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Genetics

Intracistronic complementation in the simian virus 40 *A* gene

(deletion mutant/large tumor antigen)

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ABSTRACT A set of eight simian virus 40 mutants was constructed with lesions in the *A* gene, which encodes the large tumor (T) antigen. These mutants have small deletions (3–20 base pairs) at either 0.497, 0.288, or 0.243 map units. Mutants having both in-phase and frameshift mutations at each site were isolated. Neither plaque formation nor replication of the mutant DNAs could be detected after transfection of monkey kidney cells. Another nonviable mutant, *dl*A2459, had a 14-base-pair deletion at 0.193 map unit and was positive for viral DNA replication. Each of the eight mutants was tested for ability to form plaques after cotransfection with *dl*A2459 DNA. The four mutants that had in-phase deletions were able to complement *dl*A2459. The other four, which had frameshift deletions, did not. No plaques were formed after cotransfection of cells with any other pair of group A mutants. This suggests that the defect in *dl*A2459 defines a distinct functional domain of simian virus 40 T antigen.

Simian virus 40 (SV40) is a small DNA tumor virus belonging to the papovavirus group (see ref. 1 for review). Its genome is a 5,243-base-pair (bp) double-stranded covalently closed circular DNA molecule and contains six genes. The tumor antigens [large (T) and small (t)] are encoded on the early strand (Fig. 1) and are expressed throughout the viral infection. The three capsid proteins (VP1, VP2, and VP3), as well as the agnoprotein (2), are encoded on the late strand and are expressed late in the lytic cycle, after onset of viral DNA replication. When nonpermissive cells are infected by SV40, only the early genes are expressed, resulting in the malignant transformation of a small percentage of the cells (3, 4).

The use of deletion and temperature-sensitive mutants has allowed the assignment of several functions to the T antigen. Among these are stimulation of host DNA synthesis (5), initiation of viral DNA synthesis (6), autoregulation of early mRNA synthesis (7–9), adenovirus helper function (10–12), and both initiation and maintenance of the transformed state in nonpermissive cells (3, 4). An ATPase activity (13, 14) and specific DNA binding activities (15, 16) of T antigen have also been demonstrated *in vitro*.

Because T antigen is a multifunctional protein, it may contain multiple functional domains. Temperature-sensitive mutants have been useful in the identification of functions associated with T antigen but, because these mutations lead to complete loss of function at the nonpermissive temperature, they provide little information about the domains of T antigen. Some of the functions of T antigen have been mapped by using deletion mutants (17), adenovirus type 2-SV40 hybrids (18), and monoclonal antibodies (14).

To map the functional domains of T antigen, we constructed a set of mutants with small deletions (3–20 bp) at the *Dde* I sites in the *A* gene (Fig. 1). We have described the properties of mutants with deletions at 0.219, 0.203, and 0.193 map unit (m.u.), near the COOH-terminus of T antigen (19). One of these, a nonviable mutant with a deletion of 14 bp at 0.193 m.u. (*dl*A2459), is positive for viral DNA replication. This mutant defines a function for T antigen that is required after the onset of viral DNA replication. We describe here the isolation and initial characterization of mutants with small deletions at either 0.243, 0.288, or 0.497 m.u. Mutants with both in-phase and frameshift deletions at each site were isolated. All of these mutants were nonviable and were negative for viral DNA replication. All mutants with in-phase deletions, but not those with frameshift deletions, complemented *dl*A2459, resulting in plaque formation.

MATERIALS AND METHODS

Cells and Viruses. CV-1 and CV-1p cells, continuous lines of African green monkey kidney cells, were cultured as described (20). The small-plaque strain (SV-S) of SV40 was used as wild type (21). Mutant *dlBC865* has a deletion at the *Eco*RI site (22) and fails to produce VP1. Mutant *dlA1209* (33) has a deletion of 329 bp (unpublished results) between 0.650 and 0.587 m.u. and does not produce T antigen.

