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Oligomerization and Origin DNA-Binding Activity of Simian Virus 40 Large T Antigen

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Simian virus 40 (SV40) large tumor antigen (T antigen) exists in multiple molecular forms, some of which are separable by zone velocity sedimentation of soluble extracts from infected monkey cells. Three subclasses of this antigen from SV40-infected monkey cells have been separated and characterized: the 5S, 7S, and 14S forms. Newly synthesized T antigen occurs primarily in the 5S form. Chemical cross-linking provided evidence that the 14S form is primarily a tetramer, whereas the 5S and 7S forms could not be cross-linked into oligomers. The DNA-binding properties of each subclass were investigated after immunopurification. The affinities of the three forms for SV40 DNA and for a synthetic 19-base-pair sequence from binding site I are very similar (equilibrium dissociation constant [K_D], 0.3 to 0.4 nM). The specific activity of DNA binding was greatest for the 5S and 7S subclasses and least for the 14S subclass. Moreover, the specific activity of the 5S and 7S subclasses increased sharply at about 40 h after infection, whereas the activity of the 14S subclass was maintained at a constant low level throughout infection. A model relating oligomerization and DNA binding of T antigen in infected cells is presented.

The large tumor (T) antigen of simian virus 40 (SV40) is the major regulatory protein governing the viral infectious cycle (35, 46). It is a multifunctional protein involved in autoregulation of early viral transcription, initiation of viral DNA replication, and stimulation of late viral gene expression. In addition, T antigen affects cellular metabolism directly or indirectly, influencing patterns of cellular gene expression and cellular DNA synthesis, levels of enzyme activities, and protein stability. Finally, T antigen is involved in the establishment and maintenance of cell transformation (35).

These data raise the fundamental question of how this single protein can fulfill these functions in a particular ordered sequence which culminates in the production of new virions. Several interrelated possibilities may be considered. Extensive genetic and biochemical evidence reveals that some of the T-antigen functions are associated with specific domains of the protein (35). Multiple posttranslational modifications of T antigen, including phosphorylation, acylation, ADP ribosylation, and adenylylation, may influence T-antigen function (5, 16, 26, 40, 41, 48). T antigen associates with several different subcellular fractions, e.g., plasma membrane, nuclear matrix, and chromatin (8, 26, 45). Finally, soluble T antigen occurs in multiple oligomeric forms, which differ in the degree of phosphorylation, and in ATPase, helicase, and DNA-binding activity, and thus could have different functions (35, 44).

Since specific binding of T antigen to sequences within and adjacent to the SV40 origin of DNA replication mediates the control of viral transcription and replication (6, 7, 9, 10, 19, 24, 25, 28, 36, 43), this biochemical property has been assayed to search for differences in function among the various forms of T antigen. However, investigation of the DNA-binding properties of different oligomeric forms of the protein has led to conflicting conclusions. Results obtained with the T-antigen-related protein D2 suggested that tetrameric T antigen was the active specific DNA-binding form of the protein, whereas monomers bound only nonspecifically to DNA (30, 32). Filter binding assays performed with transformed cell T antigen separated by zone velocity sedimentation at high ionic strength were interpreted to suggest that the 16S and 7 to 8S forms, but not the 5.5S form, had DNA-binding activity (4). In contrast, studies carried out with lytic T-antigen subclasses separated by zone velocity sedimentation or phosphocellulose chromatography showed that newly synthesized 5 to 7S T antigen bound more efficiently to SV40 origin DNA than a 14 to 16S subclass did (12, 14, 15, 33, 41). Furthermore, the stoichiometry of lytic T-antigen binding to SV40 DNA was shown to differ from that of D2 T antigen; one monomer mass of lytic T antigen bound to each 5′-GAGGC sequence in the template, i.e., three molecules on site I and four on site II of SV40 DNA (29), whereas tetramers of D2 were reported to bind site I (32).

Thus in an attempt to resolve some of these contradictions, we separated and characterized three subclasses of soluble lytic T antigen from freshly prepared cell extracts and reexamined their affinity and specific activity of binding to SV40 DNA by using immunopurified protein under a variety of experimental conditions. The results demonstrate that all T-antigen subclasses bind with similar affinity to site I. However, the specific activity of binding differs markedly between the 5S and 7S forms, which have high activity, and the tetrameric form, which has little activity. Moreover, the specific activity of binding of the 5S and 7S forms is dependent on time after infection, rising sharply in midinfection.

MATERIALS AND METHODS

Cells, virus, and antibodies. TC7 monkey cells (37) were cultured in Dulbecco-modified Eagle medium supplemented with 8% newborn calf serum (Boehringer Mannheim Biochemicals) and antibiotics. Propagation of SV40 (strain SVS) and infection of TC7 cells were as described previously (3). Hybridoma cell culture and purification of Pab 108 immunoglobulin G (IgG) was described previously (22). Pab 108 binds to a denaturation-resistant epitope located between SV40 map units 0.62 and 0.65 and precipitates essen-

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tially all subclasses of T antigen (18, 22). Hamster tumor serum was prepared as described previously (13).

DNA. The plasmid pSV-wt, which carries the SV40 genome in the *Bam*HI site of pAT153, was described previously (14). The plasmid pON-wt (38) carries a chemically synthesized 19-base-pair sequence from SV40 T-antigen binding site I (nucleotides 5191 to 5209, BBB numbering [46]), flanked by *Bam*HI linkers and cloned into the *Bam*HI site of pAT153 (47). Preparation of DNA, restriction enzyme digestion, and end labeling were described previously (14).

