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Biochemistry

Thermolabile T (tumor) antigen from cells transformed by a temperature-sensitive mutant of simian virus 40

(DNA binding protein/simian virus 40 *A* gene)

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ABSTRACT **Partially purified tumor (T) antigen from a strain of Chinese hamster lung cells transformed by wild-type simian virus 40 (SV40) and either of two temperature-sensitive SV40 mutants has been studied as a DNA binding protein. The DNA binding activity present in the T-antigen-containing fractions is inhibited by purified hamster anti-T IgG but not by equivalent amounts of nonimmune hamster IgG. T from either wild-type- or *tsC219*-transformed cells is relatively stable during heating at 44° compared to T prepared from *tsA239*-transformed cells. These results strongly suggest that T is a product of the SV40 *A* gene.**

The genome of the tumor virus, simian virus 40 (SV40), is small, containing all of its information in a DNA molecule of molecular weight (M_r) about 3.6×10^6. Complementation studies with temperature-sensitive mutants of this virus (1), all unable to replicate at the nonpermissive temperature, have strongly suggested a minimum gene number of three. Mutants of the *A* complementation group (1–5) seem to be characterized by a common phenotype. All are temperature sensitive in viral DNA replication and likely cannot initiate (as opposed to complete) rounds of viral DNA synthesis at the nonpermissive temperature (3, 4). Moreover, these viral mutants cannot initiate transformation at this temperature (3, 5, 6). Those which have been studied will transform certain cells at about 31–33°, which regain some growth properties associated with nontransformed cells when shifted to the nonpermissive temperature (5, 6, 8, 9). Moreover, Chou and Martin (7) have shown that these mutants have a reduced ability to induce host cell DNA synthesis at the restrictive temperature in permissive cells, a viral function (10) long hypothesized to be related to the virus' ability to transform. All of the *A* group mutants so far tested appear to map in the early region of the SV40 chromosome (11). Of the four complementation groups so far detected, only *A* mutants are defective in an early function (1–3). Taken together, these results have been viewed as consistent with the notion that a single early gene product or a derivative(s) thereof might be active in both the initiation of viral DNA synthesis and the maintenance of neoplastic transformation. The early region of SV40 DNA contains only enough information to encode approximately 100,000 daltons of protein (12, 13). One prominent early gene product is tumor (T) antigen (14–17), and various investigators have ascribed a molecular weight to this protein of 70–100,000 (18, 19). Thus, it is reasonable to suspect that this protein might be an or the *A* gene product. Such a conclusion is consistent with the earlier results of Osborn and Weber (20), Tegtmeyer *et al.* (19), and of Kuchino and Yamaguchi (21).

The detailed biochemical function of T antigen is unknown. However, recent reports have indicated that T antigen binds to calf-thymus DNA (22), that partially purified T antigen will bind SV40 DNA in solution (23–25), and that it is capable of binding to SV40 DNA at or near the origin of replication, as demonstrated by electron microscopy (23). Therefore, it is possible that T antigen may function by interacting with SV40 DNA, perhaps as part of the initiation step in viral DNA replication.

The present experiments were undertaken to determine whether partially purified T antigen from a *tsA* mutant transformed cell is more thermolabile in its SV40 DNA binding property than the wild-type protein. The evidence presented here supports the notion that T antigen is a product of the *A* gene.

MATERIALS AND METHODS

Cells. All cells were grown in Dulbecco's modification of Eagle's minimal essential medium containing 10% fetal calf serum (GIBCO, N.Y.), 100 μg/ml of streptomycin, and 200 units/ml per penicillin. A Chinese hamster lung cell strain derived from a single animal (CHL), wild-type SV40-transformed CHL (WT-SV CHL), CHL cells transformed by SV40 *tsA239*, and CHL cells transformed by SV40 *tsC219* (1, 5) were all the generous gift of Dr. Robert G. Martin. All cells were grown in glass roller bottles at 33°.