Plasmids and Bacteria. All recombinant DNA plasmids were maintained in the HB101 strain of *Escherichia coli*. Previously described methods were used for bacterial transformations (24) and for preparation of minilysates (25) and maxilysate DNA (26). Cesium chloride gradients containing ethidium bromide at 300 μg/ml were used to purify all DNA preparations. DNA for sequence analysis was purified through two cesium chloride/ethidium bromide gradients. Mutants *dlBC865* and *dlA1209* were cloned into the *Bam*HI site of pBR322.

Preparation of Mutants. Plasmid pCC2, a recombinant DNA clone containing the complete SV40 genome inserted at its *Eco*RI site into the *Eco*RI site of pBR322, was the starting material for these experiments. Form I plasmid DNA was digested with *Dde* I in the presence of ethidium bromide at 180 μg/ml at 37°C for 45 min (27, 28). Under these conditions, most molecules were cleaved once. After phenol extraction and ethanol precipitation, the DNA was suspended in nuclease S1 buffer (50 mM NaOAc/150 mM NaCl/1 mM $ZnSO_4$) and digested with nuclease S1 under conditions that allowed limited removal of nucleotides (3–25 bases) from each terminus (29). The treated DNA was subjected to electrophoresis through a 1% agarose gel in TBE buffer (89 mM Tris base/89 mM boric acid/2.5 mM EDTA, pH 8.3). The largest class of linear molecules was iso-

Abbreviations: bp, base pair(s); m.u., map unit; SV40, simian virus 40; T and t antigen, SV40 large and small tumor antigen, respectively; pfu, plaque-forming unit(s).

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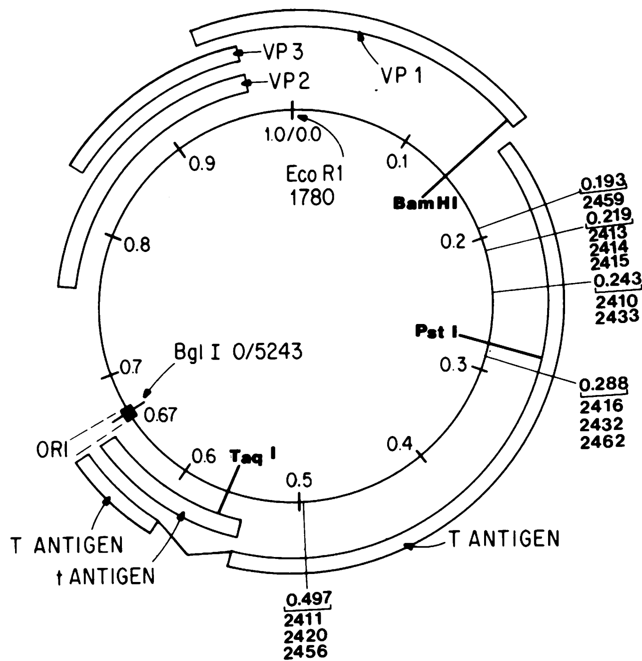

FIG. 1. The SV40 genome. Map coordinates are expressed as the fractional length of wild-type SV40 where 0.00 is the map position of the single *Eco*RI site. Also shown are the locations of the coding regions for the viral gene products and the *Dde* I sites absent in deletion mutants described in this report.

lated by electroelution. This DNA was ligated at a concentration of 5 μg/ml, to form circular molecules, and used to transform *E. coli*. Minilysate DNAs were digested with *Dde* I to determine the deletion site.

Plaque Assays. Mutant DNAs were separated from pBR322 DNA by digestion with *Eco*RI (or *Bam*HI in the case of *dl-BC865* and *dlA1209*) and ligated at low concentration (5 μg/ml) with T4 DNA ligase. Agarose gel electrophoresis was used to monitor the completeness of the digestion and ligation reactions. The mutant DNAs were used to infect CV-1p cells as described (20). The cells were stained with neutral red after 10–14 days and scored for plaques.