Separation of T-antigen subclasses. Labeling of proteins with [^{35}S]methionine or $^{32}PO_4^{3-}$ (Amersham) was performed as described previously (13). Zone velocity sedimentation of whole-cell extracts was carried out as described previously (13), except for the following changes. Extraction was carried out for 30 min instead of 1 h. Sucrose density gradients were 15 to 30% sucrose in buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.8], 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM $MgCl_2$) containing 150 mM KCl. Centrifugation was carried out in a Beckman SW55Ti rotor at 4°C and 45,000 rpm for 16 or 21.5 h. Sedimentation markers were β-galactosidase (16S), calf alkaline phosphatase (6.3S), and bovine hemoglobin (4.3S). Fractions of 4 drops (ca. 90 μl) were collected from the bottom of the gradients. Five fractions of each subclass were combined for DNA-binding assays. The pooled fractions were separated by at least five discarded fractions.

Immunoprecipitation of labeled proteins and SDS-PAGE. T antigen was immunoprecipitated either with hamster tumor serum and fixed *Staphylococcus aureus* cells exactly as described previously (13) or with excess purified Pab 108 IgG instead of tumor serum. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (27), with proteins of known molecular weights as markers (13).

Chemical cross-linking. Chemical cross-linking of $^{32}PO_4^{3-}$-labeled T-antigen subclasses was performed with 0.5 to 50 mM dimethylsuberimidate (DMS) (Pierce Chemical Co.) in 0.2 ml of 50 mM triethanolamine (pH 8.5)–1 mM $MgCl_2$ for 20 min at 20°C and stopped by addition of 2 μl of 1 M Tris (pH 8.5) (34). After immunoprecipitation with hamster tumor serum, cross-linked products were analyzed by electrophoresis in 3.5% Weber-Osborn SDS-polyacrylamide gels (51) and autoradiography.

Immune dot blot. Samples of T-antigen subclasses (usually 5 μl) were applied to a nitrocellulose filter (BA85; Schleicher & Schüll) in a microfiltration apparatus (Bio-Rad Laboratories). The filter was incubated with 3% bovine serum albumin in phosphate-buffered saline for 1 h at 37°C, washed extensively in phosphate-buffered saline and then treated with Pab 108 IgG (1.5 to 2 μg/ml in phosphate-buffered saline) for 1 h at 20°C. After extensive washing, the filter was incubated for 1 h with biotin-labeled anti-mouse IgG (1:500; Amersham), washed, incubated for 1 h with preformed streptavidin-horseradish peroxidase complexes (1:400; Amersham), washed, and developed with a fresh solution of 0.5 mg of diaminobenzidine per ml–0.02% hydrogen peroxide–0.03% $NiCl_2$ in phosphate-buffered saline for 20 min (1). The stained filters were quantitated by microdensitometry (Elscript 400; Hirschmann Gerätebau, Unterhaching).

DNA-binding assays. DNA-binding tests were carried out by using a target-bound assay with T antigen immunopurified by binding to Pab 108 IgG and *S. aureus* (20). No quantitative or qualitative effect of Pab 108 on SV40 DNA binding has been observed (38; E. Vakalopoulou and E. Fanning, unpublished data). T-antigen subclasses, typically 0.1 ml, were immunoprecipitated with 5 μg of Pab 108 IgG and fixed *S. aureus* cells, washed, and suspended in 0.11 ml of buffer A containing 80 mM KCl, 1 mg of bovine serum albumin per ml, and 0.2 mg of glycogen per ml. Excess end-labeled pSV-wt *Hin*dIII fragments or pON-wt *Eco*RI-*Sal*I fragments, typically 0.25 μg, were added, and the mixture was incubated at 0°C for 2 h to equilibrium. Immune complexes were pelleted and washed, and bound DNA was analyzed by electrophoresis in 2% agarose gels and autoradiography (14).

RESULTS

Separation and characterization of T-antigen subclasses. Three subclasses of T antigen from $^{32}PO_4^{3-}$-labeled SV40-infected monkey cells were fractionated by zone velocity sedimentation: 5S, 7S, and 14S (Fig. 1A). The distribution of immunoreactive T antigen was determined from immune dot blots of each fraction. Most T antigen was found in the 14S form; the 7S form varied in amount from one-fifth to about half as much T antigen as the 14S form (Fig. 1A; data not shown). The 5S form contained least T antigen. Similar subclasses were separated previously by sedimentation and also by gel filtration (4). These T-antigen subclasses were reported to be stable at high ionic strength, whereas in another study, a 16S form of T antigen was shown to dissociate in 1 M NaCl to a 5 to 7S form (15). Thus, to characterize the subclasses separated by the present procedure, zone velocity sedimentation was also carried out with 1 M NaCl (Fig. 1B). Most of the $^{32}PO_4^{3-}$-labeled T antigen sedimented at 5S, with a shoulder at 7S (Fig. 1B). Since $^{32}PO_4^{3-}$ labeling does not distinguish between newly synthesized (young) and accumulated (old) T-antigen molecules (41), we conclude that the bulk of oligomeric T antigen is unstable at high ionic strength.

