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Nucleotide Sequence Changes in Polyoma Virus *A* Gene Mutants

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The mutational alterations in polyoma virus mutants *ts-a* and *ts*-25E which cause their large T-antigens to be thermolabile have been identified. In *ts-a*, a G → A transition at nucleotide 2193 causes the replacement of Ala (GCT) by Thr (ACT). In *ts*-25E, a G → T transversion at nucleotide 2883 causes the replacement of Gly (GGC) by Cys (TGC). Revertants of both mutants have been isolated and shown to have the original nucleotides restored at these positions.

The polyoma virus *A* gene is defined by the *ts-a* mutant and other mutants which it fails to complement (10, 11, 17). These mutants encode thermolabile large T-antigens (but thermostable middle and small T-antigens) (20, 21, 25, 30). Marker rescue experiments with restriction endonuclease fragments have localized the altered sequences in these mutants to the origin-distal half of the polyoma genome early region (Fig. 1) (12, 13, 23). Nucleotide sequence analyses indicate that these sequences uniquely encode the COOH-proximal portion of the polyoma large T-antigen (19, 32).

The mutation in *ts-a* occurs between the *Hin*dIII site at nucleotide 1671 and the *Hha*I site at nucleotide 2331. The alteration in mutant *ts*-25E occurs between the *Hha*I site at nucleotide 2331 and the *Hha*I site at nucleotide 2913 (23). We determined the nucleotide sequences of *ts-a* and *ts*-25E throughout these regions. The DNA of mutant *ts-a* was sequenced from nucleotides 1689 → 2021 and 2072 → 2370 (Table 1). Only one difference was noted between its sequence and that of the polyoma large-plaque strain 3 virus sequenced by Friedmann et al. (19). A G → A transition at nucleotide 2193 causes the replacement of Ala (GCT) by Thr (ACT) in large T-antigen. The DNA of mutant *ts*-25E was sequenced from nucleotides 2329 → 2629 and 2649 → 3009 (Table 1). One difference was noted between its sequence and that of the virus sequenced by Friedmann et al. (19). A G → T transversion at nucleotide 2883 causes the replacement of Gly (GGC) by Cys (TGC).

Previously, we reported the isolation of spontaneously occurring polyoma virus mutants lacking *Hin*dIII site 1, which borders the *A* gene region (Fig. 1) (1). Among the mutants that we characterized were several which could grow without helper virus and which contained either single-base changes or very small deletions. We have sequenced two of these mutants. Both (1-10-1 and 1-12-1) contain a single G → A transition at nucleotide 1673. This mutation causes the loss of the *Hin*dIII site, but has no effect upon the amino acid sequence of large T-antigen (AAG [Lys] → AAA [Lys]). One of the mutants (1-12-1) has a normal phenotype; the other (1-10-1) grows poorly and gives small plaques. This must be due to a secondary mutation elsewhere in the viral genome.

Some variability in sequence occurs among wild-type strains of polyoma virus (32; Thomas and Folk, unpublished data). For instance, in

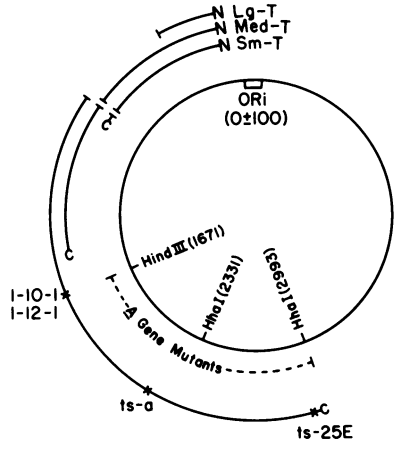

FIG. 1. *Polyoma virus early region. The HindIII site I and two HhaI sites are indicated on the circular polyoma genome. Numbers in parentheses refer to nucleotide numbers from the ori region (8). The sequences encoding the large, middle, and small T-antigens are drawn from references 19 and 32. The dashed line indicates the region in which A gene mutants have been mapped by marker rescue (12, 13, 23). The locations of the ts-a and ts-25E mutants and the two mutants with alterations at HindIII site 1 (1-10-1 and 1-12-1) are specified by a ×.*

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TABLE 1. *Sequencing strategy for various DNAs*

