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Biochemical Activities of T-Antigen Proteins Encoded by Simian Virus 40 *A* Gene Deletion Mutants

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We have analyzed T antigens produced by a set of simian virus 40 (SV40) *A* gene deletion mutants for ATPase activity and for binding to the SV40 origin of DNA replication. Virus stocks of nonviable SV40 *A* gene deletion mutants were established in SV40-transformed monkey COS cells. Mutant T antigens were produced in mutant virus-infected CV1 cells. The structures of the mutant T antigens were characterized by immunoprecipitation with monoclonal antibodies directed against distinct regions of the T-antigen molecule. T antigens in crude extracts prepared from cells infected with 10 different mutants were immobilized on polyacrylamide beads with monoclonal antibodies, quantified by Coomassie blue staining, and then assayed directly for T antigen-specific ATPase activity and for binding to the SV40 origin of DNA replication. Our results indicate that the T antigen coding sequences required for origin binding map between 0.54 and 0.35 map units on the SV40 genome. In contrast, sequences closer to the C terminus of T antigen (between 0.24 and 0.20 map units) are required for ATPase activity. The presence of the ATPase activity correlated closely with the ability of the mutant viruses to replicate and to transform nonpermissive cells. The origin binding activity was retained, however, by three mutants that lacked these two functions, indicating that this activity is not sufficient to support either cellular transformation or viral replication. Neither the ATPase activity nor the origin binding activity correlated with the ability of the mutant DNA to activate silent rRNA genes or host cell DNA synthesis.

Large T antigen, a 90,000- to 96,000-dalton phosphoprotein, is the product of the simian virus 40 (SV40) *A* gene. This multifunctional protein is expressed early in the lytic cycle of SV40-infected monkey cells and in nonpermissive cell lines that have been transformed by SV40 (for review see reference 67). The variety of biological functions that have been ascribed to T antigen include: (i) the induction of SV40 DNA replication (60); (ii) the autoregulation of SV40 early region transcription (1, 25, 51, 62); (iii) the initiation and maintenance of neoplastic transformation in nonpermissive cells (2, 27, 33, 43, 61); (iv) the induction of host cell DNA synthesis (4, 13, 21, 22, 53, 65); (v) the stimulation of rRNA transcription (35, 47, 57); (vi) the ability to allow adenovirus type 2 to grow in monkey cells (helper function) (6, 17, 26, 49); and (vii) possibly a role in the assembly of infectious virions (8). There is considerable evidence that at least some of these functions are genetically distinct and can be mapped to different portions of the *A* gene (3, 7, 8, 12, 13, 16, 39, 57, 58). This suggests that T antigen may possess a variety of distinct biochemical activities that carry out these functions and that T antigen may be composed of several protein domains that have some degree of independent function.

T antigen has been shown to possess at least three biochemical activities. It binds specifically to SV40 DNA at the origin of viral DNA replication (23, 38, 50, 64), possesses an ATPase activity (5, 14, 66), and binds tightly to a 53,000-dalton host protein (28, 32, 36). Genetic and biochemical evidence indicates that the DNA binding activity is required for the induction of viral DNA replication and for the autoregulation of early viral transcription (10, 11, 18, 41, 42, 51, 54, 55). The other two activities, however, have not yet been linked to a T antigen function.

To learn more about how the biochemical activities of T antigen are related to its biological function, and to identify possible protein domains that are associated with these activities, we have tested a set of mutant T antigens for ATPase and SV40 origin binding activity. These

mutant proteins were produced in permissive cells by a series of SV40 deletion mutants that have been characterized with respect to structure and function as described in the accompanying papers (46, 56). The observed activities are compared to the functional and structural features of the mutants.

MATERIALS AND METHODS

Cells. COS7 and CV1 monkey cells were cultured in plastic dishes containing Dulbecco modified Eagle medium plus streptomycin (0.1 mg/ml), penicillin (0.1 mg/ml), and fetal calf serum (5% for CV1 and 10% for COS7), Cells were infected at 70 to 90% confluency on 9-cm plates with 1 ml of viral stock plus phosphate-buffered saline (PBS) for 30 to 60 min. After infection, the serum concentration was reduced to 2%.

Immunological reagents. Cell lines PAb419 and PAb405 were a gift of Ed Harlow (Imperial Research Cancer Fund, London, England). Purified monoclonal antibodies PAb204 and PAb205 were donated by David Lane. Goat immunoglobulin G (IgG) directed against mouse IgG light chain, a gift of John Kimura and Leon Wofsy (both at University of California-Berkeley, Berkeley, Calif.), was affinity-purified by chromatography on PAb419-Sepharose (CNBr coupled). Goat anti-mouse beads for T antigen immobilization were prepared by coupling the affinity-purified goat anti-mouse IgG to Bio-Rad 702 or Immunobeads with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide.