In previous studies, it was assumed that the three subclasses corresponded to T-antigen monomers, dimers, and tetramers (4, 15, 41). Since protein conformation or modification could influence the sedimentation or chromatographic behavior of T antigen, we wished to confirm the identity of these species by chemical cross-linking. Several cross-linkers with different bridge lengths were used: DMS (1.1 nm), dithiobis(succinimidyl propionate) (1.2 nm), and bis(2-[succinimidooxycarbonyloxy]ethyl) sulfone (1.3 nm). The highest efficiency of cross-linking was obtained with DMS (Fig. 2). The 14S subclass reacted to yield an SDS-stable form whose electrophoretic migration corresponded to that predicted for a tetramer (Fig. 2). Neither the 5S nor the 7S subclass was cross-linked in the presence of DMS (Fig. 2) or the other cross-linking reagents under a variety of experimental conditions (data not shown). Thus these data confirm that the 14S and 5S T antigens represent primarily a tetramer and a monomer, respectively. Although the cross-linked tetramer was obtained with good recovery, the yield of 5S and 7S forms was reduced after cross-linking. Thus the data do not distinguish whether the 7S form represents a dimer not detectable by immunoprecipitation or an altered form of monomeric T antigen.

Oligomerization of newly synthesized T antigen. Pulse-chase experiments have demonstrated that newly synthesized T antigen appears in a 5 to 7S form, which then serves as a precursor for the 14S tetramer (13, 17). However, in these studies the 5S and 7S subclasses were not resolved. We therefore conducted pulse-labeling experiments to determine the form of newly synthesized T antigen (Fig. 3). A

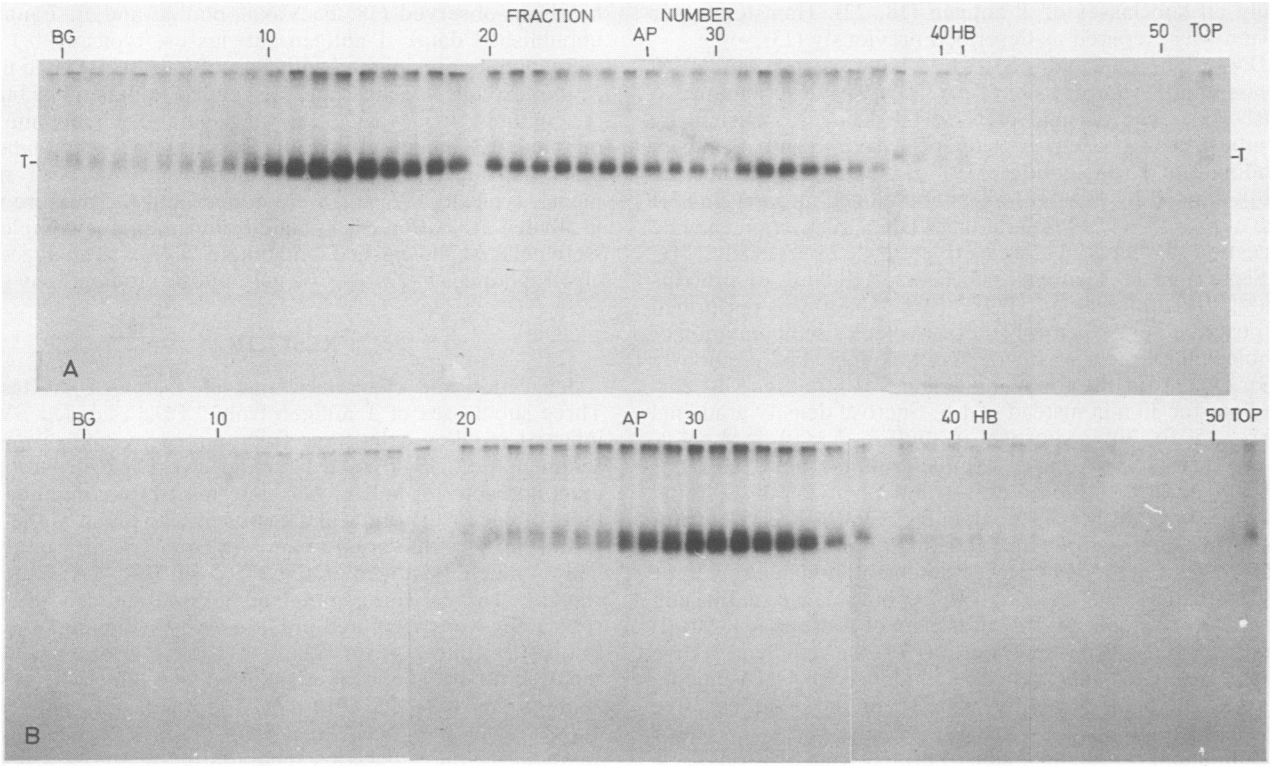


FIG. 1. Separation of T-antigen subclasses by zone velocity sedimentation. An extract of $^{32}PO_4^{3-}$-labeled SV40-infected TC7 cells (44 h postinfection) was fractionated by zone velocity sedimentation (SW55, 45,000 rpm, 16 h) in sucrose gradients made up in buffer A containing 150 mM KCl as described in Materials and Methods (A) or in a gradient also containing 1 M NaCl (B). Each fraction was immunoprecipitated with hamster tumor serum and *S. aureus*, either directly (A) or after dilution to 300 mM NaCl (B), and then analyzed by SDS-PAGE (27) and autoradiography. Sedimentation markers in parallel gradients were β-galactosidase (BG), alkaline phosphatase (AP), and hemoglobin (HB), as indicated.

prominent peak of T antigen labeled in a 15-min period sedimented at 5S (Fig. 3A), whereas T antigen labeled for 2.5 h was found in both the 5S and 14S forms (Fig. 3B). A peak of T antigen in the 7S form was not observed after the 15-min labeling period, but a trace of 7S T antigen was detectable after the 2.5-h labeling period (Fig. 3B). These results demonstrate that newly synthesized T antigen occurs primarily in a monomeric form, which is subsequently assembled into tetramers, but the identity of the 7S form remains puzzling. If the 7S T antigen were an assembly intermediate with a brief half-life, it might be detected by pulse-chase experiments. SV40-infected cells were thus labeled early or late after infection for 15 min and chased for 15, 30, or 60 min (not shown). Although traces of 7S T antigen were detected in the 60-min chase, no prominent peak of 7S T antigen was observed with any of the chase periods tested. However, it was interesting that oligomerization of T-antigen monomers proceeded significantly faster at 24 h after infection than at 48 h (data not shown), as reported recently (50).

