-free reticulocyte extract with unlabeled extracts of infected or uninfected cells, the in vitro-synthesized T antigen existed only as the 5 to 7S form. The results shown in Fig. 2, in which 10 fractions per gradient were collected, were confirmed in experiments with 20 fraction gradients, such as those normally used in other experiments. This supports the idea that the 16S form of T antigen is a property of native, functional protein. Either a specific post-translational modification or some aspect of the biological function of the T antigen protein is responsible for the ability of this viral protein to form rapidly sedimenting complexes.

16S T antigen formation. Neither *tsA* mutant T antigen at restrictive temperature nor in vitro-synthesized T antigen forms oligomers. Both are nonfunctioning T antigens, the former due to its conditional defect, the latter because of its lack of opportunity to function or lack of proper post-translational modification or both. Another link between these two non-oligomerizing T antigens is the absence of DNA replication. *tsA* mutant-infected cells failed to initiate viral or cellular DNA replication at the nonpermissive temperature, and clearly there was no DNA replication

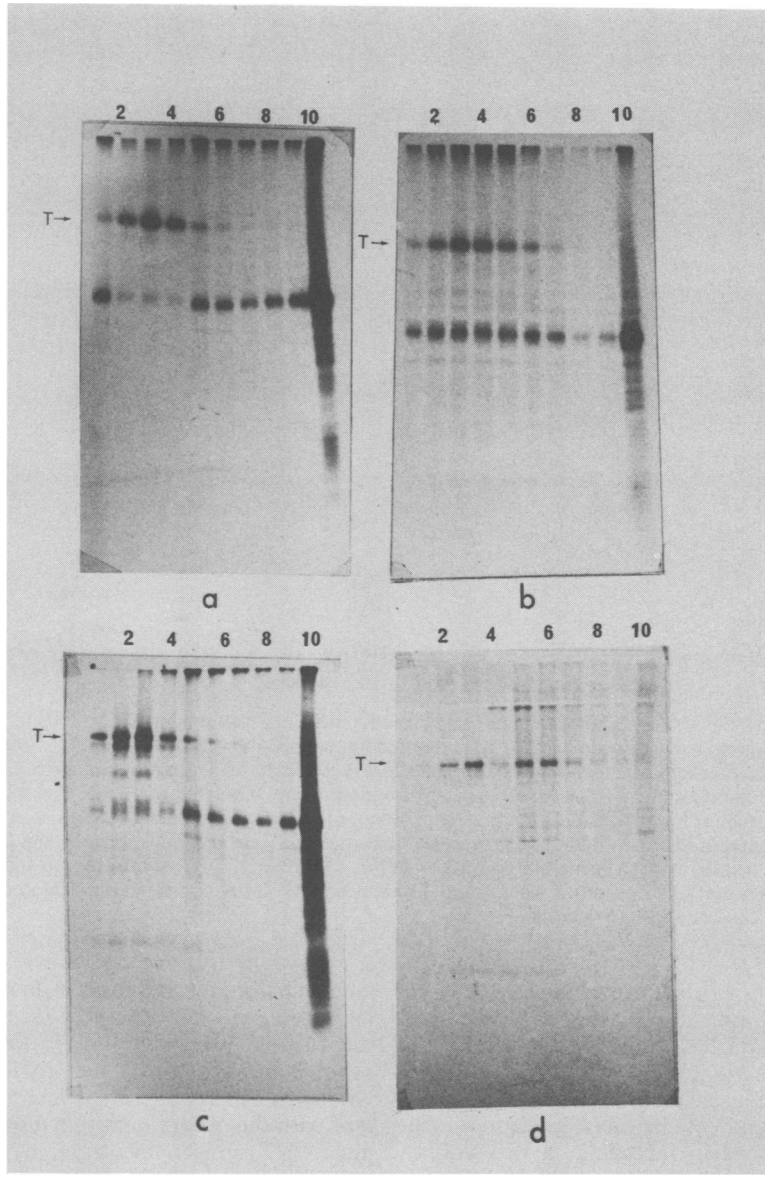

FIG. 2. Sedimentation of T antigen synthesized in vitro. SV40 T antigen was synthesized in the reticulocyte translation system (0.5-ml reaction volume) in response to mRNA from SV40-infected cells. Translation products (100 μl) were mixed with 100 μl of HIP buffer (a); 100 μl of infected unlabeled cell extract (b); 100 μl of uninfected unlabeled cell extract (C) for 1 h at 4°C followed by sucrose gradient centrifugation, immunoprecipitation, and analysis as described in the legend to Fig. 1. [^{35}S]methionine-labeled T antigen (100 μl) from SV40-infected extract was similarly analyzed on sucrose gradient (d). Ten fractions were collected from each gradient. That 5 to 7S and 16S T antigen forms could be distinguished under these sedimentation and fractionation conditions is seen in (d). Peak tubes of 5S, 18S, and 28S rRNA markers run in parallel were 3, 6, and 9, respectively.