Mutant virus stocks. SV40 deletion mutants were constructed and cloned in bacteria as described (46). Mutant DNAs were excised from recombinant plasmids by digestion with *Bam*HI endonuclease and recircularized with T4 DNA ligase at DNA concentrations of 5 to 10 μg/ml. COS7 cells (40 to 60% confluent on 6-cm plates) were transfected with the ligated DNA (50 to 150 ng) in the presence of DEAE-dextran for 4 to 6 h at 32°C (37). After 72 h at 37°C, the transfected cells were frozen and kept overnight at −20°C. The cells were thawed, suspended in the medium (5 ml), and sonicated to produce a virus stock. This virus was repassaged in COS7 cells until a majority of the cells showed cytopathic effects within 3 days of infection (one of two passages).

Immunofluorescence. CV1 cells on 6-cm plates were fixed with 3.5% formaldehyde in PBS followed by cold ethanol 18 to 24 h after infection. The fixed cells were treated first with hamster anti-SV40 tumor serum and then with fluorescein isothiocyanate-conjugated rabbit anti-hamster IgG (48).

Restriction analysis of viral DNA. Infected CV1 cells on 9-cm plates were lysed with 1 ml of Hirt extraction buffer (10 mM Tris, pH 7.9, 50 mM EDTA, 0.6% sodium dodecyl sulfate [SDS], 1 M NaCl), kept overnight at −20°C, and centrifuged at 12,000 × *g* for 15 min (20). The supernatant was phenol-extracted, and the DNA was precipitated with an equal volume of isopropanol. The DNA was redissolved in 10 mM Tris (pH 8)–0.1 M NaCl–1 mM EDTA and reprecipitated with 2.5 volumes of ethanol. The ethanol pellet was dried, redissolved in 10 mM Tris (pH 8)–1 mM EDTA, and digested with *Bst*NI or *Alu*I endonuclease. The resulting DNA fragments were resolved by electrophoresis on 1.7% agarose gels.

Immunoprecipitation of ^{35}S-labeled infected-cell extracts. Infected cells on 6-cm plates were incubated for 2 to 4 h with 50 μCi of [^{35}S]methionine in methionine-free medium. Extracts were prepared by washing the cells with PBS, lysing them with 0.5 ml of 200 mM LiCl–20 mM Tris (pH 8)–0.5% Nonidet P-40 (NP40)–0.01% phenylmethylsulfonyl fluoride, and centrifuging the lysate for 10 min in an Eppendorf 5412 microfuge to remove cellular debris. Extracts were incubated for 2 h at 0°C with 1 to 2 μg of purified monoclonal antibody and immunoprecipitated with 8 μl of a 10% solution of formaldehyde-fixed *Staphylococcus aureus* Cowan I strain (24). Immunoprecipitates were washed twice with 0.5 ml of 1 M LiCl–20 mM Tris (pH 8)–0.5% NP40 and once with 0.5 ml of 20 mM Tris (pH 8)–0.1 M NaCl–0.5% NP40 and extracted with 12 μl of SDS sample buffer (59).

Isolation of mutant T antigens. CV1 cells on 9-cm plates were infected with sufficient virus stock (~2 to 5 PFU/cell) to infect 60 to 80% of the cells, as determined by immunofluorescence (50 to 500 μl), or with 10 PFU of SV40 strain 776 per cell. At 24 to 32 h after infection the cells were washed with PBS, lysed with 0.5 ml of 200 mM LiCl–20 mM Tris (pH 8)–0.5% NP40–0.5 mM dithiothreitol, and centrifuged for 5 min in an Eppendorf microfuge to pellet cell debris. The lysates were incubated with monoclonal antibody (1 to 2 μg per plate of cells) for 2 to 4 h at 0°C and then centrifuged for 15 min in the microfuge, and the supernatant was incubated for 1 h with 2 to 4 μl of goat anti-mouse beads. The beads were pelleted and washed twice with 1 M LiCl–20 mM Tris (pH 8)–0.5% NP40 and once with 0.1 M NaCl–10 mM Tris (pH 8)–0.5% NP40. The beads were suspended in 10 mM Tris (pH 8)–200 mM NaCl–10% glycerol–0.05% NP40–0.5 mM EDTA–0.5 mM dithiothreitol for storage at −20°C before assay. To determine the amount of T antigen protein on the beads, the proteins were eluted with SDS sample buffer, resolved on gradient SDS-polyacrylamide gels (59), stained with Coomassie blue, and quantified with a scanning densitometer.

