Characterization of a New Simian Virus 40 Mutant, *tsA3900*, Isolated From Deletion Mutant *tsA1499*

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The simian virus 40 (SV40) mutant *tsA1499* contains an 81-base-pair deletion in the region of *A* gene encoding the C-terminal portion of the large *T* antigen. This mutant is particularly interesting, since it is a temperature-sensitive mutant that is apparently able to separate the lytic growth and transforming functions of the SV40 large *T* antigen at 38.5°C. We report the isolation of a *tsA1499* revertant (*tsA1499-Rev*) which is no longer temperature sensitive for lytic growth but still contains the 81-base-pair deletion of *tsA1499*. Marker rescue experiments with *tsA1499-Rev* or wild-type strain 830 (wt830) DNAs revealed that the original *tsA1499* mutant contained a second mutation within the *HindIII-Fnu4HI* restriction fragment between 0.425 and 0.484 map units. Sequencing of this DNA fragment from the *tsA1499*, *tsA1499-Rev*, and wt830 viruses revealed that *tsA1499* contained a single-base transversion (A to G) at 0.455 map units (nucleotide 4261). This transversion resulted in the creation of a new *RsaI* cleavage site in the *tsA1499* DNA and predicts an arginine-to-threonine substitution at amino acid position 186 in the mutant large *T* antigen. The DNA sequence of the *tsA1499-Rev* *HindIII-Fnu4HI* fragment was identical to that of wt830. To determine whether *tsA1499* was temperature sensitive for lytic growth solely as a result of the newly discovered point mutation or because of a combination of the point and deletion mutations, a series of viruses were constructed which contained the point mutation, the deletion mutation, both mutations, or neither. This was done by ligating the *PstI* A and B DNA fragments from either *tsA1499* or wt830 and transfected the ligated DNA into BSC-1H monkey kidney cells. This experiment revealed that all viruses containing the point mutation (the *tsA1499 PstI A* DNA fragment) were temperature sensitive for lytic growth, regardless of the presence of the 81-base-pair deletion (the *tsA1499 PstI B* DNA fragment). This newly discovered point mutation, at nucleotide 4261, is therefore unique, since to our knowledge it is the first *tsA* mutation to be described in the 0.455-map-unit region of the SV40 genome. We then tested the effect of this unique mutation on the ability of the SV40 virus to transform cultured rat cells to anchorage independence. We found that a mutant virus that contained only the point mutation, *tsA3900*, was able to transform F111 rat cells to anchorage independence equally well at the permissive and nonpermissive temperatures for lytic growth with an efficiency of transformation similar to that of a wild-type strain of SV40 (wt830). Since the *tsA* lytic phenotype of *tsA3900* is tight and the anchorage independence assay for transformation is stringent, the apparent separation of the lytic growth and transforming functions of *tsA3900* is striking.

The large *T* antigen, encoded by the simian virus 40 (SV40) *A* gene, is a multifunctional protein that is required for lytic growth of the virus in permissive cells as well as for the neoplastic transformation of nonpermissive cells (38). Several biological and biochemical activities associated with large *T* antigen include binding to the SV40 origin of replication (19, 30, 42, 44, 55), induction of SV40 DNA replication (52), stimulation of cellular DNA synthesis (5, 11, 17, 18, 45, 49, 56), autoregulation of SV40 early gene transcription (1, 20, 43, 54), increased host transcription (28, 38, 50), adenovirus helper function (7, 14, 21, 40), ATPase activity (6, 12, 57), and initiation and maintenance of transformation in nonpermissive cells (3, 22, 26, 35, 53).

In an effort to determine whether specific portions of the large *T* antigen are involved with specific functions, numerous mutants with spontaneously derived or biochemically engineered lesions in the SV40 *A* gene have been functionally characterized and physically mapped. The results indicate that large *T* antigen does indeed contain several functional domains (for a partial summary, see Rawlins et al. [41]).