in reticulocyte lysates. This led us to question whether or not there is a requirement for ongoing DNA replication in order for the oligomeric T antigen to form. To test this, extracts of [^{35}S]methionine-labeled cells were prepared 10 h postinfection, at a time considered to precede the onset of virus-induced cellular and viral DNA replication. After centrifugation of these early extracts and anti-T immunoprecipitation of gradient fractions, we observed no detectable difference in the relative proportions of 16S and 5 to 7S forms when compared with those of the

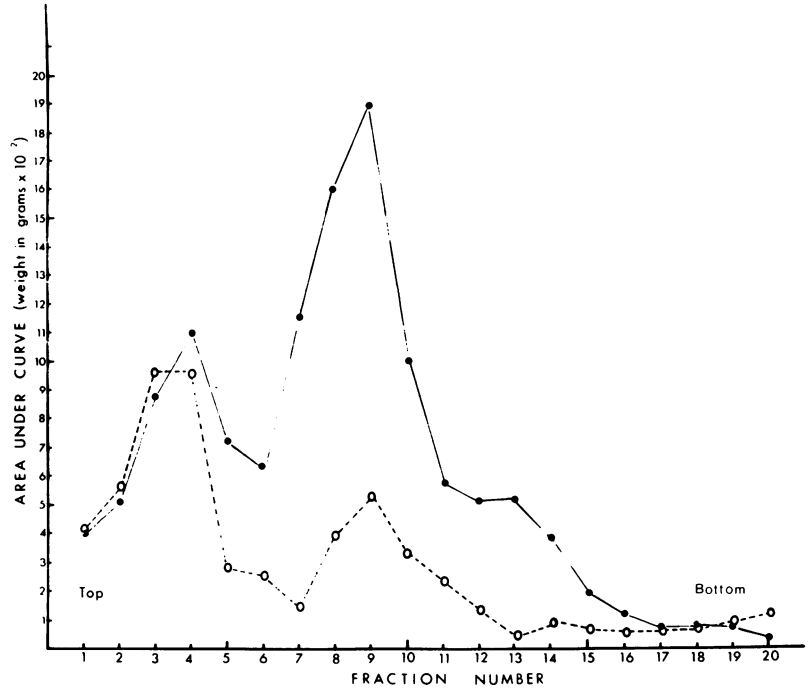

FIG. 3. Effect of cytosine arabinoside on T antigen sedimentation. Cultures of BSC-1 cells to which cytosine arabinoside (20 μg/ml) had been added at the time of infection with SV40 were labeled with [^{35}S]methionine 42 to 44 h postinfection, extracted and centrifuged through sucrose gradients as described in the text and in the legend to Fig. 1. Anti-T immunoprecipitates of 20 gradient fractions were subjected to PAGE and autoradiography, using Kodak XR-5 film. Each slot, representing the immunoprecipitate of a gradient fraction, was scanned with a Gilford densitometer, and areas under curves representing large T antigen gel bands were cut out and weighed. In parallel cultures treated with cytosine arabinoside as in this experiment, DNA synthesis was inhibited by 95%. 5S and 18S rRNA marker peak tubes were 4 and 10, respectively. Symbols: ●, culture treated with cytosine arabinoside; ○, control untreated culture.

typical 44-h late extract. However, as the cells were not synchronized and therefore there may have been some DNA replication induced even at 10 h postinfection, several inhibitors of DNA synthesis were used to further ensure the absence of viral and cellular DNA replication. All compounds tested, including hydroxyurea, 2′-deoxy-2′-azidocytidine (1), aphidicolin, and cytosine arabinoside yielded no reduction in the relative amount of labeled 16S T antigen, under conditions where DNA synthesis was reduced by 90 to 95%. Indeed, in many cases there was a significant enhancement of the 16S form of T antigen. A particularly dramatic example of this increase is seen in Fig. 3. One possible interpretation of these results is that T antigen oligomerization may, in fact, be an alternative to its functioning in DNA replication.