ATPase assay. T antigen-containing beads (0.1 to 0.5 μl) were suspended in 20 μl of a buffer containing 25 mM 1,4-piperazinediethanesulfonic acid (PIPES) (pH 7.0), 0.1 M NaCl, 5 mM $MgCl_2$, 0.01% NP40, and 0.2 to 0.5 mCi of [γ-^{32}P]dATP (~5,000 Ci/mmol), and incubated for 10 to 15 min at 20 to 23°C. Free $^{32}P_i$ was then determined as described previously (66).

Origin binding assay. T antigen-containing beads (0.2 to 0.5 μl) were incubated for 5 min at 20 to 23°C in 10 μl of DB buffer (25 mM PIPES [pH 7.0], 0.15 M NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.05% NP40) containing 100 ng of sonicated calf thymus DNA and 25 ng of ^{32}P end-labeled SV40 origin-containing DNA (pSV07) or control DNA (pPLAC or pPM1). The pSV07 plasmid contains three copies of the origin-containing SV40 RII G fragment at the RI site of pPLAC (63). pPM1 was derived from pSV010 (31) by deleting the SV40 sequences by using *Hin*dIII. pSV07 and pPM1 were constructed by Richard Myers, and pPLAC was constructed by Carl Thummel (both at University of California-Berkeley, Berkeley, Calif.). End-labeled DNA was prepared by digesting the plasmid DNA with *Eco*RI and phosphorylating the ends with T4 polynucleotide kinase and [γ-^{32}P]ATP. The beads were pelleted after adding 100 μl of DB buffer (200 mM NaCl) and washed once with 200 μl of DB buffer (200 mM NaCl). Retention of [^{32}P]DNA on the beads was determined by Cerenkov counting. The average experimental variation in counts retained was

typically less than 15%. End-labeled, *Bst*NI-digested SV40 DNA (see Fig. 3), a gift of Bill Dynan (University of California-Berkeley, Berkeley, Calif.), was prepared as described previously (30) by using [α-^{32}P]dATP and [α-^{32}P]dTTP. Differential incorporation of these two nucleotide accounts for the variation in ^{32}P specific activity of the fragments.

RESULTS

Generation of SV40 *A* gene deletion mutants. The construction, isolation, and sequence analysis of the mutants used in this study have been described previously (44–46). Briefly, SV40(pBR322) recombinant DNA was randomly nicked at a single site with DNase I, gapped with DNA polymerase I, linearized with S1 nuclease, and recircularized with DNA ligase. The mutant DNAs were then isolated and amplified by cloning in bacteria. The sequences of the deletion junctions were determined by the Maxam-Gilbert method (34) (see reference 46 and Table 1).

Production of mutant T antigens. The mutant viral DNA was excised from pBR322, circularized with DNA ligase, and transfected into subconfluent monolayers of COS7 cells to produce infectious virus. The permissive, SV40-transformed COS7 cell line produces wild-type T antigen (15) and thus complements SV40 *A* gene mutant viruses. The virus was passaged in COS7 cells until a majority of cells showed cytopathic effects by 3 days after infection. Viral stocks obtained from transfected COS cells are essentially free of helper virus, ensuring that only mutant T antigens are produced after subsequent infection of CV1 cells.

To determine whether the mutant genomes had rearranged or recombined with the host *A* gene during passage in COS7 cells, viral DNA was purified and analyzed by digestion with restriction enzymes. The predicted patterns of DNA fragments were observed for all mutant viruses (data not shown).

CV1 cells infected with the mutant viruses were analyzed for T antigen by observing nuclear immunofluorescence. All of the mutants except *dl*1136, *dl*1137, and *dl*1138 showed specific staining of the infected cell nuclei. This finding is in agreement with results obtained when transfection or microinjection was used to introduce the mutant viral DNA into monkey cells (46, 56). It was observed that 0.25 ml of viral stock was sufficient to produce detectable nuclear fluorescence in 50 to 90% of the cells on a 6-cm plate (~5 × 10^5 cells).