These data could be interpreted to suggest that 7S T antigen may be a very transient intermediate in the assembly of tetramers. However, this possibility seems unlikely, because the 7S subclass represents a major fraction of the $^{32}PO_4^{3-}$-labeled T antigen and of the total mass of immunoreactive T antigen (Fig. 1A and 4B; data not shown). The other, more likely, alternative is that the 7S subclass consists primarily of accumulated T antigen, possibly arising through dissociation of 14S tetramers, either in the cell or in vitro.

Binding of T-antigen subclasses to SV40 DNA. Some of the conflicting results obtained in previous studies described in the Introduction can be explained by the use of frozen or partially purified materials rather than fresh cell extracts and by the use of mutant (D2 and SV80) T antigens rather than the wild-type protein. However, it is also possible that the assays used are responsible for some of the disagreement. For example, most assays were performed by the method of McKay (30) in crude extract with excess unlabeled nonspecific DNA. Binding was generally carried out at 0°C for periods too short for equilibrium to be reached (49; R. Runzler and E. Fanning, unpublished data), and T-antigen concentrations differed from one subclass to another. Finally, the relation between the reported efficiency of binding and the commonly used parameters, specific activity of binding and binding affinity, was not defined.

Thus, to rule out the DNA-binding assay as the source of the conflicting results, we used a sensitive target-bound assay carried out in the absence of unlabeled competitor DNA and cellular proteins (20). Equal volumes of each subclass were immunopurified on Pab 108 IgG, an antibody that has no detectable effect on DNase footprints of T antigen (38; Vakalopoulou and Fanning, unpublished data). The amount of SV40 DNA bound at equilibrium in DNA excess was determined by using two templates, pSV-wt and pON-wt DNA, either separately or together (Fig. 4A). A comparison of the amount of bound origin DNA with the amount of immunoreactive T antigen in each subclass (Fig. 4B) revealed that 5S T antigen bound considerably more

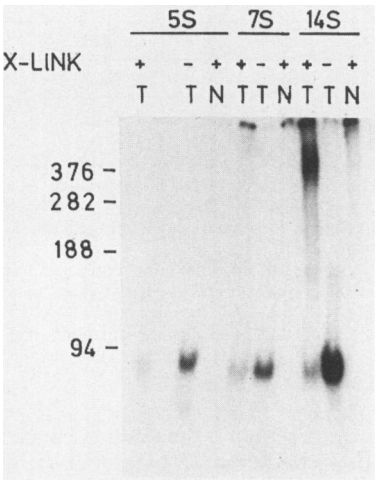


FIG. 2. Chemical cross-linking of separated T-antigen subclasses. $^{32}PO_4^{3-}$-labeled T-antigen subclasses were separated as described for Fig. 1A. Appropriate fractions were combined, divided into three samples, and adjusted to 50 mM triethanolamine (pH 8.5)–1 mM $MgCl_2$. Two samples (+) were cross-linked with 1 mM DMS as described in Materials and Methods. The samples were immunoprecipitated with nonimmune hamster serum (N) or with hamster tumor serum (T) as indicated and analyzed by SDS-PAGE (50) followed by autoradiography for 15 days. The molecular masses (in kilodaltons) of DMS–cross-linked phosphorylase *a* subunits are indicated on the left.

origin DNA than an equivalent amount of the 14S form did. Thus the results obtained with the new assay confirm previous reports that monomeric T antigen binds more origin DNA (12, 14, 15, 33, 41). Moreover, pON-wt DNA appeared to compete with pSV-wt DNA for T antigen, suggesting that their binding affinities may be similar. Differences between subclasses either in binding affinity or in the fraction of T-antigen molecules active in origin binding, i.e., specific activity of binding, could account for the observed results.

Affinity of T-antigen binding to SV40 DNA. DNA binding of immunopurified T-antigen subclasses was assayed as a function of DNA concentration by using pON-wt and pSV-wt DNA. The binding data were treated by the method of Scatchard (39) to determine an equilibrium dissociation constant (K_D) for DNA binding of each form of T antigen. Multiple determination of K_D carried out with subclasses prepared at 39 to 40 h and at 48 h after infection yielded similar values, which were averaged to give a K_D of about 0.4 nM for all three subclasses on pSV-wt DNA (Table 1). The dissociation constants for the three subclasses measured on pON-wt DNA were also very similar to each other and to that determined on pSV-wt DNA (Table 1). Thus within experimental error, T-antigen binding affinity to site I in SV40 DNA does not differ among subclasses. These results indicate that the observed differences in DNA-binding activity of the subclasses (Fig. 4) must derive primarily from their different specific activities of origin binding.