DNA	Fragment labeled	Secondary cleavage	Nucleotide site labeled	Sequence read from → to
ts-a	*Hin*dIII-2	*Hha*I	1671[a]	1689 → 1917
ts-a	*Hin*fI-3	*Sst*I	2059	2021 → 1888
ts-a	*Hin*fI-1	*Hpa*II	2059	2072 → 2370
ts-25E	*Hpa*II-2	*Eco*RI	2636	2629 → 2329
ts-25E	*Hpa*II-6	*Hha*I	2636	2649 → 2859
ts-25E	*Hpa*II-6	*Hph*I	3013	3009 → 2839
Revertant *ts-a* 13L	*Hin*fI-1	*Hpa*II	2059	2073 → 2280
Revertant *ts-a* 14F	*Hin*fI-1	*Hpa*II	2059	2084 → 2214
Revertant *ts-a* 5D	*Hin*fI-1	*Hpa*II	2059	2072 → 2306
Revertant *ts*-25E 3D	*Hpa*II-6	*Hph*I	3013	2918 → 2872
Revertant *ts*-25E 21D	*Hpa*II-6	*Hph*I	3013	2990 → 2825
Revertant *ts*-25E 23D	*Hpa*II-6	*Hph*I	3013	2990 → 2845

[a] The numbering system used throughout is that of Deininger et al. (8).

the large T-antigen encoding region described above, there are six sites at which the sequence of polyoma virus determined by Soeda et al. (32) is different from that determined by Deininger et al. (8). Because of this variability and the possibility that other mutations might have been introduced by the chemical mutagenesis used to generate mutants *ts-a* and *ts*-25E, we decided to confirm that the nucleotide changes in mutants *ts-a* and *ts*-25E are indeed the source of their thermosensitive phenotypes by isolating and sequencing several spontaneous revertants.

Numerous single plaques of mutants *ts-a* and *ts*-25E were picked from whole mouse embryo (WME) monolayers infected at 33°C and were used to prepare stocks of virus by infecting 60-mm dishes of WME cells at 33°C. Each stock was passaged through WME cells at 39.5°C to enrich for spontaneous revertants. These stocks were then plaqued on WME cells at 37°C. Approximately one-third of the stocks contained viruses capable of forming distinct plaques at this temperature. One plaque was picked from each independent stock, and its viruses were plaque purified at 37°C twice more. Virus stocks were prepared, tested for growth at 33 and 39.5°C, and then used to infect mouse 3T6 cells for viral DNA.

Three independent revertants of each mutant (which were normal in their capacity to replicate in mouse cells at 39.5°C) were characterized with respect to their sequence alterations. The *ts-a* revertants were found to have the G restored at nucleotide 2193, and the *ts*-25E revertants had the G at nucleotide 2883 restored (Fig. 2). Although not all of the T-antigen regions of these revertants were sequenced, because they are spontaneous revertants, it is unlikely that they incurred more than one mutation. Furthermore, a reversion at the same site in three independent revertants of each mutant strongly implicates that site as the cause of the phenotype of the virus and lessens the possibility that they are revertants by changes at secondary sites. Thus, it is possible to conclude that these amino acid replacements cause the thermolability of the polyoma large T-antigen.

Previous analyses by marker rescue experiments indicated that the majority of the *A* gene mutants are altered near the COOH-terminal coding sequences of the large T-antigen (12, 13, 23). Our results, together with the experiments of others (9), confirm this. The *ts-a* mutation occurs 242 amino acid residues from the COOH terminus, and the *ts*-25E mutation occurs only 11 amino acid residues from the COOH terminus. For these mutants, elevated temperatures cause instability of the large T-antigen and result in a cessation of viral DNA replication (16), impaired regulation of early mRNA synthesis (5), and a block in virus-mediated cell transformation (11, 18, 24).

The replacements of Ala by Thr and Gly by Cys in the large T-antigen of *ts-a* and *ts*-25E, respectively, are clearly destabilizing events. The sequence in the polyoma large T-antigen surrounding the site of the *ts-a* change (Ala-Gly-Val-*Ala*-Trp) is conserved between the T-antigens of polyoma virus, simian virus 40 (SV40), and BK virus proteins (14, 26, 28, 34) (Fig. 3). These amino acids and others nearby are α-helix formers, but the replacement of Ala by Thr (a helix breaker) may destabilize that structure (4). It is noteworthy that the amino acid replacements in four (and possibly more) *A* gene mutants of SV40 occur in the homologous region of the SV40 T-antigen (within an area containing 36 amino acid residues) (29). The replacement in SV40 mutant *tsA*239 adjoins the residue altered in the polyoma *ts-a* mutant (29). Similarly, the mutation in polyoma *ts*-48 that renders its large T-antigen unstable is a Ser → Ile replacement in the same area (49 amino acid residues toward the COOH terminus from the *ts-a* mu-