One particular deletion mutant, *tsA1499*, appeared to separate the lytic and transforming functions of SV40 (36, 37). This mutant contains an 81-base-pair deletion around 0.21 map units (mu), which is in the region of the *A* gene encoding the C-terminal portion of the large *T* antigen. At 38.5°C, *tsA1499* is a tight temperature-sensitive (*ts*) mutant for lytic growth; however, it is able to transform F111 rat cells to anchorage independence at the nonpermissive temperature for lytic growth (36, 37). In addition, clones of *tsA1499*-transformed F111 cells appeared to be more cold sensitive for the maintenance of anchorage independence at 32°C than did another *tsA* mutant (*tsA58*) and a wild-type strain (wt648) of SV40 (36). We report the isolation of a spontaneous revertant of *tsA1499*, *tsA1499-Rev*, which is able to productively infect permissive cells at 38.5°C. Marker rescue experiments with *tsA1499-Rev* or wt830 DNAs revealed that the original *tsA1499* mutant contained a second mutation between 0.425

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and 0.484 mu. In subsequent experiments, the precise nature of this mutation was determined by DNA sequencing and its effects on the lytic growth and transforming phenotypes of SV40 were determined.

MATERIALS AND METHODS

Cell lines and viruses. CV-1P or BSC-1H monkey kidney cell lines were used for the growth and titrating of all virus stocks. These cells were routinely grown in Dulbecco modified Eagle medium (DME) supplemented with 10% donor calf serum. Complementation assays and growth and titrating of viruses were performed in DME medium supplemented with 2% donor calf serum as described previously (31). F111 Fisher rat cells were used in the transformation assays. These cells (before passage 25) were grown in DME supplemented with 10% fetal bovine serum.

The deletion mutant tsA1499, derived from the wild-type strain wt830, has been described by Pintel et al. (37). A spontaneous revertant of tsA1499, namely tsA1499-Rev, was isolated as described below. The mutant tsA3900 was derived from tsA1499 and is described below.

Recombinant plasmids. Viral DNA was prepared by the method described by Hirt (16) and purified from CsCl-ethidium bromide gradients.

In several of the experiments to be described, cloned restriction DNA fragments were used as source of wt830, tsA1499, or tsA1499-Rev DNAs. Table 1 provides a brief list of each cloned DNA fragment, the plasmid vector into which each fragment was cloned, and the abbreviated designation assigned to each recombinant plasmid. The plasmids pBR322 and pML have been described previously (2, 24).

The ligation of each SV40 DNA fragment into its plasmid vector was performed with T4 ligase. After ligation, each DNA mixture was used to transform Escherichia coli HB101 cells, which were then screened for antibiotic resistance.

For extraction of plasmid DNAs, plasmid-containing bacteria were grown in ampicillin- or tetracycline-containing minimal growth medium. When bacterial growth reached mid-log phase, chloramphenicol was added to the suspension. The cultures were incubated for an additional 15 to 20 h at 37°C with gentle aeration. The bacteria were then ice chilled, concentrated, and lysed with lysozyme and a nonionic detergent. The plasmid DNAs were then purified from the resultant lysate by CsCl-ethidium bromide gradients.

Marker rescue experiments. Marker rescue experiments were performed by the method of Lai and Nathans (23). When cloned DNA fragments were used to rescue tsA1499 at 38.5°C, the plasmid DNA was first digested with the appropriate restriction enzyme (Table 1) to release the SV40 DNA segment from the plasmid vector. When individual restriction enzyme fragments, cut from either viral DNA or larger cloned DNA fragments, were used the individual DNA fragments were first isolated at least twice from agarose gels, either by electroelution or by extraction from low-melting agarose.

DNA transfections. For lytic growth experiments, DNA transfections were performed by the DEAE-dextran method (29). The concentration of DEAE-dextran was 400 μg/ml in DME medium, with 100 μg each of penicillin and streptomycin per ml. The DNA-DEAE-dextran mixture (0.5 ml per 100-mm dish; 0.3 ml per 60-mm dish) was incubated with subconfluent BSC-1H cells for 15 min at 37°C.

For transformation experiments, DNA transfections were performed by the calcium-phosphate coprecipitate technique (60) with sheared F111 DNA as carrier.

DNA sequencing. DNA sequencing was performed by the method described by Maxam and Gilbert (27). The DNA fragments sequenced are described in the text. The mutant DNA was isolated from the plasmid 1499-pML. The wild-type DNA was isolated from the plasmid wt-PA-pBR. The revertant DNA was isolated from the plasmid Rev-TA-pML (Table 1).

The results are expressed by the SV40 nucleotide numbering system described previously (58).

Anchorage independence transformation assay. This transformation assay was based on the assay developed by Macpherson and Montagnier (25) and was performed as described previously for viral transformations (36) and DNA transfections (4). The percentage of agar used to suspend the F111 cells was 0.31%.