T Antigen Isolation Procedure. T antigen was partially purified from SV40 transformed cell lines through the ammonium sulfate, DEAE-cellulose, and Bio-Gel agarose A-1.5m steps, as described previously (24, 26, 30). All chromatographic operations were at 4°. Nuclei from fresh cells served as the source of antigen in every instance. Those fractions representing the peak of the antigen eluting from agarose were pooled, rendered 0.02% in Triton X-100, and 10^{-3} M in dithiothreitol and then concentrated 4- to 6-fold by ultrafiltration through a PM-10 membrane (Amicon Corp., Bedford, Mass.) at 0°. Concentrated fractions were stored at −70°, for at least 2 months, without loss of activity.

In the case of the sham purification of material from CHL cells, all maneuvers were comparable to those performed with the SV40-transformed CHL lines. In the absence of significant T immunoreactivity, DEAE fractions corresponding to those which were pooled from columns to which SV40-transformed CHL extract was applied were selected and pooled.

Antisera. Hamster sera obtained from animals carrying SV40 tumors and bearing T antigen antibody titers of ≥1:60 by complement fixation were generously provided by Dr. Roger Wilsnack through the Virus Cancer Program, National Cancer Institute. We are grateful to Drs. David Howell and Jack Gruber for their assistance in obtaining this reagent.

Abbreviations: SV40, simian virus 40; T antigen, tumor antigen; CHL, Chinese hamster lung.

