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Temperature-Sensitive Mutants Identify Crucial Structural Regions of Simian Virus 40 Large T Antigen

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We have completed the cloning and sequencing of all known temperature-sensitive, amino acid substitution mutants of simian virus 40 large T antigen (*ts*A mutants). Surprisingly, many of the mutants isolated from distinct viral strains by different laboratories are identical. Thus, 17 independently isolated mutants represent only eight distinct genotypes. This remarkable clustering of *ts*A mutations in a few "hot spots" in the amino acid sequence of T antigen and the temperature-sensitive phenotypes of the mutations strongly suggest that these amino acids play crucial roles in organizing the structure of one or more functional domains. Most of the mutations are located in highly conserved regions of T antigen that correlate with DNA binding, protein-protein interactions, or ATP binding. With the exception of one mutant with a lesion in the putative ATP-binding region, all the mutants are temperature sensitive for DNA replication.

Simian virus 40 (SV40) large tumor antigen, the product of the viral *A* gene, has crucial functions both in productive infection of permissive cells and in oncogenic transformation of nonpermissive cells (13, 43). These functions include binding to specific sites in the origin of DNA replication (12, 42), melting origin DNA (8, 47), unwinding DNA at replication forks during replication (37, 45), and interacting with cellular proteins such as DNA polymerase α (36). T antigen also coordinates the transcription of viral genes by repression of its own synthesis (28, 40) and by activation of the late promoter (2, 7). In nonpermissive cells, expression of T antigen alone is sufficient to induce and maintain a transformed phenotype (43). Although the mechanisms of transformation are not yet understood, some mechanisms may be related to induction of cellular transcription (19), cellular DNA synthesis (4), and interaction with cellular proteins such as p53 (22) or the Rb protein (9).

Much information about the lytic and transforming functions of the SV40 large tumor antigen has been obtained with temperature-sensitive, amino acid (AA) substitution mutants of T antigen (*ts*A mutants). These mutants allow viral replication in permissive cells and transformation of murine cells at a permissive temperature of 32°C but fail to do so at higher temperatures (3, 4, 18, 39, 41). Marker rescue experiments have mapped the mutations which cause the *ts* phenotype to restriction fragments in the viral genome (20, 41). Many *ts*A mutants, however, have never been characterized in detail. Here we report the DNA sequencing of all known *ts*A mutants and relate the mutations to structural and functional domains of the protein. Surprisingly, many of the mutants isolated independently in different laboratories have identical mutations. We also compare the effect of temperature on DNA replication in vivo for each *ts*A mutant.

To clone the *ts*A mutants, viral stocks of *ts*A7, *ts*A28, *ts*A30, *ts*A40, *ts*A47, *ts*A57 (39), *ts*A207, *ts*A276 (4), *ts*A1609, and *ts*A1637 (41) were used to infect CV1 cells at the permissive temperature of 32°C. Three days after infection, viral DNA was extracted by the method of Hirt (17). The DNAs were linearized with *Bam*HI and cloned into the *Bam*HI site of the phagemid Bluescript KS(+) (Stratagene, La Jolla, Calif.). The cloned DNA of the *ts*A mutants produced viable virus when excised and transfected to CV1 cells at 32°C but not at 41°C (data not shown). By the method of Sanger et al. (30), we determined the nucleic acid sequence of the *Hin*dIII restriction fragments known to contain the *ts* mutations (20, 41).

Table 1 shows the sequence alterations of all known *ts* mutants in comparison with their parental strains. In most of the coding region for large T antigen, SV40 776 and 4554 had only minor differences, such as conserved AAs or silent mutations. At the extreme carboxy terminus, however, SV40 4554 had an insertion and deletion, unlike SV40 776 (data not shown). All mutations affect regions highly conserved in the T antigens of polyomaviruses that include JC virus (14), BK virus (33), lymphotropic papovavirus (27), hamster papovavirus (10), mouse polyomavirus (16), and even budgerigar fledgling disease virus (29), an avian polyomavirus. It is remarkable that *ts*A mutants independently isolated in different laboratories from different parental strains have identical mutations. Thus, the 17 *ts* mutants isolated to date can be reduced to eight different genotypes. All six mutants in the H fragment of a *Hin*dII-*Hin*dIII digest of the SV40 genome shared the same arginine-to-lysine substitution at AA 357 in T antigen. Four mutants had an identical Trp-to-Cys exchange at AA 393, and two mutants shared an Ala-to-Val exchange at AA 438. To avoid future confusion about the identity of these mutants, we propose a change in the nomenclature of the *ts*A mutants which allows an unambiguous identification (Table 1). The name would consist of the position of the affected AA in SV40 776 and the AA exchange. For example, *ts*A30 would be renamed *ts*A357R-K. Such a pattern for nomenclature would be ideal for all single AA substitutions in T antigen.