Characterization of mutant T antigens. The expression of T antigen by *A* gene deletion mutants was detected by immunoprecipitation of T antigens in [^{35}S]methionine-labeled extracts prepared from infected CV1 cells. The mutant proteins were immunoprecipitated with various monoclonal and polyclonal antibodies directed against T antigen and resolved on polyacrylamide gels as shown in Fig. 1. With the exception of *dl*1136, the infected CV1 extracts contained T antigens which corresponded in size to those predicted from the DNA sequence of the mutants (Table 1). In addition, bands corresponding to T antigens of substantially lower than predicted molecular weight appeared in some of the immunoprecipitates (lanes b and e, Fig. 1). It is likely that these bands represent proteolytic cleavage products because their relative intensity varied from experiment to experiment. The absence of full-length T antigen in mutant-infected lysates (Fig. 1) indicates that the mutant virus stocks are essentially free of wild-type virus. T antigens with C-terminal deletions, including the *dl*1140 T antigen (internal deletion near the C terminus), migrated faster than expected during SDS gel electrophoresis, whereas mutant T antigens with full-length C termini migrated more slowly than expected. This effect was most dramatic for the *dl*1140 T antigen, in which an 11-amino acid deletion results in a 4,000-dalton mobility shift. The size of T antigen as determined by SDS-polyacrylamide gel electrophoresis exceeds the predicted molecular weight of the polypeptide (82,000) by approximately 10,000. The results shown in Table 1 support the idea that the C terminus of T antigen contains the sequences which are responsible for the decreased mobility (9). The low mobility of T antigen could result from modifications of amino acid residues near the C terminus of the molecule.

Additional evidence that the mutant viruses

TABLE 1. Apparent molecular weight of T antigens produced by SV40 deletion mutants

Deletion no.	Deletion limits[a]	Protein products		
		Type[b]	Predicted size[c] (×10^3)	Observed size[d] (×10^3)
1135	5114–5082	I	91	90
1136	5067–4262	C	8	—
1137	4453–4423	C	17	16
1138	4339–4311	C	23	20
1047	4007–3570	I	73	78
1151	3798–3472	I	78	82
1055	3620–3566	C	54	51
1058	3290–3148	C	67	63
1061	3048–2907[e]	C	77	73
1066	2809–2730[e]	C	87	85
1140	2792–2763	I	91	88

[a] Numbers refer to the nucleotides deleted.
[b] I, Internal deletion; C, Carboxy-terminal deletion.
[c] Based on an apparent molecular weight of 92,000.
[d] As determined by SDS-polyacrylamide gel electrophoresis.
[e] Additional sequence present.

produce the expected proteins was obtained by immunoprecipitation with monoclonal antibodies of known specificity. The monoclonal antibody PAb419 (L19 in reference 19), which recognizes the amino terminus of large T (19), was found to precipitate all but one of the mutant proteins, indicating that the corresponding amino-terminal antigenic determinant of these proteins is intact (Fig. 1 and 2). The *dl*1135 T antigen, which lacks 11 residues near its amino terminus, was not precipitated by PAb419. Furthermore, antibodies that react with the central and carboxy-terminal regions of T antigens PAb204 (3C4 in reference 29) and PAb405 (L5 in reference 19), respectively, failed to precipitate mutant T antigens that lack these regions.

Quantitation of mutant T antigens resolved on polyacrylamide gels and stained with Coomassie blue indicated that cells infected by all of the mutants except *dl*1066 and *dl*1140 accumulated at least threefold less T antigen than cells infected with wild-type SV40 (Fig. 2 and unpublished data). Cells infected by the viable mutants *dl*1066 and *dl*1140, however, accumulated wild-type levels of T antigen. In contrast, the incorporation of [^{35}S]methionine into proteins synthesized by nonviable mutants was generally greater than into wild-type T antigen (Fig. 1), indicating that the mutant proteins turn over more rapidly than their wild-type counterparts, probably as a result of increased rates of both synthesis and degradation. In addition, the rate of small t synthesis appeared to be severalfold higher in cells infected with nonviable mutants *dl*1047, *dl*1055, *dl*1058 and *dl*1061 than in cells infected with wild-type SV40 (Fig. 1). It is possible that these differences in the rate of early viral protein synthesis reflect differences in the multiplicities of infection (only approximately equal as determined by immunofluorescence) which could determine the level of viral template available for transcription. However, as a result of viral DNA replication, the amount of viral template in wild-type SV40-infected cells is expected to be much higher (10^4- to 10^3-fold) than in cells infected with the nonviable mutants. Therefore, the most likely explanation for these results is that the repression of early viral mRNA which has been observed in vitro and in wild-type SV40 infections is reduced or absent because of the *A* gene mutations. This could be a result either of the observed instability of the mutant proteins or of an alteration in an intrinsic property of T antigen such as its ability to interact with binding sites at the origin of DNA replication.