Analysis of Viral DNA Replication. The mutant DNAs were introduced into CV-1 cells using DEAE-dextran (500 μg/ml). After a 45-min incubation period at 37°C, cells were washed once with Tris-buffered saline and then fed with 10 ml of Dulbecco's modified Eagle's medium (DME medium)/2% fetal calf serum/100 μM chloroquine phosphate (30) per 100-mm plate for 4 hr. After this treatment, chloroquine-containing medium was replaced with DME medium/2% fetal calf serum. This treatment resulted in infection of 50–60% of the cells, estimated by immunofluorescence staining for T antigen (unpublished results). Viral DNA was then extracted and analyzed as described (20).

Marker Rescue by Fragment Substitution. The locations of two deletions were confirmed by replacement of a portion of the mutant viral genomes with the corresponding portion of wild-type SV40 DNA. After gel purification, DNA fragments were ligated with T4 DNA ligase and tested for plaque-forming ability in CV-1p cells.

Chemical and Enzymes. Restriction endonucleases and DNA polymerase I were purchased from New England BioLabs; T4 DNA ligase, from Collaborative Research (Waltham, MA) or New England BioLabs; nuclease S1, from Boehringer Mannheim; proteinase K, from Beckman Instruments (Fullerton, CA); chloroquine phosphate, from Sigma; and all radiochemicals, from Amersham.

RESULTS

This report describes the initial characterization of eight nonviable mutants of SV40 (Table 1). Three had deletions at 0.497 m.u., 3 had deletions at 0.288 m.u., and 2 had deletions at 0.243 m.u. No viable mutants that had deletions at these sites were isolated.

Characterization of the Mutants. Mutant DNAs were subjected to DNA sequence analysis. The results are summarized in Fig. 2. All of the mutants had deletions of 3–20 bp. In-phase and frameshift mutations were isolated at each of the three sites. The predicted amino acid sequence for each mutant T antigen is also shown. Four of the mutants had deletions that were multiples of 3 bp, leaving the T antigen reading frame intact. The other four had frameshift mutations that should cause termination of translation.

These predictions were confirmed by analyzing the size of each mutant T antigen by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 3). The four mutants with in-phase deletions, *dl-2411*, *dl-2433*, *dl-2432*, and *dl-2462*, produced T antigens that migrated to approximately the same position as wild type T antigen (M_r 90,000). In several different experiments, the T antigen produced by *dl-2433* was barely detectable, suggesting that it is unstable. Truncated T antigen polypeptides of the predicted sizes were produced in cells infected by *dl-2420* (M_r 18,000), *dl-2456* (M_r 18,000), and *dl-2410* (M_r 72,000). The fragments produced by *dl-2420* and *dl-2456* comigrate with t antigen. The T antigen produced by *dl-2416* was probably unstable, since no polypeptide of the predicted size (M_r 68,000) was observed.

Plaque assays of each of the mutant DNAs indicated that all were nonviable. We tested the ability of each mutant to complement both a mutant defective for capsid protein VP1 (*dl-BC865*) and a mutant defective for production of T antigen (*dlA1209*). All eight mutants formed plaques when plated with *dlBC865* DNA, while none were able to complement *dlA1209*. Therefore, all belonged to the A complementation group (Table 1).

Intracistronic Complementation. Genetic complementation analyses were also done with pairs of nonviable A group deletion mutants. We tested 10 mutants: the 8 described above, *dlA2459* (a 14-bp deletion at 0.193 m.u.), and a mutant with a 22-bp deletion at 0.219 m.u. (*dlA2413*; ref. 19). Plaques were observed when each of the four mutant DNAs with in-phase deletions (*dlA2411*, *dlA2432*, *dlA2433*, and *dlA2462*) was plated with *dlA2459* DNA (Table 1). Approximately the same level of complementation [plaque-forming units (pfu)] was obtained with

Table 1. SV40 mutants with deletions at *Dde* I sites

Mutant	*Dde* I site deleted, m.u.	Deletion size, bp	Plaques formed with *dlA2459** (pfu/μg)
dl-2411	0.497	12	5×10^4
dl-2420	0.497	20	0
dl-2456	0.497	17	0
dl-2416	0.288	10	0
dl-2432	0.288	12	2×10^4
dl-2462	0.288	3	2×10^4
dl-2410	0.243	10	0
dl-2433	0.243	9	4×10^3
dl-2413	0.219	22	0
dl-2459	0.193	14	

All mutants were of complementation group A. Viability and complementation analyses were done in CV-1p cells by using plaque assays. All mutants formed $>1 \times 10^3$ pfu/μg when plated with 10 ng of *dlBC865* DNA. No plaques were formed when mutants were plated with 10 ng of *dlA1209* DNA.