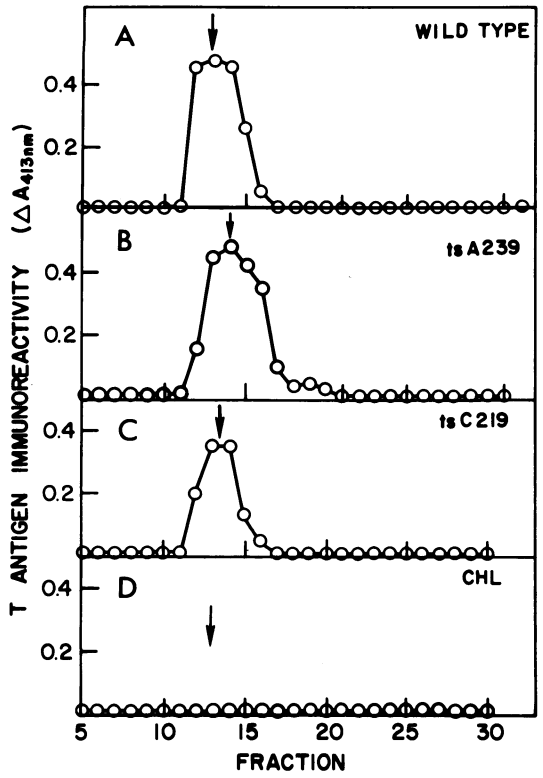

FIG. 1. Behavior of wild-type-, *tsA239*-, and *tsC219*-transformed CHL cell T antigens on agarose Bio-Gel A-1.5m. (A) Nuclei from 15 g of fresh SV40-transformed CHL cells grown at 33° were prepared, disrupted, and the DEAE-cellulose-purified T antigen fraction was isolated as noted in *Materials and Methods*. Peak T-antigen-containing fractions (19 mg of protein) eluting between 0.15–0.25 M NaCl from DEAE-cellulose were brought to 60% saturation with $(NH_4)_2SO_4$ and the pellet was redissolved in 0.17 volume of 0.05 M Tris·HCl, pH 7.8, 10^{-3} M dithiothreitol, 20% glycerol (Buffer B), dialyzed against two changes of 100 volumes of Buffer B for 3 hr, at 4°, and then 10 mg were applied to a (1 × 38 cm) column of agarose A-1.5m, equilibrated in the standard ethylene-glycol-containing buffer noted previously (24, 26). One milliliter fractions were collected and 2 μl were assayed for T antigen by the micro method reported earlier (24, 26). (B) Nuclei from 25 g of fresh *tsA239*-transformed CHL cells, grown at 33°, were prepared in a manner identical to that described in part A. The antigen eluted from DEAE in a comparable fashion, and the peak fractions (25 mg of protein) were pooled and dialyzed. An aliquot of this fraction (15 mg of protein) was then applied to an agarose A-1.5m column identical to that described above. The column fractions were also assayed identically to those noted above. (C) Nuclei from 7 g of fresh *tsC219*-transformed CHL cells were prepared, extracted, and the dialyzed 60% ammonium sulfate fraction was chromatographed on DEAE-cellulose in a manner identical to that described in A and B. The T-antigen-positive fractions eluting between 0.15–0.25 M NaCl (3.2 mg of protein) were pooled and 3 mg were applied to a column of agarose A-1.5m identical to that described in A and B. One milliliter fractions were collected and 5 μl were assayed for T antigen as described previously. (D) Nuclei from 7 g of fresh virus-free CHL cells were extracted, $(NH_4)_2SO_4$ precipitated, and the dissolved pellets were chromatographed on DEAE-cellulose exactly as in sections A–C above. The DEAE column was assayed for T immunoreactivity, with negative results. Fractions eluting between 0.15–0.25 M NaCl were pooled, $(NH_4)_2SO_4$ treated, and the protein pellet was dissolved in Buffer B as above. After dialysis against Buffer B, 13 mg of protein were applied to a (1 × 38 cm) agarose A-1.5m column. Fractions (1.2 ml) were collected and 2 μl were assayed for T immunoreactivity, as noted above.

Purification of Hamster Anti-T and Nonimmune IgG. Nonimmune and anti-T-containing hamster sera were separately brought to 60% saturation with solid ammonium sulfate at room temperature. The precipitate was collected by centrifugation and then dissolved in a volume of 0.01 M potassium phosphate, pH 8.0 (Buffer A) equivalent to the volume of serum originally fractionated. This solution was then dialyzed against two changes of 100 volumes of Buffer A, diluted with an equal volume of this buffer, and then applied to columns of DEAE-cellulose (DE-52, Whatman, Piscataway, N.J.)—5 ml bed volume per ml of original serum—equilibrated in Buffer A. The wash-through fractions, containing IgG, were identified by scanning at 280 nm and pooled for future use.

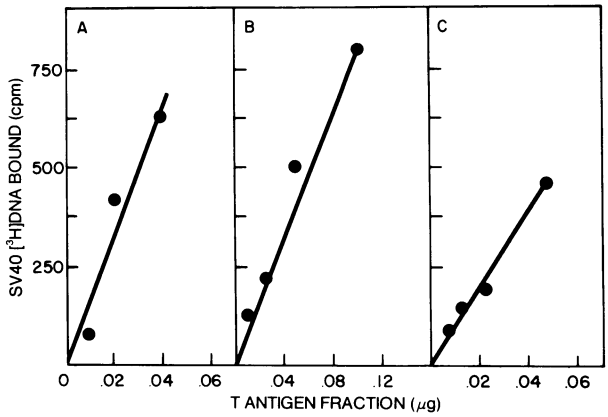

FIG. 2. Binding of SV40 [^{3}H]DNA by T antigen protein from various transformed cell lines. Increasing quantities of agarose-purified T antigen were added to the standard DNA binding reaction mixture. Each assay mix contained 1500–1600 cpm of SV40 [^{3}H]DNA I (2×10^5 cpm/μg). The background radioactivity was ≤45 cpm in each instance. Assays were performed as described in *Materials and Methods*. T antigens added were: Panel A—from SV40-transformed CHL; Panel B—from SV40 *tsA239*-transformed CHL; Panel C—from SV40 *tsC219*-transformed CHL.