Specific activity of T-antigen DNA binding. A valid comparison of the specific activities of origin binding of these forms requires that binding experiments be performed with equal concentrations of T antigen from each subclass. Dilu-

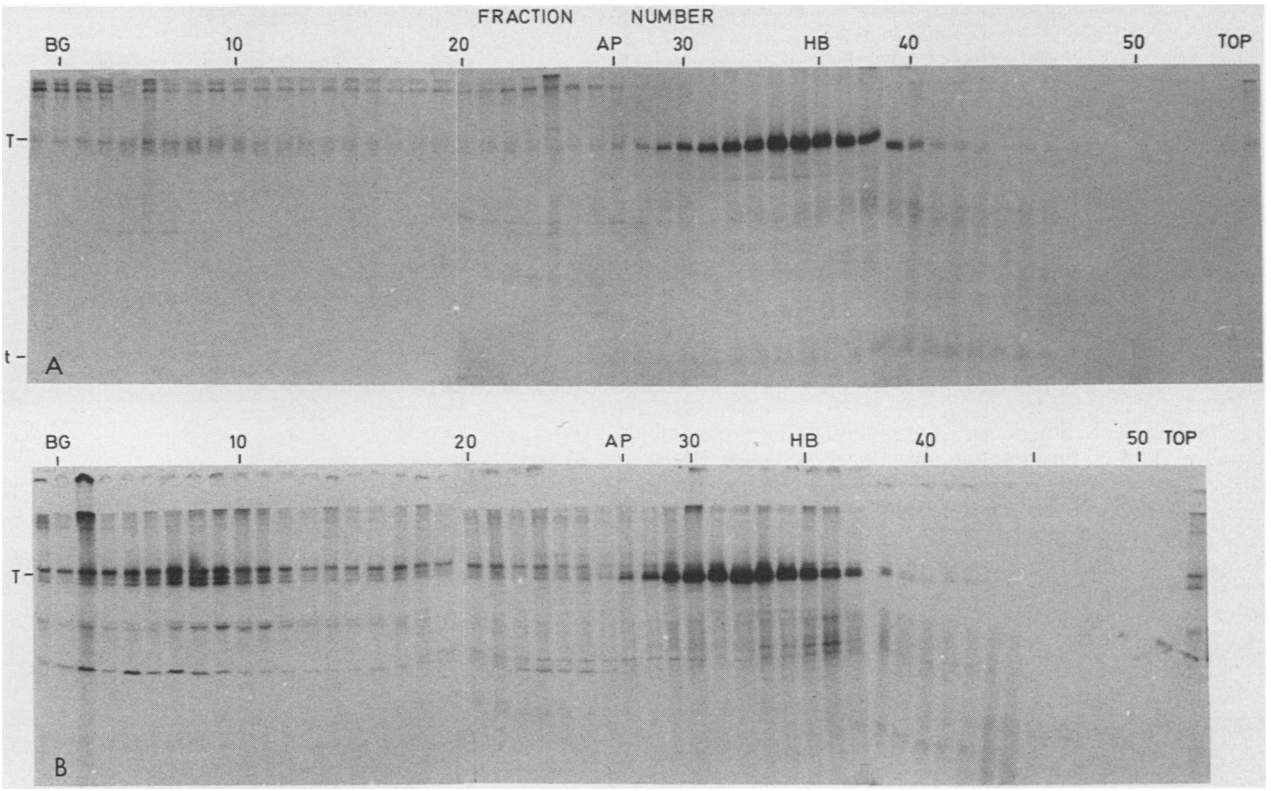


FIG. 3. Fractionation of newly synthesized T antigen by zone velocity sedimentation. SV40-infected TC7 cells were labeled at 42 h after infection with [^{35}S]methionine (250 μCi/ml, 800 to 1,000 Ci/mmol) for 15 min (A) or 2.5 h (B). Cell extracts were fractionated by zone velocity sedimentation as in Fig. 1A, except that sedimentation was for 21.5 h. T antigen in each fraction was immunoprecipitated with hamster tumor serum and analyzed by SDS-PAGE (27) and fluorography. The positions of sedimentation markers as in Fig. 1A are indicated.

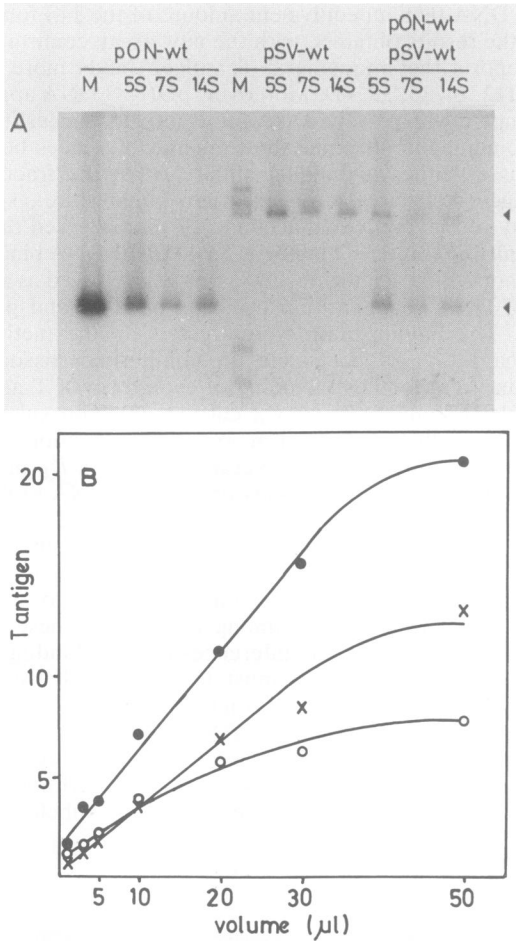


FIG. 4. T-antigen binding to SV40 DNA. T-antigen subclasses were prepared from SV40-infected cells at 48 h postinfection. (A) Samples of each subclass (0.1 ml) were immunopurified on Pab 108 and assayed for binding to equimolar amounts of pSV-wt, pON-wt, or a mixture of the two DNA fragments for 2 h at 0°C as described in Materials and Methods. Binding assays included pAT153 vector DNA fragments when necessary to make a total of 250 ng of DNA in each assay. Marker DNA (M) was 12.5 ng of the labeled input DNA. Arrows indicate fragments containing T-antigen-binding sites. (B) T-antigen subclasses of the indicated volumes were applied to nitrocellulose and analyzed by immune dot blot and microdensitometry. Symbols: ○, 5S; ×, 7S; ●, 14S.