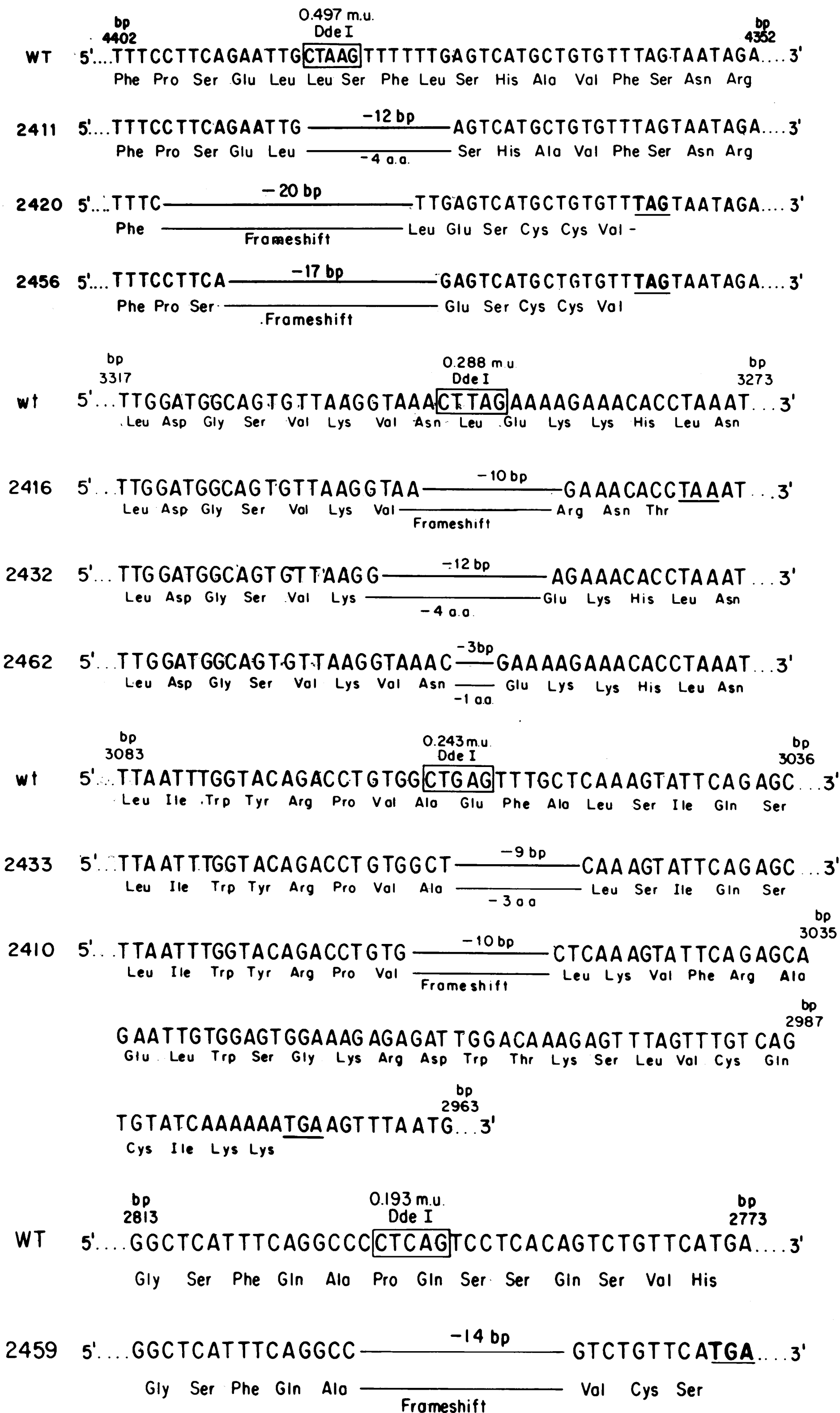

FIG. 2. Nucleotide sequences of deletion mutants. The nucleotide sequence was determined for each of the mutants by the chemical method of Maxam and Gilbert (31). The amino acid sequences of the mutant T antigens, inferred from the DNA sequences, and for wild-type T antigen, are also shown.