DNA Binding Assay. SV40 [^{3}H]DNA I was prepared and the nitrocellulose filter binding assay was performed as described earlier (24). All incubations were at 0° for 10 min. Each reaction mixture (0.25 ml) contained Tris·HCl, 0.01 M, pH 7.4; EDTA, 0.1 mM; dithiothreitol, 1.1 mM; dimethylsulfoxide, 3%; manganese acetate, 0.2 mM; and bovine serum albumin, 10 μg/ml.

T Antigen Assay by Complement Fixation was performed as described previously (26) by the micro method of Wasserman and Levine (27).

Protein Determinations were performed by the method of Lowry *et al.* (28).

RESULTS

Chromatographic behavior of T antigens from wild-type-, *tsA239*-, and *tsC219*-transformed CHL cells

Aliquots of T antigen purified from isolated nuclei of wild-type-, *tsA239*-, and *tsC219*-transformed CHL through the ammonium sulfate and DEAE steps, were applied to identical size columns of Bio-Gel A-1.5m. As noted in Fig. 1, antigen appeared in the excluded volume of the column in all three instances. An aliquot of DEAE-purified protein isolated from uninfected CHL cells, comparable to that from WT-SV CHL, was also applied to a column of Bio-Gel A-1.5m identical in size to those noted above. No detectable T immunoreactivity developed at any point from this column.

DNA binding properties of agarose-purified antigens

Pooled, concentrated agarose antigen (Fig. 1), was tested in

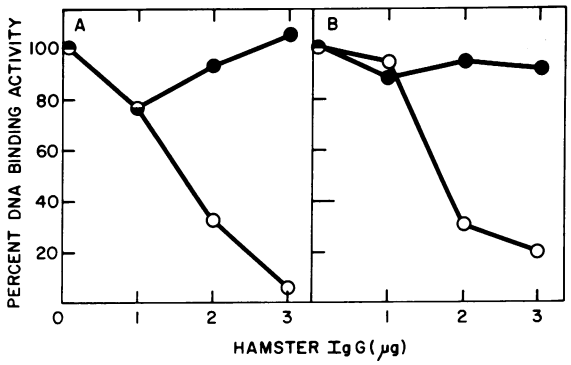

FIG. 3. Effect of hamster anti-T and control IgG upon the DNA binding activities present in various T-antigen-containing fractions. Anti-T (○) and nonimmune (●) IgG were mixed at the indicated concentrations with either wild-type (WT) or *tsA239*-derived T protein in a 0.02 ml reaction mixture and incubated for 10 min at 30°. One-half of the preincubation cocktail was transferred to a standard DNA binding mixture (0.10 ml total volume). The amounts of T present in the binding mixture were: SV40, 0.04 μg, and *tsA239*, 0.09 μg. These aliquots bound comparable amounts of SV40 [^{3}H]DNA (600–650 cpm). Components of the preincubation mixture were Tris·HCl, pH 7.4, 0.10 M and Triton X-100, 0.01%. T was added as the DEAE-cellulose and agarose A-1.5m purified material, in a volume of 5 μl, contained originally in 0.03 M imidazole, pH 6.8, 10^{-3} M dithiothreitol, 10% ethylene glycol, 0.02% Triton X-100 and diluted in Buffer B to the indicated concentration prior to addition. The standard DNA binding assay mixture contained 1000–1100 cpm of SV40 [^{3}H]DNA I (2×10^5 cpm/μg). The background radioactivity in all instances was <60 cpm. Panel A—wild-type T; Panel B—*tsA239* T.

the nitrocellulose filter DNA-binding assay reported previously (24). As shown in Fig. 2, concentrated wild-type, *tsA239*, and *tsC219* agarose-purified T antigen binds DNA to an extent proportional to the amount of protein added.