ATPase activity of mutant T antigens. T antigen ATPase activity in mutant-infected cell lysates was determined by using a modification of a procedure described previously (5). Extracts

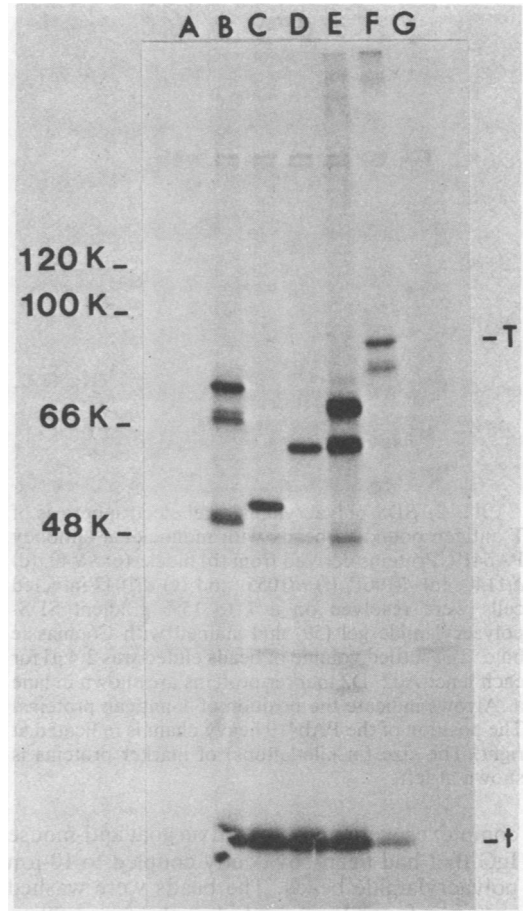

FIG. 1. Immunoprecipitation of mutant T antigens. CV1 cells were infected with mutant virus stock (200 μl), wild-type SV40 stock (100 μl), or mock-transfected stock (200 μl). All stocks were derived from the second passage of the initial (transfection) stock in COS7 cells. The infected cells were labeled with [^{35}S]methionine 24 h after infection. T-antigen proteins were immunoprecipitated from 50 μl of labeled extract by monoclonal antibody PAb419 and subjected to electrophoresis in a 7 to 15% gradient SDS-polyacrylamide gel (59). The autoradiograph shown was exposed to the dried gel for 24 h (Kodak SB film). The T-antigen proteins were derived from cells infected with (A) *dl*1136, (B) *dl*1047, (C) *dl*1055, (D) *dl*1058, (E) *dl*1061, (F) SV40, or (G) mock virus. The positions of large T (T) and small t (t) are indicated at right. The size (in kilodaltons) and position of Ad2$^+$D2 marker proteins are shown at left.

derived from CV1 cells infected with each deletion mutant, except *dl*1135, were incubated with PAb419, a monoclonal IgG that does not inhibit the ATPase activity of purified large T antigen (E. Harlow and R. Clark, unpublished data). T antigens from mutants *dl*1135 and Ad-SVR280 (see below) were complexed with PAb205 (3C5 in reference 5) or PAb405. The resulting immune

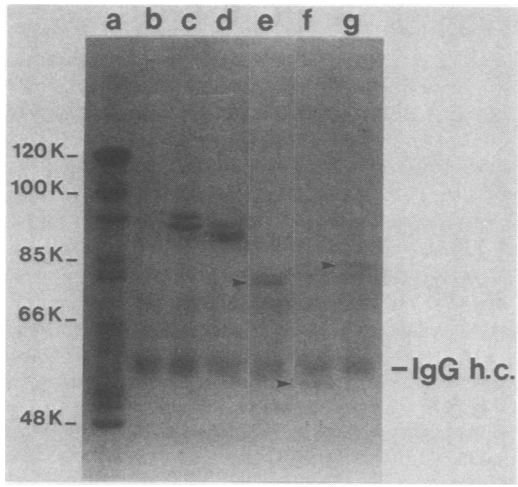

FIG. 2. SDS-polyacrylamide gel electrophoresis of T antigen bound to beads with monoclonal antibody PAb419. Proteins derived from (b) mock, (c) SV40, (d) *dl*1140, (e) *dl*1061, (f) *dl*1055, and (g) *dl*1047-infected cells were resolved on a 7 to 15% gradient SDS-polyacrylamide gel (59) and stained with Coomassie blue. The settled volume of beads eluted was 2.4 μl for each lane. $Ad2^+D2$ marker proteins are shown in lane a. Arrows indicate the position of T-antigen proteins. The position of the PAb419 heavy chain is indicated at right. The size (in kilodaltons) of marker proteins is shown at left.

complexes were immobilized on goat anti-mouse IgG that had been covalently coupled to 10-μm polyacrylamide beads. The beads were washed extensively and assayed directly for ATPase activity. That the hydrolysis of ATP was catalyzed by T antigen was confirmed by treating the beads with PAb204, a monoclonal antibody known to strongly and specifically inhibit SV40 T antigen ATPase activity (5). Results scored as positive for T antigen ATPase activity showed at least 50 to 90% inhibition by PAb204. The average experimental variation in ATPase activity is typically less than 2% of the wild-type activity. In all cases, the ATPase activity on beads prepared from mock-infected cell extracts was determined and subtracted from the results. This value was approximately 2% of that found in extracts of cells infected with wild-type virus.