tion of T-antigen oligomers, for example, might be predicted to promote dissociation into subunits more active in DNA binding. Thus the concentration of T antigen in each subclass was first determined by immune blot, and the oligomeric forms were diluted to the concentration of the monomer. Immunopurified T antigen from each subclass was then assayed for binding to SV40 origin DNA at several concentrations of T antigen.

The specific activity of DNA binding was greatest for 5S and 7S T antigens at all concentrations tested (Fig. 5), but the relative specific activity of binding of the 5S and 7S forms varied slightly among experiments. The 5S and 7S forms bound five- to sevenfold more origin DNA than an equivalent amount of 14S T antigen did. Similar results were obtained with pON-wt DNA (data not shown). The data are consistent with previous results (Fig. 4) (12, 14, 15, 33, 41) and suggest that the fraction of the 14S T antigen active in origin DNA binding is much smaller than that of the 5S and 7S subclasses. Interestingly, the binding curve with all three subclasses, but particularly the 14S subclass, was reproducibly nonlinear when a wide range of T-antigen concentrations was tested; interpretation of this result will require further work.

TABLE 1. Affinity of T-antigen subclasses for SV40 DNA and a synthetic 19-base-pair sequence from binding site I

Template	K_D (nM)[a]		
	5S	7S	14S
pSV-wt	0.4 ± 0.1[b]	0.5 ± 0.3[b]	0.3 ± 0.2[b]
pON-wt	0.3 ± 0.2[c]	0.3 ± 0.2[c]	0.2 ± 0.05[c]

[a] The ratio of bound to unbound DNA was plotted as a function of the concentration of bound DNA (39). Slopes (K_D^{-1}) were determined by using a Hewlett-Packard program for linear regression analysis. Correlation coefficients were 0.9 or greater.

[b] Mean of five independent determinations, two at 39 h after infection and three at 48 h after infection. The error is reported as the average deviation from the mean.

[c] Mean of three or four independent determinations, one at 40 h and two (7S) or three (5S, 14S) at 48 h after infection. The error is reported as the average deviation from the mean.

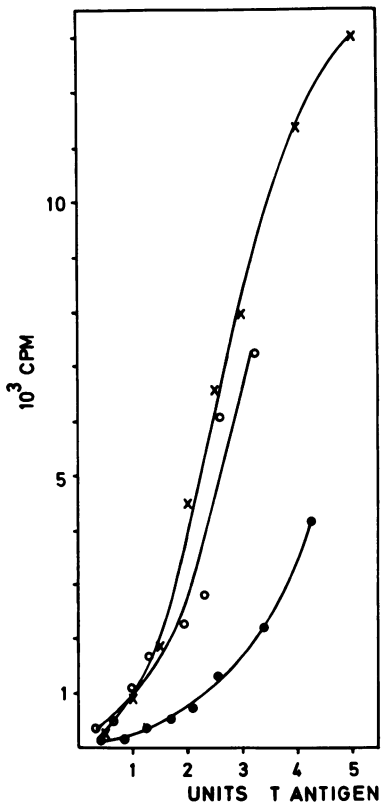
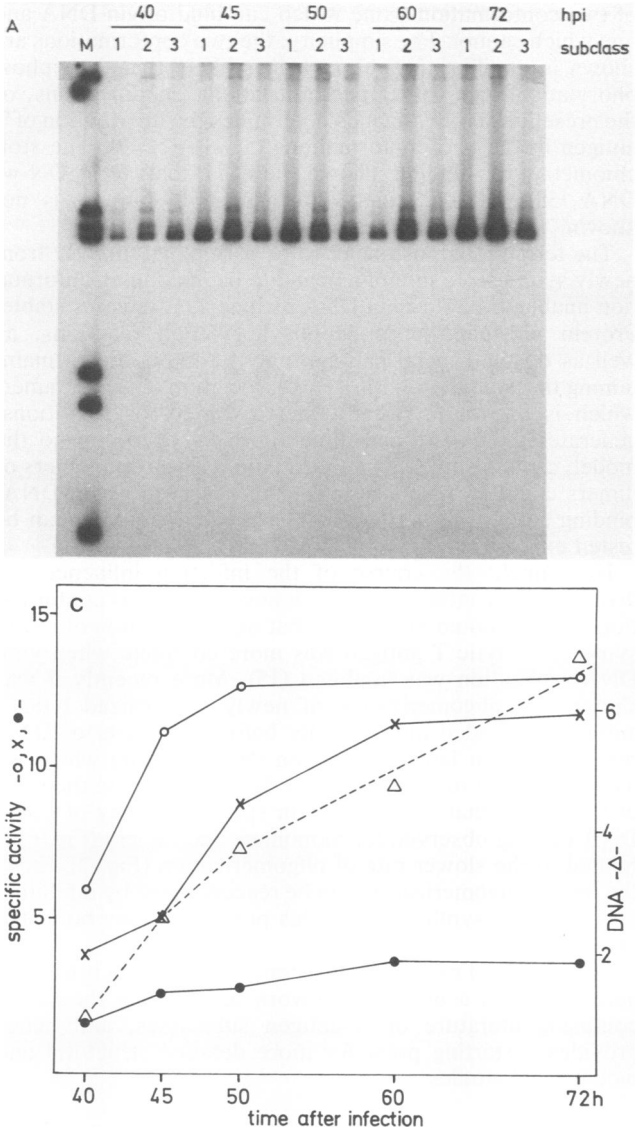