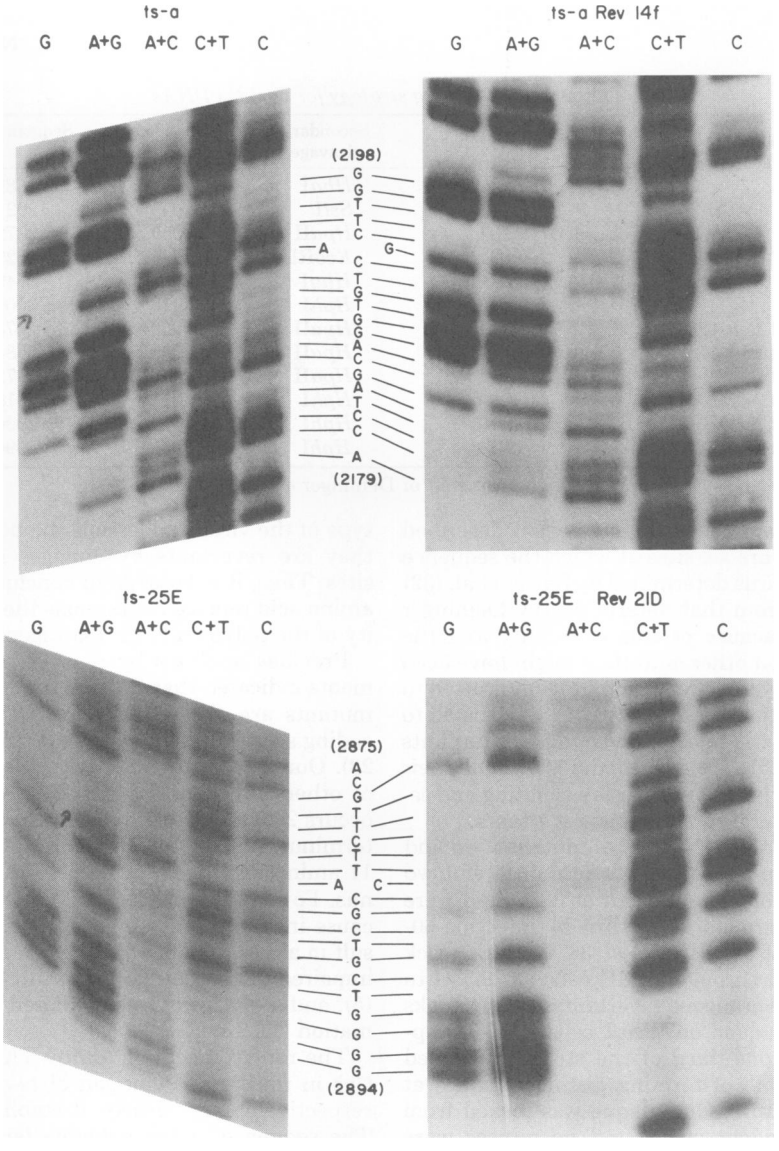

FIG. 2. *Autoradiograms of chemically degraded, end-labeled DNAs. Viral DNAs produced in mouse 3T6 cells were purified through cesium chloride-ethidium bromide gradients after a modified Hirt lysis extraction procedure (15). They were cleaved with endo BamHI (ts-a, ts-25E, 1-10-1, and 1-12-1 DNAs) or endo HindIII (revertants of ts-a and ts-25) and ligated to endo BamHI- or HindIII-cleaved pBR322 DNA as previously described (3). After transfection into Escherichia coli HB 101, Amp' clones were selected and purified. Their plasmid DNAs were extracted and checked for polyoma pBR322 sequences by restriction enzyme digestion. Polyoma pBR322 DNAs were extracted from chloramphenicol-amplified cultures by a modified cleared lysis procedure, deproteinized, and banded in cesium chloride-ethidium bromide gradients as previously described (3). After the ethidium bromide was removed by isopropanol extraction, the DNAs were ethanol precipitated and then digested with endonucleases. The terminal phosphates on the DNA fragments were removed with alkaline phosphatase, and the DNAs were deproteinized and ethanol precipitated. The digests were then fractionated on 6% polyacrylamide gels containing 0.32% diallytartardiamide. The appropriate fragments were solubilized in sodium periodate (2), recovered on DEAE columns, and phosphorylated with [γ-^{32}P]ATP and polynucleotide kinase (3). Digestion with a second restriction enzyme produced two asymmetrically labeled fragments which were purified by polyacrylamide gel electrophoresis. If the second cleavage occurred near a labeled terminus and produced a small labeled oligonucleotide, frequently it was not necessary to separate it from the labeled oligonucleotide being sequenced. The sequencing strategy employed for the various DNAs is given in Table 1. The labeled fragments were sequenced by the chemical degradation procedure of Maxam and Gilbert (22). The products were fractionated on 0.4-mm polyacrylamide gels (8%) as described by Sanger and Coulson (27). Portions from sequencing gels, including the sites of mutation or reversion of a ts-a allele and the ts-25E allele, are shown.*