Effect of anti-T IgG upon the DNA binding activity associated with SV40 and SV40 *tsA239* T antigens

Either purified hamster anti-T IgG or nonimmune IgG was added in increasing quantities to comparable amounts of wild-type and *tsA239* CHL T-antigen-associated DNA binding activity. As shown in Fig. 3, hamster anti-T IgG inhibits the DNA binding activity in each instance, while equivalent amounts of nonimmune IgG have no inhibitory effect.

Effect of temperature on the survival of T antigen DNA binding activity from wild-type-, *tsA239*-, and *tsC219*-transformed CHL cells

Equivalent aliquots of wild-type and *tsA239* DEAE-cellulose- and agarose-purified T antigens were heated for identical periods at both 30° and 44° (Fig. 4). Notably, the antigen from the mutant transformant loses DNA binding activity in geometric fashion at 44° while the wild-type antigen is more stable. At 30°, the mutant cell antigen also loses activity but less rapidly than at 44°, while the wild type is again stable. In contrast to the effects of heat upon the *tsA239* antigen, the *tsC219* protein(s) is as stable at 44° as wild type (Fig. 5). A mixture of equal aliquots of wild type and *tsA* mutant transformed cell antigen loses activity at 44° in a fashion which is predictable based upon the activities of each antigen analyzed separately (Fig. 6). Thus, the wild-type antigen fraction does not contain a recognizable factor which can stabilize the mutant antigen's DNA binding activity, and the *tsA* mutant fraction does not contain a discernible element which can inactivate the wild-type antigen.

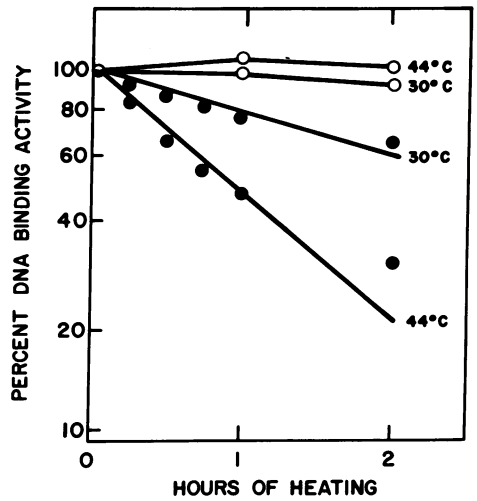

FIG. 4. Thermolability of wild-type- and *tsA239*-derived T antigens. Wild-type- and *tsA239*-derived, agarose-purified proteins were diluted in fresh Buffer B to concentrations of 8 and 20 μg/ml, respectively. At these levels, 10 μl of each bound approximately 6.8 ng (1360 cpm) of SV40 [^{3}H]DNA. A total of 12.5 ng (2500 cpm) of SV40 [^{3}H]DNA was present in each assay mixture. Each antigen was then heated in a water bath at 44° or 30° for the indicated times. At these points, each was transferred to an ice bath for 10 min at which time 10 μl was removed and tested in the standard DNA binding assay (2500 cpm/tube of SV40 [^{3}H]DNA I: 2×10^5 cpm/μg). The master aliquot was then reincubated at the indicated temperature for the indicated time period. The values employed as the 100% standard for the unheated DNA binding activities in each instance are those of the appropriately diluted wild-type and *tsA239* antigens which are unheated and remain on ice. Activity of wild-type T antigen (○); activity of *tsA239* T antigen (●).

DISCUSSION

The results described here strongly suggest that the observed DNA binding activity and SV40 T antigen immunoreactivity present in a specific agarose chromatographic fraction are properties of the same macromolecule or molecular complex. That the T protein is SV40 specific is indicated by the observation that virus-free CHL cells do not contain quantities of T antigen which can be measured under present conditions. The finding that the DNA binding activity can be inhibited by anti-T antibody supports the hypothesis that the observed DNA binding activity is associated with the SV40 T antigen molecule or complex.