either *dlA2459* or *dlBC865*. Therefore, the efficiency of intracistronic complementation was comparable with that observed for intercistronic complementation. None of the mutants with frameshift deletions was able to complement *dlA2459* for plaque formation. No complementation occurred when other pairs of A mutants were tested.

Analysis of Viral DNA Replication. All nonviable mutants were tested for viral DNA replication. No progeny DNA was detected in cells transfected by *dlA2411* (Fig. 4) or any other nonviable mutant DNAs (data not shown). This analysis would have been able to detect a level of replication approximately 1% that of wild-type SV40 DNA. We conclude that the DNA replication function of T antigen is substantially reduced or eliminated by the deletions at 0.243, 0.288, and 0.497 m.u. Approximately 1/4th as much progeny viral DNA is formed in cells transfected with *dlA2459* as in cells transfected with wild-type DNA (19). The level of progeny DNA produced when cells were cotransfected with *dlA2459* and *dlA2433* (or other A group de-

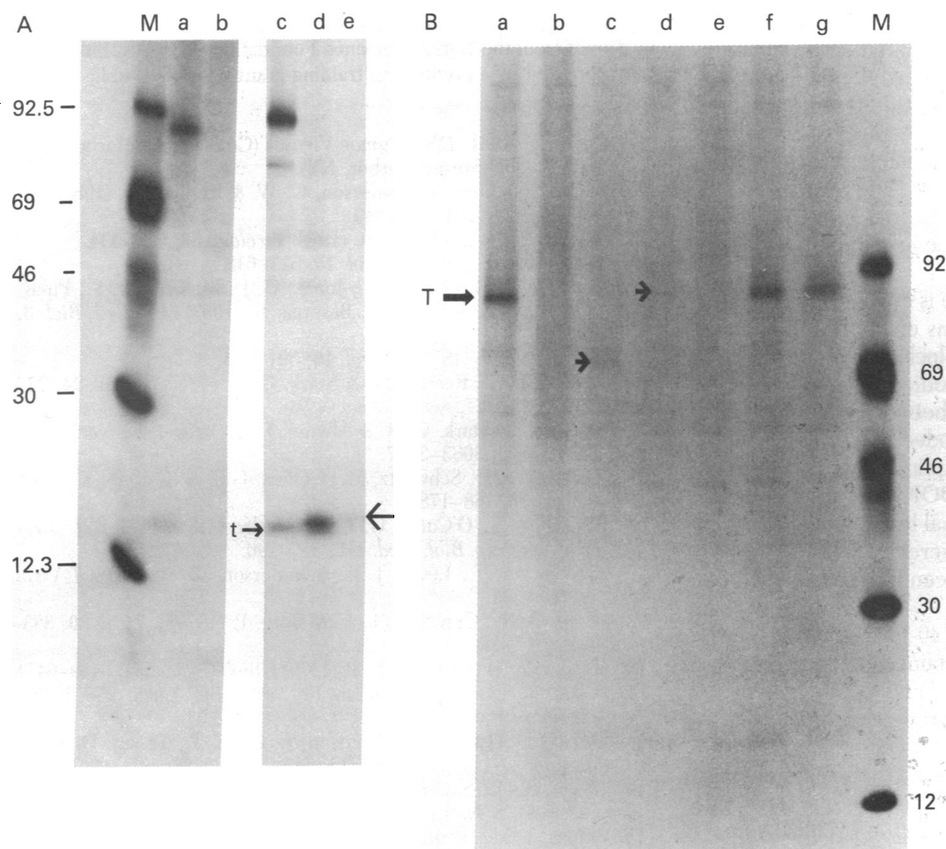

FIG. 3. Autoradiogram of [^{35}S]methionine-labeled T antigens immunoprecipitated from cytoplasmic extracts. CV-1 cells (in 35-mm dishes) were transfected with mutant or wild-type DNAs. Cultures were labeled for 1 hr beginning 30 hr after infection, using 75 μCi of [^{35}S]methionine (1 Ci = 37 GBq) in 0.25 ml of methionine-lacking medium. Extracts were immunoprecipitated, alkylated, and electrophoresed on 7–20% $NaDodSO_4$/polyacrylamide gradient gels. Gels were fixed, fluorographed, dried, and exposed to Kodak XAR-5 film with an intensifying screen for 3 days at −70°C. (*A*) Lanes: M, markers; a, wild-type SV40; b, mock infected; c, *dl-2411*; d, *dl-2420*; e, *dl-2456*. (*B*) Lanes: a, wild-type SV40; b, mock infected; c, *dl-2410*; d, *dl-2433*; e, *dl-2416*; f, *dl-2432*; g, *dl-2462*; M, markers. Numbers on the left and right represent $M_r \times 10^{-3}$.