![FIG. 1. Restriction enzyme digests of tsA1499-Rev viral DNA.](http://jvi.asm.org/Downloaded from http://jvi.asm.org)
RESULTS

Revertant genome contains an 81-base-pair deletion. The SV40 marker, tsA1499 contains an 81-base-pair deletion around 0.21 mu (36, 37) and is a tight ts mutant for lytic growth at 38.5°C. A revertant of tsA1499 was selected by plating plaque-purified tsA1499 on BSC-1H cells and incubating these cultures at 38.5°C for 2 to 3 weeks. The virus isolated at 38.5°C (tsA1499-Rev) was plaque purified twice more and used to infect BSC-1H cells at 38.5°C. Titers of up to \(2 \times 10^8\) PFU/ml of lysate were obtained at the originally nonpermissive temperature for tsA1499.

To demonstrate that this tsA1499-Rev was not a wild-type contaminant and still contained the 81-base-pair deletion of the original mutant, viral DNA from tsA1499-Rev was mapped with the restriction enzymes HindIII and HincII. If tsA1499-Rev contained the deletion, then its HindIII A and HincII C fragments should be appropriately smaller than those of the wild type (wt830) and similar to those of tsA1499.

The results of such an analysis are shown in Fig. 1. Note that the revertant still contained the same large deletion in the HindIII A and HincII C fragments. Therefore, tsA1499-Rev probably differs from tsA1499 in some other area of the genome.

Marker rescue experiment with revertant DNA. To determine the region of the tsA1499-Rev genome that was modified to enable it to productively infect monkey kidney cells at 38.5°C, a series of marker rescue experiments were performed. In these experiments, segments of the tsA1499-Rev genome were hybridized to whole, denatured tsA1499 DNA and transfected into BSC-1H cells, which were then incubated at 38.5°C. If a particular DNA segment contained the region of the tsA1499-Rev genome that enabled the revertant to productively infect BSC-1H cells at 38.5°C, a productive infection should result.

To facilitate the separation of individual revertant DNA segments and to prevent low-level cross-contamination of the individual DNA segments, several restriction endonuclease-generated fragments of the tsA1499-Rev genome were cloned into plasmid vectors (Table 1). For the marker rescue experiments, the tsA1499-Rev DNA was released from each plasmid clone by digestion with the appropriate restriction enzyme(s). Once digested, the tsA1499-Rev DNA segments were either hybridized directly to denatured tsA1499 DNA or redigested with additional restriction enzymes to generate smaller DNA segments, which were then isolated and separately hybridized to denatured tsA1499 DNA. The results obtained when these hybridized DNAs were transfected into BSC-1H cells incubated at 38.5°C are shown in Table 2. The smallest tsA1499-Rev DNA fragment that was able to rescue tsA1499 at 38.5°C was the HindIII B-Fnu4HI B fragment located between 0.425 and 0.484 mu on the SV40 physical map (58). Thus, tsA1499-Rev had undergone some change from tsA1499 in this area of the genome that caused it to lose its ts phenotype for lytic growth.

Marker rescue experiments with wt830 DNA. As a control to the above experiment, a similar experiment was performed with restriction DNA fragments from wt830 DNA. Again, cloned PstI A and B DNA fragments (from wt-PA-pBR and wt-PB-pBR) were either used directly for hybridization to tsA1499 or first digested into smaller DNA segments. Only DNA segments derived from wt-PA-pBR were able to rescue tsA1499 (data not shown; for similar data, see Table 3). Although these data coincided well with those from the tsA1499-Rev experiment, it was unexpected because it had previously been believed that the 81-base-pair deletion was responsible for the ts phenotype of tsA1499 (37).

To eliminate the possibility that there was something unusual about the cloned wt830 DNA fragments, another marker rescue experiment was performed with DNA segments isolated directly from wt830 viral DNA. The results are shown in Table 3. Note that tsA1499 was again rescued by the 311-base-pair HindIII B-Fnu4HI DNA fragment located between 0.425 and 0.484 mu. It therefore appears that the original tsA1499 virus had a second mutation between 0.425 and 0.484 mu which was involved in the ts phenotype for lytic growth.