Sensitivity of 16S T antigen form to increased ionic strength. To elucidate the properties of the bonds forming the oligomers of T antigen, we tested different extraction and centrifugation procedures. Pulse-labeling and pulse-chase experiments showed that the 5 to 7S form is labeled before the 16S form (data not shown), as has been reported by others (5, 6). Therefore, the relative proportions of [^{35}S]methionine-labeled T antigen 5 to 7S and 16S forms changed with the duration of the labeling period, consistent with the observation that most of the accumulated T antigen measured by the enzyme-linked immunosorbent assay exists in rapidly sedimenting complexes. Under similar labeling, extraction, and centrifugation conditions as described for Fig. 1, the relative amount of the 16S form was not appreciably affected by the presence or absence of 0.5% Nonidet P-40, 5 mM $MgCl_2$ or $CaCl_2$, or 5 mM dithiothreitol or β-mercaptoethanol. Moreover, the amount of the 16S form was not diminished when cell extracts were incubated with quantities of micrococcal nuclease sufficient to digest 40 μg of rRNA markers. This indicates that the T antigen in the 16S form is not associated with long, nuclease-sensitive stretches of nucleic acid.

However, some treatment did affect the integrity of the 16S form. When extracts of infected cells were adjusted to 1.0 M NaCl and centri-

fuged through sucrose gradients containing this salt concentration, the oligomeric forms of T antigen vanished (Fig. 4). Indeed, the 5 to 7S T antigen form was also seen to sediment even more slowly than normally observed, suggesting that this form may itself be heterogeneous, perhaps consisting of monomers and dimers, the latter being similarly dissociated at 1.0 M NaCl. Thus, T antigen oligomers are unstable at higher salt concentrations and must be generated through bonds sensitive to increase in ionic strength. The 45,000-molecular-weight protein tentatively identified as VP-1 is also apparently sensitive to the increased salt concentrations. In addition, when extracts were adjusted to pH 6.0 and centrifuged through sucrose gradients at this pH, there was a considerable reduction but not an entire disappearance of the amount of the [^{35}S]methionine-labeled 16S T antigen form (data not shown). This pH effect was not observed above pH 6.5.

Stability of 5 to 7S and 16S T antigen forms in vitro. Several lines of evidence point to the possibility that SV40 T antigen is a multifunctional protein whose various biological activities may reflect different domains or forms of the molecule. As the protein exhibits heterogeneity with respect to its sedimentation properties, it was of interest to test whether these forms differed with respect to their biological and biochemical properties. To assess any such differences, assurance was sought that these forms were not interconvertible in vitro. For example, as oligomerization of many proteins can be a concentration-dependent event, it might be suspected that dilution of the 16S form may cause its reconversion to the 5 to 7S form. To test this possibility, as well as to test the stability of the 5 to 7S form, extracts of SV40-infected cells were subjected to sucrose gradient centrifugation as described in Fig. 1. The peak fractions of the 5 to 7S and 16S forms were then diluted twofold and threefold, respectively (to dilute the sucrose concentration to below 5%), and each was separately subjected to a second centrifugation through sucrose gradients (Fig. 5). Both 5 to 7S and 16S T antigen forms, even after dilution, retained their original sedimentation properties; 5 to 7S T antigen remained as such, whereas virtually no 16S T antigen was converted to the more slowly sedimenting forms. Nor did there appear to be any conversion of the 5 to 7S to 16S form in vitro: coincubation of isolated [^{35}S]methionine-labeled 5 to 7S peak fraction with an unlabeled 16S peak fraction did not yield any labeled 5 to 7S T antigen forming heavier complexes upon re-centrifugation (data not shown).