letion mutants tested) was approximately the same as in cells transfected only with *dlA2459* (data not shown).

Marker-Rescue Analysis. To show that the complementation was due to an interaction between mutants whose only lesions were in the *A* gene, marker-rescue experiments were conducted on one complementing pair of mutants. When the *Taq* I (0.564 m.u.)–*Nde* I (0.391 m.u.) fragment from *dlA2411* DNA was replaced with the same fragment from wild-type DNA, plaque-forming DNA resulted. Similarly, plaque-forming viral DNA was created by the replacement of the *Bam*HI (0.143 m.u.)–*Pst* I (0.271 m.u.) fragment from *dlA2459* DNA with the analogous wild-type DNA fragment. In both cases, no plaques were obtained with mutant DNA, while the reconstructed DNA produced more than 5×10^4 pfu/μg. This indicated that the lesion in *dlA2411* was confined to the region of the *A* gene between 0.535 m.u. (the acceptor splice junction) and 0.391 m.u. Because *dlA2459* complements *dlBC865*, it must retain a functional *BC* gene (encoding VP1) and hence does not have a lethal lesion in the portion of the rescuing fragment that encodes the COOH-terminus of VP1 (0.143–0.154 m.u.). Therefore, the lesion in *dlA2459* is located between 0.271 m.u. and the 3′ end of the *A* gene (0.174 m.u.). DNA sequence analysis of the region surrounding the deletion did not reveal any lesion other than the deletion described (Fig. 2).

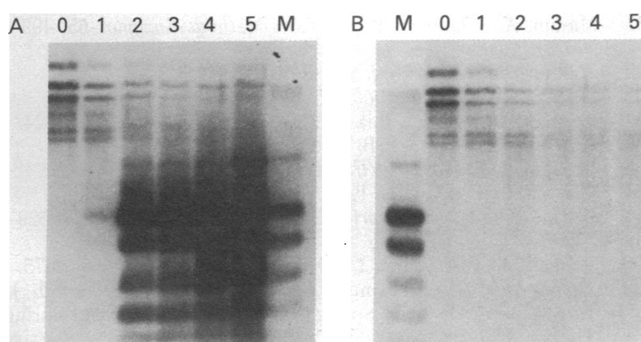

FIG. 4. Autoradiograms of Southern blots (32) showing DNA replication in cultures of CV-1 cells transfected with wild-type (*A*) or *dlA2411* (*B*) DNA. Hirt extracts (33) were prepared 0, 1, 2, 3, 4, and 5 days (lanes 0–5) after transfection. Each sample was digested with *Mbo* I, which will digest only progeny DNA. The marker lanes (lanes M) contained 20 ng of *Mbo* I-digested wild-type SV40 DNA. The multiple bands seen at the top of the gel represent input SV40 and pBR322 DNA.