Heating the wild-type antigen at 30° and 44° results in minimal inactivation of its DNA binding activity over a 2-hr period. This was a reproducible observation. Prior and more recent published reports have pointed to the lability of this molecule at 37° (24, 31). We cannot be sure why this antigen is more stable, but the strain of virus employed, the host cell of origin, the high concentrations of dithiothreitol present, the temperature at which the cells were grown (i.e., 33°), and the operational conditions before and during heating are, in part, or wholly, different than those employed previously by us and others where heat lability has been expressly studied (24, 31).

Comparably purified T antigen, isolated from *tsA239* transformed cells, is substantially more heat labile at 44° than wild type. There is also a difference at 30°, but of less dramatic proportions. Chou and Martin (32) have suggested, on the basis of their temperature shift data, that the *A* gene product of many of their *A* mutants, including *tsA239*, is relatively thermolabile, even at the permissive temperature. This is consistent with our observations.

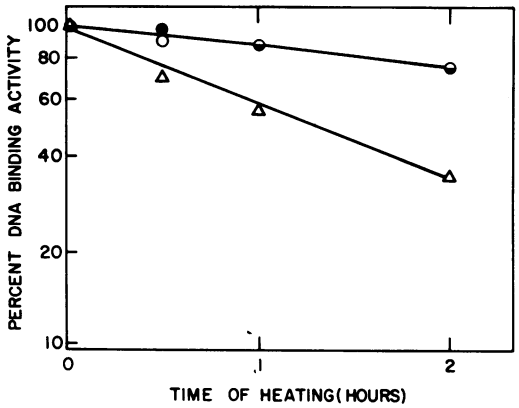

FIG. 5. Thermolability of wild-type-, *tsA239*-, and *tsC219*-derived antigens. Wild-type-, *tsA239*-, and *tsC219*-derived agarose-purified antigens were diluted in fresh Buffer B to the point at which an aliquot of each (10–15 μl), assayed prior to being heated at 44°, bound 1400–1700 cpm of SV40 [^{3}H]DNA (input, 2120 cpm). The concentration of the wild-type T protein was 8 μg/ml. The *tsA239* concentration was 14 μg/ml, and the *tsC219* concentration was 17 μg/ml. At various times during the heating period, the heated samples were removed to an ice bath for 10 min; aliquots were then removed and assayed at 0° in the standard DNA binding mixture (SV40 [^{3}H]DNA input—2120 cpm, specific activity 2 × 10^5 cpm/μg). The remainder of the operational details are identical to those reported in the legend to Fig. 4 and in *Materials and Methods*. Activity of wild-type T (○); activity of *tsC219* T (●); activity of *tsA239* T (△).

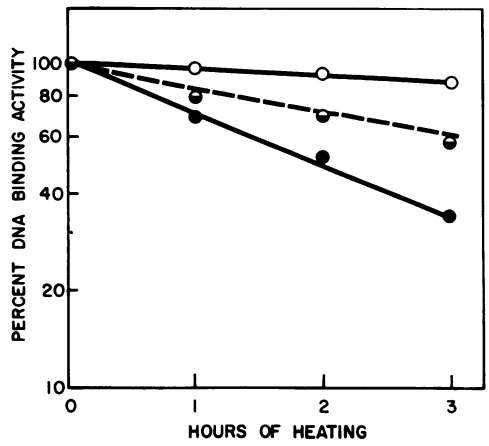

FIG. 6. Effect of heating upon a mixture of wild-type and *tsA239* antigens. Wild-type (8 μg/ml) and *tsA239* (20 μg/ml) T antigens diluted to these assigned concentrations with fresh Buffer B were heated at 44° alone and in a mixture—wild-type (8 μg/ml) plus *tsA239* (20 μg/ml). After heating for the indicated times, and removing samples to an ice bath for 10 min, volumes (10 μl) of each unmixed sample and of the mixture (5 μl) which, in the unheated state, bind 1100 cpm of SV40 [^{3}H]DNA I (specific activity 2 × 10^5 cpm/μg) were tested in the standard DNA binding assay (input, 2100 cpm). The background radioactivity in each instance was 50 cpm. Activity of wild-type T (○); activity of *tsA239* (●); activity of the wild-type plus *tsA239* T mixture (◒). The broken line represents the theoretical reduction in activity with time, at 44°, of a noninteracting mixture of wild-type and *tsA239* T, at the concentrations indicated above, based upon the actual heat inactivation rates of the individual, unmixed samples.