FIG. 5. DNA-binding activity of T-antigen subclasses as a function of T-antigen concentration. T-antigen subclasses prepared as for Fig. 4 were assayed for immunoreactive T antigen by immune dot blot (data not shown). Stock solutions of all three subclasses were adjusted to 10 mM HEPES (pH 7.8)–80 mM KCl–0.5 mM $MgCl_2$–1 mM dithiothreitol and equal concentrations of T antigen. Increasing volumes of each subclass were immunopurified and then tested for specific binding to pSV-wt DNA fragments in 0.11-ml assays. Bound origin DNA fragments were excised from the gels, counted, and plotted as a function of the amount of immunoreactive T antigen in the assay. One unit of T antigen is empirically defined as the amount of T antigen in 0.1 ml of the stock solutions. Symbols: ○, 5S; ×, 7S; ●, 14S.



Specific activity of T-antigen–DNA binding varies with time after infection. The DNA-binding activity of immunopurified T antigen subclasses prepared at various times after infection was monitored by using pSV-wt DNA in excess (Fig. 6A). The amount of origin DNA bound by duplicate samples of T antigen (equal volumes of each subclass) was determined by scintillation counting of the excised gel bands (data not shown). The amount of T antigen in each sample estimated by immune blot was then used to calculate the specific activity of DNA binding as a function of time after infection (Fig. 6C). The amount of viral DNA accumulated at each time after infection (Fig. 6B) was estimated in arbitrary units by microdensitometry (Fig. 6C).

The results demonstrate that although the specific activity of DNA binding of T-antigen tetramers remains at a relatively constant low level throughout infection, the specific activity of 5S T antigen increases sharply between 40 and 50 h after infection. A similar sharp increase in the specific activity of 7S T antigen occurred with a delay of about 5 h. The rise in binding activity was temporally correlated with the onset of viral DNA replication and the late phase of infection (Fig. 6C).

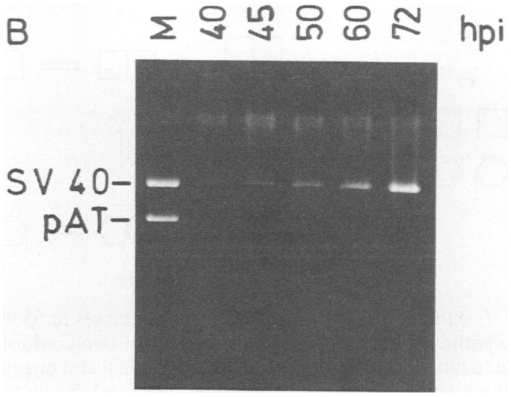


FIG. 6. Specific activity of T-antigen binding to SV40 DNA as a function of time after infection (hpi). (A) T-antigen subclasses were prepared at the indicated times after infection. Samples (0.1 ml) of 5S (lane 1), 7S (lane 2), and 14S (lane 3) T antigen were immunopurified and assayed for specific binding to pSV-wt *Hin*dIII fragments as for Fig. 5. (B) A parallel dish of infected cells was harvested at each time after infection. The low-molecular-weight DNA was extracted (21), linearized by *Bam*HI digestion, and analyzed by agarose gel electrophoresis. *Bam*HI-cleaved pSV-wt DNA served as marker (M). (C) The bound *Hin*dIII-C DNA was cut from the gel in panel A, solubilized, and counted. The amount of T antigen in a sample of each subclass was determined by immune dot blot in arbitrary units. The counts per minute bound per unit of T antigen (specific activity) was calculated for each subclass at each time after infection. Symbols: ○, 5S; ×, 7S; ●, 14S; △, accumulated SV40 DNA determined by microdensitometry of the gel in panel B.

DISCUSSION

Relationship among different subclasses of T antigen. We have demonstrated that T antigen occurs in three oligomeric subclasses separable by zone velocity sedimentation: a 5S monomer, a 14S tetramer, and a 7S form which could be either a dimer or a modified monomer (Fig. 1 and 2). Preliminary results on the DNA-binding kinetics and stoichiometry of the 7S form, however, are consistent with a dimeric mass (R. Runzler, E. Vakalopoulou, and E. Fanning, unpublished data). Newly synthesized T antigen occurs as a monomer which serves as a precursor for a tetrameric form. The bulk of the 7S T antigen, a major fraction by mass and by incorporation of phosphate, is probably not an intermediate in the assembly of tetrameric T antigen (Fig. 1A, 3, and 4B; unpublished data). The tetrameric form appears to be quite stable in vivo (13, 15, 41) and in vitro in solutions of neutral pH and moderate concentrations of salt and divalent cations (12, 31, 41). However, dissociation of the tetramer is observed at high ionic strength (15; Fig. 1B), in the presence of chelating agents (31), and upon chromatography (12). Taken together, the data support the idea that a portion of the highly phosphorylated tetrameric T antigen may dissociate in vitro or in the cell, generating 7S and possibly even 5S forms. Consequently, the 5S and 7S subclasses each comprise a heterogeneous population, some representing newly synthesized T antigen and some derived by dissociation from tetramers. The heterogeneity of the 5S to 7S T-antigen phosphorylation pattern (41) provides further evidence for this interpretation.