The *ts*A mutants can be divided into topologically distinct groups, which correlate with different functional domains of the protein (Fig. 1). The unique *ts*A186R-T (18) maps in the region of the protein that is sufficient for specific binding to the SV40 origin of DNA replication (38). The region around AA 186 is particularly important for DNA binding; mutants

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TABLE 1. DNA and AA sequence alterations of all known temperature-sensitive mutants of SV40 large T antigen

Mutant	Parent strain	*Hin*dII-*Hin*dIII fragment[a]	Codon change (position)[b]	AA change (position)	Proposed new name
*ts*A3900[c]	WT 830	A	AGG→ACG (4261)	Arg→Thr (186)	*ts*A186R-T
*ts*A30	VA 4554	H	AGA→AAA (3748)	Arg→Lys (357)	*ts*A357R-K
*ts*A40	VA 4554	H	AGA→AAA (3748)	Arg→Lys (357)	*ts*A357R-K
*ts*A47	VA 4554	H	AGA→AAA (3748)	Arg→Lys (357)	*ts*A357R-K
*ts*A57	VA 4554	H	AGA→AAA (3748)	Arg→Lys (357)	*ts*A357R-K
*ts*A1609	VA 4554	H	AGA→AAA (3748)	Arg→Lys (357)	*ts*A357R-K
*ts*A1637	VA 4554	H	AGA→AAA (3748)	Arg→Lys (357)	*ts*A357R-K
*ts*A28	VA 4554	I	TGG→TGC (3639)	Trp→Cys (393)	*ts*A393W-C
*ts*A207	776	I	TGG→TGC (3639)	Trp→Cys (393)	*ts*A393W-C
*ts*A239[d]	776	I	TGG→TGC (3639)	Trp→Cys (393)	*ts*A393W-C
*ts*A241[d]	776	I	TGG→TGC (3639)	Trp→Cys (393)	*ts*A393W-C
*ts*A255[d]	776	I	TGG→TGC (3552)	Trp→Cys (422)	*ts*A422W-C
*ts*A209[d]	776	I	CCA→CTA (3538)	Pro→Leu (427)	*ts*A427P-L
*ts*A58[e]	VA 4554	I	GCT→GTT (3505)	Ala→Val (438)	*ts*A438A-V
*ts*A276	776	I	GCT→GTT (3505)	Ala→Val (438)	*ts*A438A-V
*ts*A1642[f]	VA 4554	B	CCC→TCC (3461)	Pro→Ser (453)	*ts*A453P-S
*ts*A7	VA 4554	B	TTT→TCT (3133)	Phe→Ser (562)	*ts*A562F-S

[a] SV40 restriction fragments that rescue *ts*A mutants (20, 41).
[b] Numbers indicate positions at which change occurred. Change at position 4261 was a spontaneous mutation, change at position 3133 was a nitrosoguanosine mutation, and all other changes were hydroxylamine mutations.
[c] For sequence, see reference 44.
[d] For sequence, see reference 34.
[e] For sequence, see reference 31.
[f] For sequence, see reference 6.

with single AA changes at positions 185 and 187 in the protein are defective for origin binding (44; D. Simmons, personal communication). Therefore, it is possible that the mutation in *ts*A186R-T causes a temperature-dependent structural change in a region directly involved in the interaction of T antigen with origin DNA.

*ts*A357R-K is located in a region in which many functions may overlap. This change from a wild-type (WT) arginine to a highly similar lysine residue alters a distinct pattern of four leucines and four arginines that are conserved among all known polyomaviruses (Fig. 2A). This region resembles a leucine zipper, a protein segment of four or more leucines in a periodic array on an alpha helix. Landschulz et al. (21) have proposed that the leucine residues extending from every seventh position of an alpha-helical region interdigitate with the leucines of a corresponding element from