To verify that the viruses produced at 38.5°C were truly derived from marker-rescued tsA1499, viral DNA preparations were made from several plaque-purified viruses and mapped with restriction enzymes to confirm the presence of

### Table 2. Marker rescue results obtained with cloned fragments of tsA1499-Rev DNA

<table>
<thead>
<tr>
<th>Recombinant plasmid</th>
<th>Cloned DNA fragment</th>
<th>Sub-fragment</th>
<th>No. of base pairs</th>
<th>SV40 physical map location (mu)</th>
<th>PFU/ml of lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rev-PA-pBR</td>
<td>PstI A</td>
<td>HindIII B-Fnu4HI A</td>
<td>4,027</td>
<td>0.272-0.039</td>
<td>3.8 \times 10^4</td>
</tr>
<tr>
<td>Rev-PA-pBR</td>
<td>PstI A</td>
<td>HindIII B-Fnu4HI B</td>
<td>858</td>
<td>0.484-0.648</td>
<td>1.6 \times 10^6</td>
</tr>
<tr>
<td>Rev-PA-pBR</td>
<td>PstI A</td>
<td>HindIII B-TaqI A</td>
<td>311</td>
<td>0.425-0.484</td>
<td>1.7 \times 10^6</td>
</tr>
<tr>
<td>Rev-PA-pBR</td>
<td>PstI A</td>
<td>HindIII B-Fnu4HI B</td>
<td>737</td>
<td>0.425-0.566</td>
<td>1.1 \times 10^6</td>
</tr>
<tr>
<td>Rev-TA-pML</td>
<td>HindIII B-TaqI A</td>
<td></td>
<td>432</td>
<td>0.566-0.648</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Rev-TB-pML</td>
<td>HindIII B-TaqI B</td>
<td></td>
<td>1,216</td>
<td>0.039-0.272</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Rev-PB-pBR</td>
<td>PstI B</td>
<td></td>
<td>2,009</td>
<td>0.373-0.74</td>
<td>4.5 \times 10^5</td>
</tr>
</tbody>
</table>

* This titer is due to the cross-contamination of the PstI B fragment with the PstI A fragment.

### Table 3. Marker rescue results obtained with viral or cloned wt830 DNA

<table>
<thead>
<tr>
<th>Viral DNA fragment</th>
<th>No. of base pairs</th>
<th>SV40 physical map location (mu)</th>
<th>PFU/ml of lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>PstI A</td>
<td>4,027</td>
<td>0.272-0.039</td>
<td>2.5 \times 10^4</td>
</tr>
<tr>
<td>PstI B</td>
<td>1,216</td>
<td>0.039-0.272</td>
<td>1.1 \times 10^6</td>
</tr>
<tr>
<td>HindIII B-TaqI A</td>
<td>737</td>
<td>0.425-0.566</td>
<td>&lt;10</td>
</tr>
<tr>
<td>HindIII B-Fnu4HI A</td>
<td>858</td>
<td>0.484-0.648</td>
<td>&lt;10</td>
</tr>
<tr>
<td>HindIII B-Fnu4HI B</td>
<td>311</td>
<td>0.425-0.484</td>
<td>2.3 \times 10^6</td>
</tr>
<tr>
<td>wt-PA-pBR-PstI A</td>
<td>4,027</td>
<td>0.272-0.039</td>
<td>9 \times 10^5</td>
</tr>
<tr>
<td>wt-PB-pBR-PstI B</td>
<td>1,216</td>
<td>0.039-0.272</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* This titer is due to a slight cross-contamination of the PstI B fragment with the PstI A fragment.
of the mutant for lytic growth was due solely to the point mutation or to a combination of the point and deletion mutations. It was therefore necessary to construct a series of viruses that contained only the point mutation, only the deletion mutation, both, or neither and to determine their abilities to productively infect BSC-1H cells at the permissive and nonpermissive temperatures for tsA1499 lytic growth.

Conveniently, the PstI restriction enzyme cuts the SV40 genome into two DNA fragments: the PstI A fragment, which extends from 0.272 to 0.359 mu and contains the region of the tsA1499 point mutation, and the PstI B fragment, which extends from 0.359 to 0.272 mu and contains the region of the tsA1499 81-base-pair deletion. The appropriate PstI A and B DNA fragments from either tsA1499 or wt830 were then ligated and transfected via DEAE-dextran into BSC-1H cells and incubated at either 32 or 38.5°C.