Since the mixture experiment (Fig. 6) strongly suggests the difference in thermolability noted between *tsA239* and wild-type antigen is not due to the action of a contaminating inhibitor in the mutant fraction or a protector molecule in the wild-type preparation, the data strongly suggest that the antigen from the *A* mutant transformed cell bears a structural lesion which renders it relatively thermolabile.

The observed thermolability of T from the *tsA* transformant is most likely a result of the *A* gene mutation as opposed to a nonspecific effect of mutagen base alteration, since *tsC219* T antigen is not as thermolabile as the *tsA239* protein(s) and is as stable as the wild-type protein. If, as seems reasonable based in part upon the experience with another *A* mutant (29), *tsA239* DNA contains a lesion in but one gene leading to the temperature-sensitive phenotype, one can strongly suggest that T antigen plays a role in the initiation of viral DNA synthesis, a step which is selectively blocked at the nonpermissive temperature upon lytic infection with *A* mutants (3, 4). Moreover, there is now evidence from multiple laboratories (5, 6, 8, 9) suggesting that a viral *A* gene product plays a role in maintaining a disturbance in growth control induced by the virus as a permanent event at the permissive temperature. In this regard, the data presented here are consistent with the notion that the T protein present in our fractions, a possible precursor or a derivative plays a role in such a process and could fall into the category of proteins involved in the maintenance of transformation. The presently isolated T antigen could be a specific cleavage product of a master molecule which itself alone is the mediator of transformation. If such a putative "zymogen" were rapidly turned over in certain cells (19), T could, in certain circumstances, be the most prevalent immunoreactive species available for isolation, yet the pro-T, despite being quantitatively a minority species, could be the active principle in transformation.

The demonstration of the DNA binding property of T protein raises certain possibilities relative to its *in vivo* biochemical function. For T to be involved in the initiation of viral DNA replication, it is reasonable that it be able to bind to SV40 DNA, which it does (23–25). Moreover, Reed *et al.* (23) have shown that it is capable of selectively binding to SV40 DNA at or near the origin of DNA replication. A question which immediately arises relates to a potential role for T (or a precursor or derivative) in promoting a major disturbance in cellular growth control. On the basis of results presented here and elsewhere (7) one can speculate that any role which it may play in maintaining transformation might require its interaction with one or more species of nucleic acid. An observation consistent with such a hypothesis is that the *A* gene product may well be central to the ability of the virus to induce host cell DNA synthesis (7). Thus, if one postulated that the role of the *A* gene in this function and the maintenance of transformation operate by a somewhat common mechanism, it would not be unreasonable to suspect that, if T antigen were involved in the latter, it might do so in the role of DNA binding protein.

In contrast, T and/or pro-T could be a bifunctional protein. Its DNA binding function could be involved in the control of viral DNA replication and another activity, unrelated to its ability to bind DNA, could be involved in the maintenance of transformation. In such a situation the temperature-sensitive lesion could be viewed as affecting both functions in parallel.

Alternatively it is conceivable that there could be two separate early gene products, one (the T antigen) involved in the initiation of replication and the other in the maintenance of transformation. The effect of the *A* gene mutation upon the maintenance of transformation could be interpreted as a result of complex formation between T and the other early protein. In such a situation, *A* mutant, thermolabile T might

be viewed as exerting a dominant lethal effect upon the latter at the nonpermissive temperature. At the present time our experimental results do not allow us to make a proper choice among these and other possibilities.

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