To determine specific activities, the T antigens were eluted from the beads with SDS sample buffer, resolved on SDS-polyacrylamide gels, and quantified by Coomassie blue staining (Fig. 2). ATPase specific activities of the mutant T antigens are listed in Table 2. The observed ATPase activity of *dl*1066 T antigen suggests that the 38 amino acids at the C terminus (residues 671 to 708) are not required for activity. In contrast, *dl*1061 T antigen lacked ATPase activity, indicating that amino acid residues included in an adjacent region (residues 590 to 669) are required for activity. Similarly, residues in the central portion of T antigen, lacking in *dl*1047 (residues 271 to 416) and in *dl*1151 (residues 341 to 449), are also required for activity. In addition, antibody inhibition data suggest that residues between these two sites are likely to be involved in ATP hydrolysis. The monoclonal antibody PAb204, which strongly inhibits T antigen ATPase activity, will immunoprecipitate *dl*1047, *dl*1058, *dl*1061, and the $AD2^+ND2$ 42,000-dalton T antigen, but not *dl*1055 T antigen, indicating that it binds in the region defined by amino acid residues 417 to 509 (data not shown, and reference 29). The inhibitory effect of PAb204 on T antigen ATPase activity suggests that these residues are also involved in ATPase activity (5). The low ATPase activity of the *dl*1135 T antigen is discussed below.

Origin binding. The ability of the mutant T antigens to specifically bind to the SV40 origin of DNA replication was determined by a modification of the solid-phase T antigen ATPase assay that is similar in principle to an assay described previously (38, 52). Mutant T antigens were immobilized on polyacrylamide beads as described earlier. The beads were incubated in a solution of ^{32}P end-labeled DNA with or without origin sequences, and DNA binding was then quantified by measuring the counts retained on the beads. In the presence of T antigen, origin-containing DNA fragments were retained 10- to 100-fold more efficiently than non-origin-containing fragments. Furthermore, the retention of

TABLE 2. Specific activities of mutant T antigens

Virus	T antigen primary structure (map units)	ATPase[a]	Origin binding[b]
SV40		1.0	1.0
dl 1140		1.0	1.0
dl 1066		1.1	1.3
dl 1061		<0.02	1.2
dl 1058		<0.01	0.7
dl 1055		<0.02	1.5
dl 1151		<0.05	<0.03
dl 1047		<0.04	<0.04
dl 1138		0[c]	0[c]
dl 1137		0[c]	0[c]
dl 1135		0.1	<0.05
$Ad2^+D2$		1.1	1.1
Ad-SVR280		1.1	1.1
	.6 .5 .4 .3 .2		

[a] Initial rate of dATP hydrolysis per mole of T antigen relative to wild-type T antigen.

[b] Amount of origin-containing DNA bound per mole of T antigen relative to wild type T antigen.

[c] No activity was detected, and there was insufficient T antigen protein for quantitation by Coomassie blue staining.

origin-containing DNA was linear with respect to the amount of wild-type T antigen on the beads. The amount of mutant T antigen on the beads was determined and used to calculate specific binding capacities of these proteins (Table 2).

When using this kind of assay it is important to determine that (i) only origin-containing fragments are retained on the beads, (ii) the retention of the origin DNA is T antigen dependent, and (iii) components of the infected cell lysate do not interfere with the assay. These features of the assay are demonstrated in the experiment depicted in Fig. 3. In addition, this experiment shows that nearly all of the origin-containing DNA fragment can be quantitatively and selectively retained on the beads by T antigen.

Our results show that the portion of T antigen required for origin binding differs from that required for ATPase activity. T antigens from mutants *dl*1140, *dl*1066, *dl*1061, *dl*1058, and *dl*1055 bound origin-containing DNA as efficiently as wild-type T antigen. The remaining mutant T antigens (from *dl*1047, *dl*1151, *dl*1138, and *dl*1135) showed little or no origin binding activity. The origin binding region is therefore defined by the peptide sequence shared by *dl*1055 T antigen and D2 T antigen (residues 83 to 399) because both retain wild-type origin binding activity. Deletions in this region (e.g., *dl*1047) eliminate the ability of T antigen to bind to the SV40 origin. Deletions C-terminal to this region do not abolish the activity but do, however, appear to affect the stability of the origin binding activity in vitro. Beads prepared from *dl*1055, *dl*1058, and *dl*1061 extract showed high capacity for origin binding only when assayed within a few hours of washing, diminishing severalfold relative to wild-type SV40 after 24 to 48 h at 0°C. The binding capacities of the *dl*1066 and *dl*1140 T antigens, however, were as stable as that of the wild-type protein.