Several plates from each transfection were used to assay either plaque formation directly or virus production in liquid medium. The results of the above experiment are shown in Table 4. Virus constructs no. 1 and 3 (viruses that did not contain the point mutation at nucleotide 4261) were able to productively infect BSC-1H cells at 38.5°C, and only virus constructs no. 2 and 4 (viruses that did contain the point mutation) were ts for lytic growth. This demonstrated that the point mutation is solely responsible for the tsA1499 ts lytic growth phenotype and that the 81-base-pair deletion apparently had no dramatic effect on lytic growth of the virus.

To verify that each virus construct was indeed what it was intended to be, each virus was plaque purified and restested for ts lytic growth and also used for the preparation of viral DNA. The ts lytic growth results demonstrated that constructs no. 2 and 4 were tight ts mutants for lytic growth at 38.5°C (at least 104-fold). The viral DNA preparations, when analyzed by restriction enzyme digestion, showed that each virus construct contained the appropriate deletion or point mutations, or both (data not shown). Virus construct no. 2, which contains the point mutation at 0.455 mu but not the 81-base-pair deletion, we now refer to as tsA3900.

Assaying the effect of the new tsA3900 point mutation on the ability of SV40 to transform F111 rat cells to anchorage independence. All tsA mutants characterized to date have been

<table>
<thead>
<tr>
<th>Virus construct no.</th>
<th>PstI fragment</th>
<th>Point mutation</th>
<th>Deletion mutation</th>
<th>Lytic growth (PFU/ml) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32°C</td>
</tr>
<tr>
<td>1</td>
<td>A wt830</td>
<td>-</td>
<td>-</td>
<td>2.3 × 10^4</td>
</tr>
<tr>
<td></td>
<td>B wt830</td>
<td>-</td>
<td>-</td>
<td>&lt;10</td>
</tr>
<tr>
<td>2</td>
<td>A tsA1499</td>
<td>+</td>
<td>-</td>
<td>8.7 × 10^4</td>
</tr>
<tr>
<td></td>
<td>B tsA1499</td>
<td>-</td>
<td>-</td>
<td>&lt;10</td>
</tr>
<tr>
<td>3</td>
<td>A wt830</td>
<td>-</td>
<td>+</td>
<td>4.1 × 10^4</td>
</tr>
<tr>
<td></td>
<td>B tsA1499</td>
<td>+</td>
<td>-</td>
<td>&lt;10</td>
</tr>
<tr>
<td>4</td>
<td>A tsA1499</td>
<td>+</td>
<td>+</td>
<td>1.6 × 10^5</td>
</tr>
<tr>
<td>tsA1499</td>
<td>B tsA1499</td>
<td>+</td>
<td>+</td>
<td>4.6 × 10^4</td>
</tr>
<tr>
<td>Mock</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

A: BSC-1H cells transfected with ligated DNA mixtures were incubated at 32°C and fed on day 27. Lysates were harvested on day 36. Viral constructs no. 1 and 3 reached maximum cytopathic effect approximately 2 weeks before viral construct no. 2 and 4, or tsA1499 at 32°C.

B: Plaque assay performed at 32°C.

C: Plaque assay performed at 38.5°C.
been at least partially defective for both lytic growth and transformation (58). Most of these tsA mutants have mutations that map between 0.32 and 0.425 µm (23), with a few between 0.16 and 0.32 µm (8, 23). The SV40 mutant tsA3900, described above, is the only tsA mutant that has its mutation map at 0.455 µm, which directly affects the amino-terminal portion of the large T antigen.

In the following two experiments, we determined the effect of this unique genetic lesion on the ability of the tsA3900 mutant virus (or its DNA) to transform cultured rat cells to anchorage independence at the permissive and nonpermissive temperatures for lytic growth. Anchorage independence is one of the most stringent tests for transformation in vitro and is the transformed characteristic most closely associated with tumorigenicity in vivo (46).

In the first experiment, the tsA3900 virus was used to transform F111 Fisher rat cells. After the cell-virus mixture was incubated for 1 h at 37°C, the cells were plated into soft agar at a concentration of 2.5 × 10^5 cells per 60-mm dish. One-half of the total number of dishes were incubated at 38.5°C and one-half were incubated at 32°C. Colonies growing in agar were counted after 4 to 6 weeks at 38.5°C or 8 to 10 weeks at 32°C. The transformation results obtained when F111 cells were infected with three different multiplicities of tsA3900 virus are shown in Fig. 3. The mutant virus tsA3900 transformed F111 rat cells to anchorage independence equally well at both the permissive and nonpermissive temperatures for lytic growth.