DNA-binding properties of different sedimenting forms of T antigen. Many of the roles of T

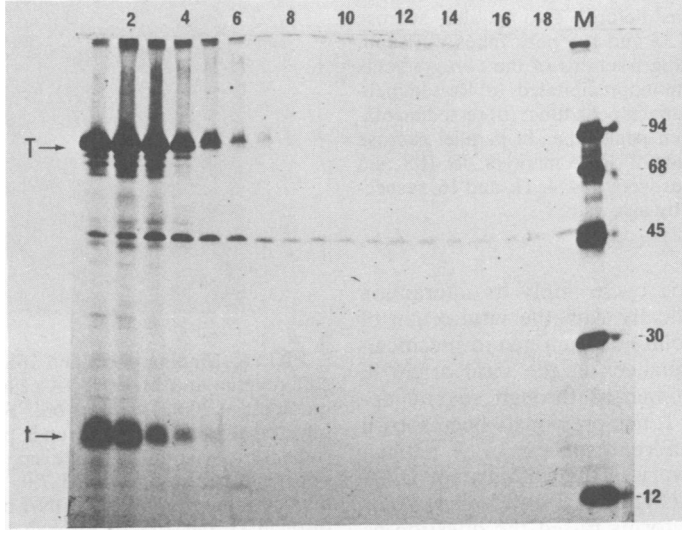

FIG. 4. Sedimentation of SV40 T antigen at high ionic strength. Extracts of [^{35}S]methionine-labeled infected cells were prepared as described in the text. After adjusting the concentration of NaCl in the extract to 1.0 M, 200 μl was centrifuged through 5 to 20% sucrose gradients in buffer containing 1.0 M NaCl, 0.02 M HEPES buffer, and 0.001 M $MgCl_2$ (pH 7.4). Nonidet P-40 was omitted from gradient solutions due to co-precipitation with 1.0 M salt in the 20% sucrose-containing solution. However, the absence of Nonidet P-40 from sucrose gradients containing 0.15 M NaCl did not measurably affect the proportions of 5 to 7S and 16S T antigen forms (see the text). The peak tubes of 5 to 7S and 16S T antigen forms run in parallel under identical conditions as in Fig. 1 were 4 and 8, respectively.

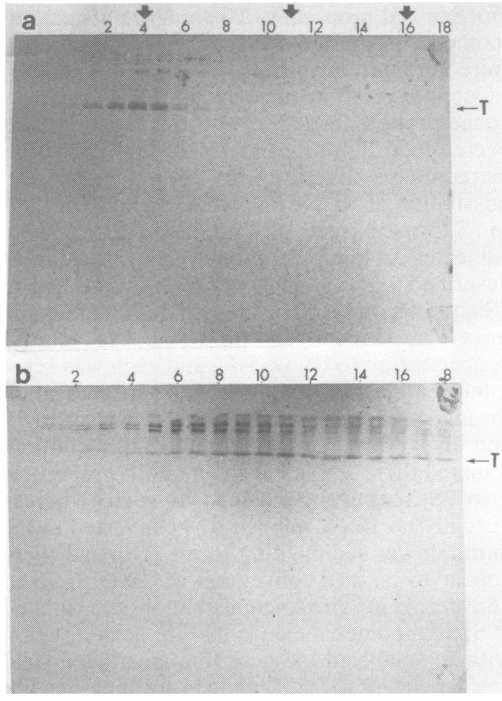

FIG. 5. Re-sedimentation of 5S and 16S T antigen forms. Sucrose gradient sedimentation of [^{35}S]methionine-labeled infected cell extracts was performed as described in the legend to Fig. 1. Peak tubes of 5 to 7S and 16S T antigen forms (generally tubes nos. 4 and 10) were diluted twofold and threefold, respectively, in HIP buffer and subjected to sedimentation through sucrose gradients as before. Two parallel sucrose gradients of diluted 5S and 16S peak tubes were run, and the corresponding fractions of the two gradients were pooled and immunoprecipitated. (a) Re-sedimentation of 5 to 7S T antigen peak tube; (b) re-sedimentation of 16S T antigen peak tube. In parallel sucrose gradient sedimentation of rRNA markers, 5S, 18S, and 28S rRNA peak tubes were nos. 4, 11, and 16, respectively, as indicated by arrows.