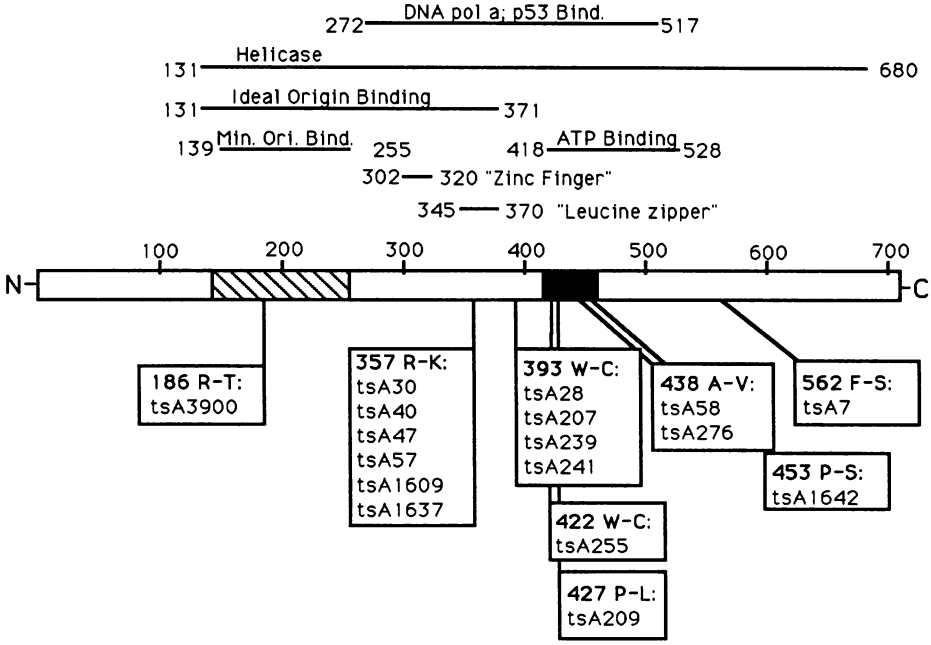

FIG. 1. Locations of the temperature-sensitive mutations in SV40 large T antigen. Shown is the location of the mutations in relationship to domains associated with helicase activity (37; D. Simmons, personal communication), minimal (Min.) (38) and ideal origin (Ori.) binding (Bind.) (35), DNA polymerase α (pol a) (36) and p53 binding (15, 32), and ATP binding (1). Symbols: ■, region matching consensus sequence for a mononucleotide-binding fold; ▨, region required for minimal binding to the origin.

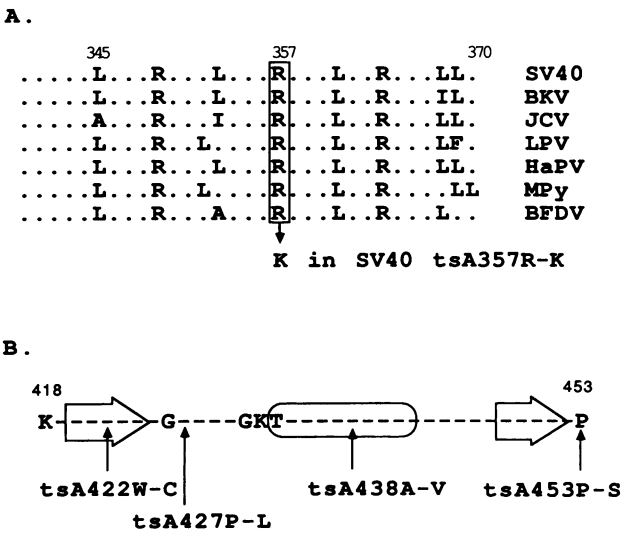

FIG. 2. Locations of *ts* mutants that cluster in regions of functional importance. (A) Repetitive arrangement of leucines and arginines in presumptive leucine zipper region of large T antigens of SV40, BK virus (BKV), JC virus (JCV), lymphotrophic papovavirus (LPV), hamster papovavirus (HaPV), mouse polyomavirus (MPy), and budgerigar fledgling disease virus (BFDV). The AA numbers above the sequence correspond to the positions in SV40 776 large T antigen. AAs are indicated by single-letter code; dots indicate positions of intervening AAs. (B) Presumptive ATP-binding fold of SV40 large T antigen between AAs 418 and 453, adapted from reference 1. Horizontal arrows represent regions with strong preferences for a beta sheet; ellipse indicates an alpha-helical conformation. Dashes represent the positions of intervening AAs. Locations of the four *ts*A mutations mapping in this region are indicated by vertical arrows.

another protein and facilitate dimerization by hydrophobic interactions. Structural predictions (5) for this region of all polyomavirus large T antigens suggest a strong preference for an alpha-helical conformation. Since one helical turn in a protein spans approximately 3.6 AA residues, the leucines and arginines of this element would be in phase on the helix (Fig. 2A).