It is generally believed that the binding of T antigen to DNA near the origin of viral DNA replication is required for the repression of early transcription in vitro and in vivo; moreover, it has been suggested that the binding itself might cause repression by hindering the binding of transcriptional proteins (41). Our results suggest that three of the mutants described here (*dl*1055, *dl*1058, and *dl*1061) fail to repress synthesis of early proteins, despite the fact that they encode a large T antigen that is capable of origin binding. We propose three possible explanations, as follows. First, the observed decrease in stability of the mutant T antigen may result in an intracellular concentration that is too low to bind enough of the DNA template to repress early viral transcription efficiently. Second, the mutant protein may bind abnormally within the origin region; for example, it might be able to bind only site I but not site II, a deficiency that would not be detected in the binding assay used here. There is, in fact, some evidence that site I binding is not sufficient to repress early transcription, and thus binding to sites II, III, or both may also be required (41; D. Rio and R. Tjian, unpublished data). Finally, the mutant protein might bind normally but lack another activity that is required for autoregulation. These alternatives could be distinguished by more extensive in vitro binding studies and by testing the ability of the mutant T antigens to repress transcription in vitro.

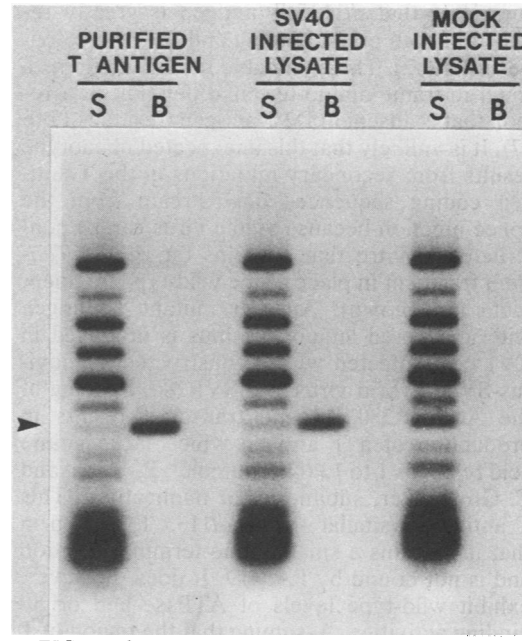

FIG. 3. Selective binding of origin-containing DNA to immobilized T antigen. T antigen from cell lysates (SV40 and mock infected) or purified D2 T antigen was coupled to beads by using monoclonal antibody PAb205 (3C5 in reference 29). The beads (0.5 μl, settled volume) were incubated with 50 ng of ^{32}P end-labeled SV40 DNA (digested with *Bst*NI) in 10 μl of DB buffer. The D2T and SV40 beads contained approximately 0.2 μg of T antigen. After the standard wash procedure, DNA fragments were eluted from the beads with 2% SDS in 10% sucrose and subjected to electrophoresis on a 1.7% agarose slab gel (lanes B) along with 5 μl of supernatant from the binding reaction (lanes S). The autoradiograph shown was exposed to the gel for 48 h (Kodak XR film). The arrow indicates the position of the origin-containing *Bst*NI G fragment.

The observation that D2 T antigen, which lacks the amino-terminal portion of SV40 T antigen (residues 1 to 82), exhibits origin binding and ATPase activities similar to those of wild-type T antigen, indicates that this region is not required for these activities (40, 66). Thus it is

surprising that *dl*1135 T antigen is greatly reduced in both origin binding and ATPase activities (Table 2). This nonviable mutant contains a small in-frame amino-terminal deletion in a region that is absent in D2 T antigen (residues 17 to 27). It is unlikely that this unexpected phenotype results from secondary mutations in the T antigen coding sequences downstream from the splice junction because viable virus can be constructed in vitro that contains the *dl*1135 *Taq-Bam* fragment in place of the wild-type fragment (data not shown). Another mutant T antigen with an altered amino terminus is produced in CV1 cells infected with a constructed adenovirus-SV40 hybrid virus, Ad-SVR280. Splicing of the Ad-SVR280 *A* gene transcript results in production of a T antigen which lacks amino acid residues 1 to 13 (C. Thummel, R. Tjian, and T. Grodzicker, submitted for publication). This T antigen is similar to the *dl*1135 T antigen in that it contains a small amino-terminal deletion and is not bound by PAb419. It does, however, exhibit wild-type levels of ATPase and origin binding activities. Assuming that the region of T antigen encoded by the first exon of the *A* gene (residues 1 to 82) is not specifically involved in either the ATPase or origin binding activities of T antigen, it appears likely that the conformation of the region encoded by the second *A* gene exon (residues 83 to 708) is somehow disrupted in *dl*1135 by its interactions with the altered T-antigen amino-terminal region.