antigen in the lytic cycle imply its interaction with DNA, specifically with the viral origin of replication. The ability of T antigen to specifically bind DNA sequences at the viral origin in vitro is well documented through several approaches (34, 41). It has previously been shown that there are different subsets of T antigen molecules which vary in their affinity for DNA (8, 23, 29). The existence of T antigen as two or more sedimenting forms raised the question as to whether these forms may differ in their affinities for DNA and thus may be related to the DNA-binding heterogeneity of T antigen. As the 5 to 7S and 16S classes, when separately isolated, retained their ability to sediment as light and heavy forms, respectively (Fig. 5), it was possible to test their individual interactions with DNA. Two DNA-binding assays were employed, both of which test for tight binding of T antigen to DNA. In the first assay, specific and nonspecific binding was examined by testing the differential salt-sensitive binding of T antigen to SV40 origin-containing (SV40 multiorigin variant [MOV] and origin-minus (calf thymus) DNA linked to cellulose (23). When this method was developed, it was found that there are different classes of T antigen which exhibit varying affinity for DNA. To examine whether these classes might be related to different sedimenting forms of T antigen, the peak tubes of the 5 to 7S and 16S form were collected after sucrose gradient sedimentation, and each bound to and eluted from calf thymus or SV40 MOV DNA-cellulose columns (Fig. 6). It has been previously shown that the amount of DNA on the calf thymus or MOV DNA-cellulose columns is in considerable excess of the bindable T antigen isolated from comparable quantities of infected cells (23, 29). Under the binding (pH 7.0; 0.15 M NaCl) and elution (pH 7.0; 0.4 M and 1.0 M NaCl) conditions used, it was found that the 5 to 7S form of large T antigen binds calf thymus and MOV

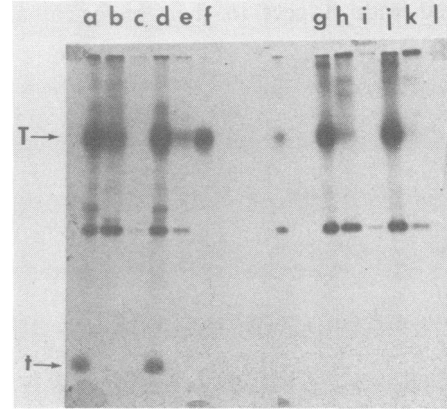

FIG. 6. Binding of 5S and 16S T antigen forms to calf thymus and MOV DNA-cellulose. Five sucrose gradients of 200-μl samples of [^{35}S]methionine-labeled infected cell extracts were run in parallel. Peak 5 to 7S (tube no. 4) and 16S (tube no. 10) fractions were pooled and adjusted to pH 7.0, and each pool was divided into two portions. DNA binding of 5 to 7S (a through f) and 16S (g through l) T antigen to calf thymus DNA-cellulose (a, b, c, g, h, i) or F-161 MOV DNA (d, e, f, j, k, l) was tested by binding and elution pH 7.0 in buffer containing 0.1 M NACl (a, d, g, j), followed by elution in buffer containing 0.4 M NaCl (b, e, h, k), then in buffer containing 1.0 M NaCl (c, f, i, l). All elution fractions were adjusted to 0.1 M NaCl by dilution, immunoprecipitated with anti-T serum, and analyzed by PAGE and autoradiography.

DNA, whereas small t antigen does not. The 5 to 7S T antigen, which bound to DNA, could be eluted from calf thymus DNA with 0.4 M NaCl, but required higher ionic strength (1.0 M NaCl) to be eluted from MOV DNA, consistent with earlier observations of whole cell extracts of T-antigen-containing infected cells (23). In contrast, the 16S form bound very inefficiently to calf thymus DNA and even more poorly to MOV DNA, in that considerably more of this T antigen class did not bind to the column at all when compared to the proportions of bound and nonbound 5 to 7S T antigen. In contrast to the 5 to 7S form, no detectable tight (1.0 M NaCl-sensitive) binding of the 16S form to MOV DNA was observed. Thus, these two forms clearly differ in their interaction with DNA immobilized on cellulose columns.

We also tested the binding of T antigen to various DNA sequences in an assay in which extracts from infected cells containing T antigen are bound to ^{32}P-labeled restriction fragments of viral SV40 DNA in solution (19; Scheller et al., submitted for publication). The T antigen-DNA fragment complex is then immunoprecipitated with anti-SV40 T serum (Scheller et al., submitted for publication). Incubation of *Bst*N1-digested SV40 DNA comprising 13 fragments, including the 311-base-pair G fragment spanning the viral origin of replication, with extracts of SV40-infected cells under suitable binding conditions, yielded considerable quantities of the origin sequence-containing G fragment specifically bound to T antigen. To test whether the different sedimenting forms of T antigen varied in their ability to specifically bind to origins, gradient fractions were bound to ^{32}P-labeled *Bst*N1-digested SV40 fragments in solution (Fig. 7).