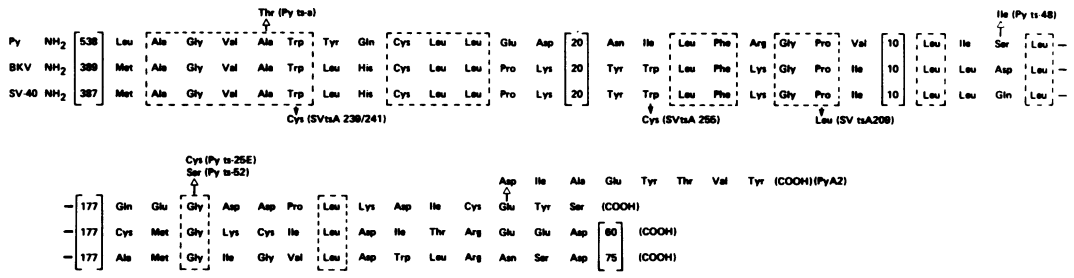

FIG. 3. *Comparison of amino acid sequences of viral large T-antigen near the sites of A gene mutations. Amino acid sequences of polyoma virus (19, 32), BK virus (28, 34), and SV40 (14, 26) from the middle (upper section) and COOH-terminal (lower section) portions of large T-antigen are displayed. Numbers in brackets refer to amino acids deleted for the purpose of clarity. Amino acids in common among the three T-antigens are enclosed in dashed boxes. Amino acid replacements in A gene mutants are indicated by arrows (9, 29).*

tation) (9). This clustering of mutations in independently isolated *A* gene mutants of both viruses (Fig. 3) emphasizes the homologous nature of their large T-antigens and suggests that this part of each protein is particularly important for stabilizing its structure.

The glycine that is altered in the polyoma *ts*-25E protein is at a site conserved between the large T-antigens of polyoma virus, SV40, and BK virus. It is interesting that this change is only 11 residues from the COOH terminus and that the alteration in polyoma *A* gene mutant *ts*-52 occurs at the same site (9) (Fig. 3). It appears that this site at the COOH terminus is critical for the stability of the polyoma large T-antigen. Because of the number of charged or polar amino acids or both in this region, it is unlikely that the COOH terminus has much α-helix content (4).

It is curious that one of the differences between the polyoma virus sequenced by Friedmann et al. (19) and the virus sequenced by Soeda et al. (32) occurs at the COOH terminus of large T-antigen. The sequence determined by Soeda et al. for the polyoma A-2 strain predicts a protein with five additional COOH-terminal amino acids. The *ts*-25E mutant has an *Hpa*II restriction pattern, suggesting that it is derived from the polyoma A-3 strain; like the polyoma strain 3 virus sequenced by Friedmann et al. (19), it does not have the sequence which might cause this larger T-antigen. Such sequence variability indicates that the structure of the large T-antigen COOH terminus is not rigidly defined. However, the glycine altered by the *ts*-25E and *ts*-52 mutations must be particularly important for the stability of this part of the protein. Curiously, the large T-antigens of SV40 and BK virus contain approximately 70 additional residues beyond the COOH terminus of the polyoma protein (14, 26, 28, 34). Deletions in the SV40 genome removing parts of this additional sequence do not appear to affect the thermostability of the large T-antigen, but diminish its adenovirus helper function (6, 7). This suggests that these sequences interact only minimally with those preceding them which are homologous in structure and presumably in function to the polyoma sequences.

Finally, several comments can be made about the nature of the mutations that have occurred in these viruses. Both *ts-a* and *ts*-25E were generated by nitrous acid mutagenesis. A G → A transition as observed in *ts-a* can be explained by deamination of the cytosine in a C·G base pair, giving rise to a T·A pair. However, a G → T transversion is not predicted as an outcome of nitrous acid treatment on straightforward chemical grounds (33). Similar G → T transversions have been found in nitrous acid-mutagenized ϕX174 (31). The mutations in the spontaneously occurring *Hin*dIII site 1 mutants and in the *A* gene revertants are both transitions (G → A; A → G) and transversions (T → G). These must be the results of mistakes in replication (or in repair) or are the result of naturally occurring damage to DNA.

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ADDENDUM IN PROOF

We have sequenced an additional revertant of *ts*-25E (*ts*-25E 4e). It is apparently a second-site revertant, for the T at nucleotide 2883 is unchanged, but a G has replaced an A at nucleotide 2864. This causes an Ile → Met amino acid substitution six residues upstream from the Cys that is the cause of the thermolability of the T-antigen.

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