DISCUSSION

In this study we have examined the relationship between the biochemical properties and the primary structure of SV40 large T antigen. Our goal has been to determine whether the ATPase and origin binding activities of T antigen can be attributed to separate functional domains, and to determine the biological functions of these activities. Our strategy has been to compare the activities and functions of mutant T antigens encoded by a set of SV40 deletion mutants that have been characterized for structure and various T antigen-related functions. This approach has taken advantage of the availability of monoclonal antibodies directed against T antigen that can be used to isolate biochemically active T antigen.

We have determind that the amino acid residues required for specific binding to the origin of viral DNA replication are encoded within the region of the SV40 genome between 0.35 and 0.54 map units. In contrast, three or more sites in the region of T antigen encoded between 0.2 and 0.42 map units are involved in the ATPase activity. It is not yet clear whether residues between 0.42 and 0.54 are also required for ATPase activity. It should be pointed out that portions of the regions required for both activities may be involved in maintaining an essential protein conformation or in promoting a required post-translational modification rather than in direct interaction with DNA or ATP. The amino-terminal 82 residues, which are shared with small t antigen, are not specifically required for either the origin binding or ATPase activities, but alteration of this region (*dl*1135) can strongly inhibit both activities, perhaps by altering the tertiary structure of the protein. These results do not exclude the possibility that separate domains of T antigen are independently responsible for the ATPase and origin binding activities. It is equally likely, however, that the folding of the peptide chain in native T antigen results in an interdependence of the two activities.

The *A* gene mutants used in this study have been tested for their ability to perform several T antigen-dependent biological functions (46, 56). To learn more about how T antigen carries out these functions, we have correlated the biological properties of the mutants with the biochemical activities of the mutant T antigens they produce. These correlations are summarized in Table 3. The most dramatic positive correlation is between ATPase activity and three biological functions of T antigen: transformation, viral DNA replication, and viability, suggesting that the ATPase activity is involved in all three of these functions. The presence of origin binding activity also correlates with these biological properties; however, three mutant T antigens (from *dl*1055, *dl*1058, and *dl*1061) that retain the ability to bind origin-containing DNA appear to lack all of these functions, indicating that the origin binding activity is not sufficient to support these T antigen-dependent phenomena.

None of the mutant T antigens described here differentiates between viral DNA replication and cellular transformation functions. The SV80 T antigen, which is a prouct of an SV40-transformed human cell line, appears to lack the ability to support viral replication while retaining its transforming function (W. Gish and M. Botchan, personal communication). As we have shown previously, the SV80 T antigen has normal levels of ATPase activity but is highly deficient in origin binding activity (40, 66). This suggests that the ATPase activity may be involved in the transformation function, but that the origin binding activity is not.

The three remaining functions that have been studied, activation of silent rRNA genes, T antigen nuclear fluorescence, and stimulation of host cell DNA synthesis, do not appear to correlate with either ATPase or origin binding activity (46, 56). This observation indicates that neither activity is involved in these three func-

TABLE 3. Correlation of biochemical and biological properties of mutant T antigens

Activity or function	T antigen from mutant:									
	*dl*1140	*dl*1066	*dl*1061	*dl*1058	*dl*1055	*dl*1151	*dl*1047	*dl*1138	*dl*1137	*dl*1135
Origin binding	+	+	+	+	+	−	−	−	−	−
ATPase	+	+	−	−	−	−	−	−	−	−
Viral DNA replication[a]	+	+	−	−	−	−	−	−	−	−
Transformation[a]	+	+	−	−	−	−	−	−	−	−
Viability[a]	+	+	−	−	−	−	−	−	−	−
Activation of rRNA synthesis[b]	+	+	+	+	−	−	−	−	−	+
Stimulation of cell DNA synthesis[b]	+	+	+	+	+	+	+	−	−	+
T antigen nuclear fluorescence[a]	+	+	+	+	+	+	+	−	−	+

[a] Results of Pipas et al. (46).
[b] Results of Soprano et al. (56).

tions and suggests that T antigen may have additional biochemical activities that have not yet been detected.

To define further the relationship between protein structure, biochemical activities, and biological functions of SV40 T antigen, analysis of a wider range of *A* gene mutants, including point mutants, will be necessary. We also plan to examine additional aspects of T antigen structure and function such as 53,000-dalton protein binding and post-translational modification.

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