Mutations causing the *ts* defects of *ts*A422W-C, *ts*A427P-L, *ts*A438A-V, and *ts*A453P-S are located in a small stretch of 30 AAs spanning a highly conserved region with structural homology to an ATP-binding structure (Fig. 2B). The mutations are located either in the structural elements (alpha helix or beta sheet) or directly adjacent to these elements, which match the "descriptor" of the ATP-ADP mononucleotide-binding fold described by Bradley et al. (1). Although the structural predictions for these mutations (5) are not significantly different from those of WT T antigen, it is evident that these mutations cause a temperature-sensitive change in the structures of these elements. Similarly, *ts*A393W-C maps near the putative ATP-binding fold and could influence its structure.

We compared the temperature-sensitive defect of all eight groups of *ts*A mutants to identify potential differences among the mutants in DNA replication (Table 2). Since *ts*A3900 (*ts*A186R-T) was isolated from a deletion mutant of viral strain 830 (18), we made a position 186 R-to-T mutation in a strain 776 background by using oligonucleotide-directed mutagenesis (23). This new construction excludes the possible effects of the parental strain and of additional mutations on the replication functions of the protein. CV1 cells were

TABLE 2. Replication efficiency of the *ts*A mutants relative to that of WT virus

Mutant	% Replication efficiency at:		
	32°C	39°C	41°C
WT 4554	100	100	100
*ts*A186R-T	44	0[a]	0
*ts*A357R-K	81	12	
*ts*A393W-C	101	0	0
*ts*A422W-C	108	16	0
*ts*A427P-L	114	0	0
*ts*A438A-V	103	<0.1	0
*ts*A453P-S	97	51	49
*ts*A562F-S	112	73	0

[a] No replication could be detected even after prolonged exposure of autoradiograms.

transfected with plasmid DNA (24) and incubated for 4 days at 32°C or for 2 days at 39°C and 41°C, and DNA was extracted by the method of Hirt (17). Replicated DNA was identified by *Mbo*I digestion and quantitated as previously described (11). While most of the mutants had little or no defect in replication at the permissive temperature, mutant *ts*A186R-T displayed a significant deficiency (44%) even at the permissive temperature. Raising the temperature to 39°C had a dramatic effect on most of the *ts*A mutants. Mutants *ts*A186R-T, *ts*A393W-C, *ts*A427P-L, and *ts*A438A-V replicated at less than 0.1% of the efficiency of WT virus. Mutants *ts*A357R-K, *ts*A422W-C, *ts*A453P-S, and *ts*A562F-S replicated at significant but reduced levels. At the restrictive temperature of 41°C, all mutants except *ts*A453P-S showed a complete replication shutdown. The minimal temperature sensitivity of mutant *ts*A453P-S for DNA replication is consistent with a previous report (6) that this mutant is temperature sensitive for the production of infectious virions but not for DNA replication.

The remarkable clustering of many *ts* mutations in a few "hot spots" in the AA sequence of T antigen and the *ts* phenotypes of the mutations strongly suggest that these AAs play crucial roles in organizing the structure of the protein for function. It remains unclear whether the mutations have global effects on many functional domains of T antigen or affect only the domains in which the substituted AAs reside. Many of the *ts*A mutations (13, 43) affect both DNA replication and transformation. This dual effect is consistent with the possibility that the mutations inactivate more than one domain in the protein. The block in DNA replication must be direct because a shift from the permissive to the restrictive temperature causes an immediate decrease in replication (39). In contrast, the temperature-sensitive defect in transformation may be the indirect effect of a more rapid turnover of mutant T antigens at elevated temperatures rather than the direct effect on a transformation domain. Thus, the apparent pleomorphic phenotypes of these mutants may be caused by changes limited to a single domain. In some cases, *ts* mutations apparently lead to focused defects in T antigen. The *ts*A186R-T mutation blocks replication functions but not all transforming functions (44). The *ts*A453P-S mutation leads to a drastic reduction in the production of infectious virus and a loss of transformation at the restrictive temperature without a significant effect on viral DNA replication (6).

The biochemical functions of the temperature-sensitive T antigens have been investigated only to a limited extent. The *ts* mutations at AAs 357, 393, and 438 reduce T-antigen oligomerization at elevated temperatures (25, 26). These

mutations and an additional mutation at AA 562 reduce the binding of T antigen to origin DNA (46). Because many of the mutations that affect oligomerization and DNA binding are in the putative ATP-binding region, altered ATP binding may interfere with protein-protein and/or protein-DNA interactions that are essential for one or more events in viral DNA synthesis. The effects of *ts*A mutations on the ATPase and helicase activities of purified T antigens have not been reported in detail. Our present results set the stage for a logical investigation of the relationship between key structural elements in various domains of T antigen and specific functions in DNA replication.

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