Origin DNA binding of T-antigen subclasses. The affinity of binding of each subclass to SV40 DNA was determined by measurement of bound DNA as a function of DNA concentration with two different templates (Fig. 4; Table 1). The results show clearly that all three subclasses, despite their

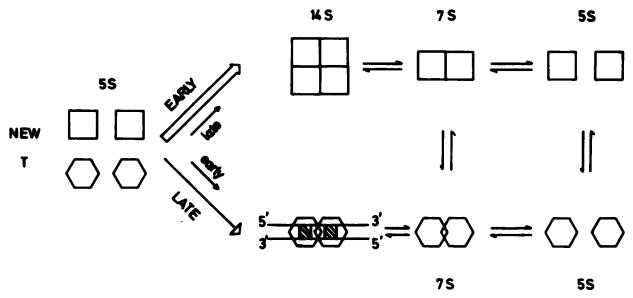


FIG. 7. Model for binding of T-antigen subclasses to SV40 DNA. Newly synthesized T antigen assumes either of two conformations, one able to bind viral DNA specifically (hexagons) and one unable to bind but which assembles into a tetramer (squares). Some tetramers can dissociate to yield 5S and 7S T antigen, which can also assume either of the two conformations. A dimer mass of T antigen binds to pON-wt DNA, which contains two consensus pentanucleotides (hatched boxes), but the binding mechanism is unknown (38). The molecular regulation of T-antigen distribution in the two conformations is the subject of speculation in the text.

heterogeneity, have similar affinities for site I DNA. The K_D measured in our work is very similar to that reported previously for highly purified wild-type T antigen (23) and T antigen from mKSA SV40-transformed mouse cells (20). A 10-fold-lower affinity calculated for purified lytic T antigen (11) was based on the assumption that all of the protein is potentially able to bind to origin DNA, an assumption that may not be valid (Fig. 5 and 6) (20, 42; A. Schmid and E. Fanning, unpublished data).

The specific activity was measured for each subclass as the amount of SV40 DNA specifically bound per immunoreactive unit of T antigen. Under the conditions of the assay, a difference among subclasses in the specific activities of binding may be attributed to a difference among subclasses in the fraction of the total protein molecules able to bind the template. The results demonstrate clearly that the specific activity of origin DNA binding on these templates is greatest for 5S T antigen and least for tetrameric T antigen (Fig. 4 to 6).

It is important to note that although the antibody Pab 108 immunoprecipitates T antigen quantitatively, not all of the N-terminal epitopes in each tetramer need be available to the antibody. Thus the amount of immunoreactive T antigen in the 14S form could be an underestimate of the mass of T antigen in the 14S subclass by as much as a factor of 4. Therefore, the specific activity of the 14S T antigen measured in this work must be regarded as a maximum value.

The specific activity of binding of 5S and 7S T antigen increased during the infection, whereas that of the tetramer remained at a constant low level (Fig. 6). This result could also be accounted for by increased affinity of T antigen for SV40 DNA at late times after infection. However, the affinity of T-antigen subclasses for SV40 DNA did not vary significantly with time after infection (Table 1). Thus the change in binding activity of the 5S and 7S forms is probably due to an increase in the fraction of molecules active in DNA binding at late times after infection, whereas the fraction of material in the tetrameric subclass able to bind origin DNA remains approximately constant.

A model for oligomerization and origin DNA binding of T antigen. We have summarized the data available into a simple model correlating the oligomerization of T antigen with its origin-binding activity (Fig. 7). We propose that 5S newly synthesized monomeric T antigen can take up either of two conformations, one which can bind origin DNA and one which cannot. For simplicity, the two conformations are shown in equilibrium, although it is likely that their phosphorylation state, other posttranslational modifications, or the presence of other ligands determines the distribution of T antigen in the two conformations (5, 41, 42, 49). The stoichiometry of binding shown here is that for pON-wt DNA (38), but the kinetic mechanism of binding is not known.

The tetramer is postulated to be assembled directly from newly synthesized monomers and to be fixed in a conformation unable to bind origin DNA as long as it remains stable. Protein phosphorylation-dephosphorylation reactions, as well as divalent metal cations, may be important in maintaining this stability (2, 31, 41). Dissociation of the tetramer, which is known to occur under a variety of conditions, generates monomers and dimers, which, according to the model, can take up either conformation. These monomers or dimers could be responsible for the observed origin DNA-binding activity of the tetramer, a prediction which can be tested experimentally.

How might the course of the infection influence the distribution of monomers and dimers in the two conformations? It was noted previously that oligomerization of newly synthesized lytic T antigen was more complete when viral DNA replication was inhibited (15). More recently it was shown that oligomerization of newly synthesized lytic T antigen proceeded more rapidly before the onset of DNA replication than later in infection (50), a finding which we have confirmed (data not shown). We speculate on the basis of these data that the increase in specific activity of origin DNA binding observed for monomers and dimers (Fig. 6) is related to the slower rate of oligomerization (Fig. 7). Since the rate of oligomerization can be reaccelerated by inhibition of viral DNA synthesis (50), this possibility may be tested experimentally.

The proposed model is consistent with the data presented here, provides a useful framework to integrate the rather confusing literature on T-antigen subclasses, and hence provides a starting point for more detailed structural and biochemical studies.

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