This experiment differed from the column-binding experiments in two major ways: first, the kinetics of binding in solution were more rapid than when DNA was immobilized on cellulose; second, whereas the column-binding experiment provided information about labeled (newly synthesized) T antigen molecules, the solution-binding experiment measured the specific-origin-binding activity of the total accumulated T antigen in each gradient fraction. The results confirmed and extended those obtained with the DNA-cellulose binding assay. Only fractions in the 5 to 7S region of the gradient bound to *Bst*N1 fragment G; more rapidly sedimenting forms exhibited little or no specific affinity for the origin-containing fragment. It is noteworthy that tube no. 4 showed considerably greater G fragment binding than did tube no. 3. Consistent with the results of the NaCl dissociation experiment (Fig. 3), which suggested that the 5 to 7S class may be heterogeneous, it can be suggested that the 5 to 7S T antigen may consist of both monomers and dimers, of which the latter are the major origin-binding form.

DISCUSSION

Our experiments indicate that different forms of T antigen vary in their affinity for DNA. Under the DNA-binding conditions that we used, the 5 to 7S T antigen form could bind to DNA with high affinity, whereas the 16S form could not. These data are somewhat at odds with those of Myers et al. (21), who provide evidence from electron microscopic analysis that preparations of purified and concentrated D-2 protein

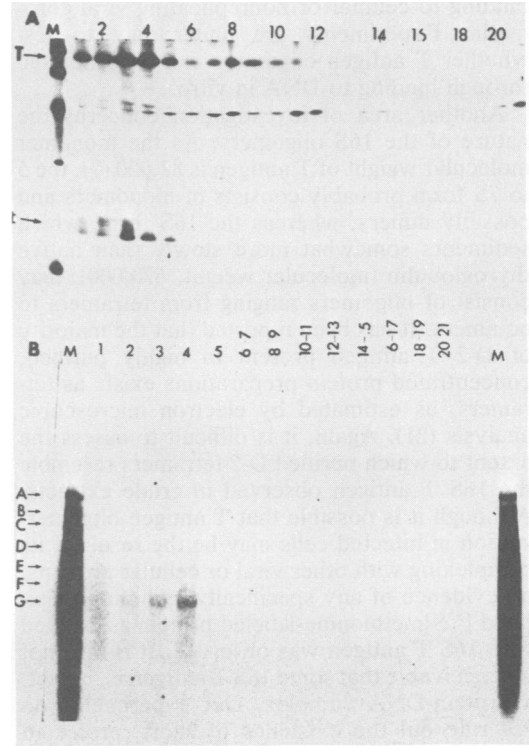

FIG. 7. Binding of various sedimenting forms of T antigen to DNA fragments in solution. Two parallel sucrose gradients of 200-μl portions of [^{35}S]methionine-labeled and unlabeled infected cell extracts were run. Gradient fractions of [^{35}S]methionine-labeled extracts were collected and subjected to immunoprecipitation and analysis as described in the legend to Fig. 1 (A), or gradient fractions of unlabeled extract were incubated with 5 ng of ^{32}P-labeled *Bst*N1 SV40 restriction fragments, followed by immunoprecipitation of the T antigen DNA complex as described in the text (B). Tubes from fractions 6 and upwards were doubled in the DNA-binding assay, as shown in the figure, to compensate for the greater quantities of [^{35}S]methionine-labeled T antigen present in the more slowly sedimenting fractions.

consist mainly of oligomers which were observed bound to sequences at the viral origin of replication. To reconcile their observations with the binding studies reported herein, it can be suggested that either highly purified D-2 protein is functionally different from 16S T antigen isolated from extracts of SV40-infected cells or that, although the active binding form of T antigen is the 5 to 7S form, the 16S oligomers are generated through DNA binding. Possibly, T antigen binds to DNA as monomer or dimer, which could not be separated in these sedimentation conditions, and then forms tetramers through juxtaposition at its binding sequences. Although cessation of DNA replication did not reduce the appearance of 16S T antigen, it is possible that these oligomers are formed by binding to cellular or nonreplicating viral chromatin. Experiments are under way to test whether T antigen oligomers can be generated through binding to DNA in vitro.