DISCUSSION

Intracistronic complementation has been observed between nonviable mutants of SV40 that contain deletions in the *A* gene. Complementation occurred between *dlA2459*, which has a 14-bp deletion at 0.193 m.u. (19), and all other group A in-phase deletion mutants that were tested. The complementing mutants have deletions at either 0.497, 0.288, or 0.243 m.u. Since mutants that contained frameshift deletions at the same three sites did not complement *dlA2459* (Table 1), recombination between mutant viral genomes cannot explain these results. We have also observed complementation between *dlA2459* and a variety of other *A* gene mutants (unpublished results). These include (*i*) three nonviable point mutants that have base changes near 0.52 m.u. (provided by D. Kalderon and A. Smith); (*ii*) *dlA1135* (17), which has a 36-bp deletion at map position 0.63; (*iii*) *tsA1642*, a temperature-sensitive mutant that is positive for viral DNA replication and has a single-base change near map position 0.31 (34); and (*iv*) a series of transformation-positive replication-negative SV40 mutants isolated by cloning the integrated viral genome from transformed cells (35–37). Most members of this last group of mutants have single-base alterations (35, 36), but one has an in-phase deletion of 219 bp between 0.47 and 0.51 m.u.

Intracistronic complementation was first observed between

β-galactosidase mutants in the *lac* operon of *E. coli* (38). This protein is divided into three regions, designated α, β, and ω. A defective β-galactosidase protein that has a deletion in the α region can be complemented by a polypeptide fragment that contains a complete α region. Kinetic studies suggest that the peptide fragment causes a conformational change that converts an inactive dimer to an active tetramer (39). The resultant enzyme has the same K_m as the wild-type enzyme (39). Complementation also occurs between a wild-type ω polypeptide fragment and ω mutants (40).

T antigen is multifunctional, and there is evidence that some of its properties require different regions of the protein. The ATPase activity of T antigen can be blocked specifically by monoclonal antibodies (14). These antibodies recognize determinants that are encoded by sequences between 0.27 and 0.33 m.u. (14). The portion of T antigen encoded by sequences between 0.43 and 0.50 m.u. has been implicated in the origin-binding property (15, 18, 28, 41). The COOH-terminal 38 amino acids of T antigen are sufficient for normal levels of adenovirus helper function (11, 12, 20, 23, 42). Therefore, at least three separate properties of T antigen have been localized to different regions of the polypeptide.

When T antigen is extracted from SV40-infected cells, both monomeric and multimeric forms are obtained (43). There is no evidence to indicate which form is active *in vivo*. T antigen may be fully functional as a monomer. A T-antigen monomer that is defective in one function might retain other essential functions. Mutant T antigens with lesions in separate functional domains would then complement each other by carrying out separate functions independently. *dlA2459* expresses the viral DNA replication function of T antigen, but is defective for a late function of it (19). If this late function is provided by monomer T antigen, the defect in *dlA2459* defines a separate functional domain. All mutants that complement *dlA2459* must contain this domain.

Alternatively, T antigen may function as a multimer. Two different mutant T antigens could complement each other in one of the following ways. (*i*) Each mutant T antigen forms separate multimers that carry out separate functions independently. Mixed multimers could exist in co-infected cells but would not be required. According to this explanation, the defect in *dlA2459* again defines a separate functional domain. (*ii*) Homogeneous multimers are partially active or inactive. Only mixed multimers would express all of the functions of T antigen. These multimers could have regained activity due to conformational changes, as was observed for β-galactosidase (39). In this case, the defect in *dlA2459* might not define a separate functional domain.

Mutant *dlA2459* is positive for viral DNA replication (19). Although none of the mutants that complemented *dlA2459* was replication competent, each was capable of supplying the function defective in the T antigen produced by *dlA2459*. All retained that portion of T antigen missing in *dlA2459* T. The simplest explanation for these observations is that the defect in *dlA2459* defines a distinct functional domain of T antigen. It is interesting to note that the portion of T antigen affected by the deletion in *dlA2459* is in the only part of SV40 T antigen showing no homology to polyoma T antigen (44). The observed intracistronic complementation is consistent with T antigen functioning as either a monomer or as a multimer.

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