In the second experiment, we compared the ability of tsA3900 to transform rat cells to anchorage independence at 32 and 38.5°C with a wild-type strain (wt830) of SV40 and another tsA mutant (tsA58). The wt830 virus is the strain from which tsA3900 was originally derived (37). The mutant tsA58 is a typical tsA mutant that is ts for both lytic growth and transformation (36, 58). To avoid possible variations in the multiplicities of infection of each virus used, as a result of differences in the ability of each virus to give accurate plaque titers, we transformed the F111 cells with equal quantities of each viral DNA. The viral DNA was transfected into the F111 cells via the calcium-phosphate coprecipitate method (60). After transfection of the F111 cells with the viral DNAs, the cells were incubated overnight at 37°C before being plated into soft agar at a concentration of 5 × 10^5 to 8 × 10^5 cells per 60-mm dish. Again, one-half of the total number of plates were incubated at 38.5°C and one-half were incubated at 31.5°C. Colonies growing in agar were counted at 4 to 6 and 8 to 10 weeks, respectively (Fig. 4). The viral DNAs from both tsA3900 and wt830 transformed the F111 cells to anchorage independence equally well at 38.5 and 32°C, and both viral DNAs resulted in similar efficiencies of transformation. As expected, tsA58 was dramatically ts for its ability to transform. These results show that tsA3900 is not ts for transformation at the nonpermissive temperature for lytic growth and is able to transform F111 rat cells.
cells to anchorage independence at a frequency similar to that of a wild-type strain of SV40.

We also isolated two tsA3900-transformed clones and two w830-transformed clones, fused each clone with BSC-1H permissive cells, and tested the rescued viruses for their abilities to productively infect permissive cells at 32 and 35.5°C. As expected, both of the tsA3900-transformed clones released viruses that were ts for lytic growth and both w830-transformed clones released viruses that were able to lytically infect permissive cells at 32 and 35.5°C (data not shown).

**Complementation test between tsA3900 and tsA58.** The genetic lesions of tsA3900 and tsA58 that cause the ts lytic phenotypes of these viruses map in different regions of the A gene (0.455 and between 0.32 and 0.425 mu [23], respectively), and both viruses behave differently from one another in their transforming functions at the nonpermissive temperature for lytic growth. Since the SV40 large T antigen is a large, multifunctional protein, we performed tests to determine whether these two mutants were indeed in the same complementation group. The results of a complementation test are shown in Table 5 and demonstrated that tsA3900 and tsA58 were in the same complementation group for lytic growth. Therefore, tsA3900 is a tight tsA mutant of SV40 that is able to separate the lytic and transforming functions of the large T antigen at 35.5°C.

**DISCUSSION**

In this paper we report the isolation of a spontaneous revertant of the tsA1499 mutant, tsA1499-Rev. Marker rescue studies with DNA from this revertant or with DNA from the original wild-type strain (w830) from which tsA1499 was derived revealed that the tsA1499 virus actually contained two mutations: the 81-base-pair deletion that had been described previously and another mutation between 0.425 and 0.484 mu. DNA sequencing revealed that this second mutation was a point mutation consisting of a cytosine-to-guanine-transversion at nucleotide 4261 (0.455 mu). This transversion generated a new RsaI restriction endonuclease recognition site on the mutant DNA and predicted an arginine-to-threonine substitution at amino acid 186 in the mutant large T antigen. In a viral reconstruction experiment, we demonstrated that a virus containing this point mutation was ts for lytic growth and that the presence of this point mutation on the tsA1499 genome was the sole cause of the ts lytic phenotype of that virus. We had thus identified a new and unique ts mutation in the SV40 A gene. This mutation is unique because it was mapped at 0.435 mu. All other tsA mutants described to date are located between 0.16 and 0.425 mu (8, 23), with the majority being located between 0.32 and 0.425 mu (23).