Another area of investigation concerns the nature of the 16S oligomers. As the monomer molecular weight of T antigen is 82,000 (7), the 5 to 7S form probably consists of monomers and possibly dimers, whereas the 16S form, which sediments somewhat more slowly than native thyroglobulin (molecular weight, 670,000), may consist of oligomers ranging from tetramers to octamers. It has been reported that the majority of D-2 T antigen present in highly purified, concentrated protein preparations exists as tetramers, as estimated by electron microscopic analysis (21). Again, it is difficult to assess the extent to which purified D-2 tetramers resemble the 16S T antigen observed in crude extracts. Although it is possible that T antigen oligomerization in infected cells may be the result of its complexing with other viral or cellular proteins, no evidence of any specifically immunoprecipitated [^{35}S]methionine-labeled protein associated with 16S T antigen was observed. It is also not inconceivable that some 16S T antigen is, in fact, a protein-DNA complex. Our experiments do not rule out the existence of short, protected sequences of nucleic acid. Experiments to determine whether there are other proteins or nucleic acid fragments associated with 16S T antigen are in progress.

SV40 T antigen has been shown to bind to (6, 9, 14, 18, 20) and stabilize (22) an immunoprecipitation-resistant complex with a 53,000-molecular-weight host protein found in transformed cells. This complex was shown to sediment more rapidly than the 16S T antigen form (18, 20, 28). Although it has been suggested that 16S T antigen may be an intermediate in the formation of the 22S T–53,000-molecular-weight host protein complex, there are substantial quantities of 16S T antigen in lytically infected cells which have considerably smaller amounts of the 53,000-molecular-weight protein (10, 20). The properties of the T antigen that is complexed to the 53,000-molecular-weight host protein are different from those of the 16S form because the former can bind to DNA sequences at the viral origin (Scheller et al., submitted for publication), whereas the latter does not.

The existence of multiple forms of T antigen differing in their state of oligomerization has been reported by several other groups. Although minor discrepancies among some of the reported studies exist, salient features of the phenomena can be summarized as follows. (i) In lytic infection the T antigen oligomers sediment at approximately 16S, whereas in transformed cells there are still heavier 22S T antigen complexes containing the 53,000-molecular-weight nonviral T antigen. (ii) Pulse-labeling studies suggest that the monomeric or 5 to 7S form is formed before the heavier forms and therefore may be their precursor. (iii) *tsA* mutant T antigens do not form oligomers at the restrictive temperature. (iv) The rapidly sedimenting T antigen is more extensively phosphorylated than the more slowly sedimenting forms. In experiments not described in this paper we have confirmed all of these observations except for the fourth, in which we did not observe a significant difference in the degree of phosphorylation of the two sedimenting forms of T antigen. However, our data are consistent with the pulse-labeling studies in that we have previously shown that newly synthesized T antigen binds more efficiently (23, 28) and tightly (23) to DNA than do older T antigen molecules. We are currently exploring the possibility that various monoclonal antibodies which react with subpopulations of T antigen that are active in DNA binding (Scheller et al.) preferentially immunoprecipitate the 5 to 7S form.

Biological and biochemical studies of SV40 T antigen suggest that this viral gene product is a multifunctional protein. Although the different functions may be due to different domains or post-transcriptionally modified states (e.g., phosphorylation) of the T antigen polypeptide, it is also possible that T antigen may exhibit heterogeneity through various degrees of oligomerization. The observation that the 5 to 7S and 16S forms differ in their DNA-binding properties may be related to different functions of the protein. We can speculate that, although the newly synthesized 5 to 7S T antigen form is the active viral origin-binding form, the subsequently formed more rapidly sedimenting T antigen oligomers may (i) bind nonspecifically to DNA and play a role in the cellular DNA replication function of T antigen; (ii) have a function unrelated to DNA binding, such as association with

the 53,000-molecular-weight nonviral T antigen, or adenosine triphosphatase activity; or (iii) be inactive forms of T antigen representing an additional mode of autoregulation of the availability of functional T antigen. We are currently carrying out experiments to test these and other possibilities.

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ADDENDUM IN PROOF

While this paper was submitted for publication, Bradley et al. (M. K. Bradley, J. D. Griffin, and D. M. Livingston, Cell 28:125–134, 1982) reported that the slowly sedimenting form of T antigen can be resolved into 5.5S and 7S species. They have reported that of the two forms only the 7S species is active in binding to DNA.

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