To determine whether this new mutation, carried on the mutant tsA3900, was unique in other ways we characterized it further. First, we determined the effect of the mutation on the ability of tsA3900 to transform cultured rat cells to anchorage independence. We chose the soft agar anchorage independence transformation assay, since it is one of the most stringent assays for transformation and anchorage independence is the in vitro characteristic most closely associated with tumorigenicity in vivo (46). Therefore, if tsA3900 were defective in transformation, we should detect such a defect with this assay. Interestingly, when we tested the mutant virus and its DNA for the ability to transform F111 cells to anchorage independence, we found that tsA3900 transformed these cells at both the permissive and nonpermissive temperatures for lytic growth and that the overall efficiency of transformation at both temperatures was similar to that of a wild-type strain of SV40 (w830). To eliminate the unlikely possibility that the transformation results were due to the presence of a non-temperature-sensitive subpopulation of virus within the tsA3900 viral stock (although the frequency of plaques obtained at 38.5°C with the viral stock used was $3 \times 10^{-4}$ with respect to the frequency of plaques obtained at 32°C), we fused tsA3900-transformed cells with BSC-1H cells and found that the rescued viruses were ts for lytic growth. Therefore we conclude that tsA3900 exhibited a strict separation of the lytic and transforming functions of SV40 at 38.5°C.

It is not a complete surprise that a mutation mapped at 0.455 mu should be able to separate the lytic and transforming functions of SV40, since two other mutations that map near this region on viruses C6-2 (13) and SVR9D (51) can also separate these two functions. However, the difference between tsA3900 and these other two viruses is that only tsA3900 is a ts mutant for lytic growth. Both C6-2 and SVR9D are nonconditional mutants.

C6-2 has been shown to be defective in binding its large T antigen to the viral origin of replication (39), and it is believed that the defect is due to an asparagine-to-threonine substitution at amino acid position 153 in the mutant large T antigen. It has also been demonstrated that a tryptic fragment consisting of approximately 130 of the amino-terminal amino acids of a wild-type large T antigen has origin-specific DNA binding activity and that in vivo phosphorylation causes a similar fragment to lose this origin binding activity (32). Both C6-2 and tsA3900 predict threonine amino acid substitutions within the adjacent 60 amino acids in the large T antigen. It is possible that these threonine residues may provide novel phosphorylation sites on the mutant T antigens that may affect their origin binding activities. Genetic and biochemical evidence indicates that the binding of the large T antigen to the regulatory region in the vicinity of the SV40 origin of replication is required for the induction of viral DNA replication and autoregulation of the SV40 early genes (9, 10, 15, 33, 34, 43, 47, 48). If tsA3900 is similar to C6-2 and is defective in its ability to bind to the SV40 origin of replication at 38.5°C, then tsA3900 might allow the biological functions associated with this binding to be controlled with temperature. However, further experimentation is required to demonstrate this possibility.

We have also attempted to use our viral constructs to determine the cause of the cold-sensitive phenotype of tsA1499 that has been reported previously (36, 37). However, we were not able to demonstrate a correlation between
the cold-sensitive phenotype and either the point or deletion mutation on that genome (data not shown). One probable conclusion is that the cold-sensitive phenotype is a consequence of the presence of both mutations in the genome.

Intracistronic complementation has been demonstrated within the SV40 A gene with small deletion mutants (59). The multifunctional nature of the large T antigen makes such complementation feasible. The mutant virus tsA3900 appeared to be unique from other tsA mutants, since its genetic lesion mapped in a region of the A gene unique from all other tsA mutants described to date and since it was able to separate the lytic and transforming functions of the large T antigen, unlike any other tsA mutants. Therefore, we tested the possibility that tsA3900 might complement other tsA mutants, for example tsA38. The results demonstrated, however, that tsA3900 was not able to complement tsA38. Therefore, tsA3900 does appear to be in the A complementation group for lytic growth.

In summary, the ts lytic phenotype of tsA1499 is due solely to the presence of a unique point mutation at nucleotide 4261 on that genome. The newly discovered mutation is unique for two reasons. First, it maps at 0.455 μm, which is further into the region of the A gene encoding the amino-terminal portion of the large T antigen than is any other tsA mutation. Second, a newly constructed virus carrying this point mutation, tsA3900, is the only tsA mutant that is able to transform cultured rat cells to anchorage independence at an efficiency similar to that of a wild-type strain of SV40 at the nonpermissive temperature for lytic growth. The ability of tsA3900 to separate the lytic and transforming functions of SV40 may make it very useful in determining how each of the multifunctions of the SV40 large T antigen are involved in lytic growth of the virus and in transformation of nonpermissive cells.

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