A Simian Virus 40 *dl884/tsA58* Double Mutant Is Temperature Sensitive for Abortive Transformation

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We have constructed a *dl884/tsA58* double mutant and a $t^{+}T^{-}$ early-region deletion mutant and have used these mutants to study the roles of the simian virus 40 tumor antigens (T and t) in transformation. Our major conclusions are that (i) although the mutant *tsA58* is not temperature sensitive for the maintenance of transformation, the *dl884/tsA58* double mutant is; (ii) small t antigen can provide at least one, but not all, of the functions required for the maintenance of transformation; and (iii) at least two different functions are required for the maintenance of simian virus 40 transformation.

To examine the roles that the simian virus 40 (SV40) large T and small t antigens play in initiating and maintaining cellular transformation, we have constructed a double mutant that combines the temperature-sensitive large T antigen mutation of mutant *tsA58* (19) with the small t antigen mutation of mutant *dl884* (14). We present evidence here that the double mutant is temperature sensitive for both initiating and maintaining transformation but that the parental mutant, *tsA58*, is temperature sensitive only for initiating transformation. From these results we conclude that the SV40 small t antigen can perform at least one of the functions required for maintaining cellular transformation.

The *tsA* mutants of SV40 were selected because of their lytic behavior, that is, because their replication is impaired at high temperature. Because their temperature-sensitive mutations map in the coding region for large T antigen, *tsA* mutants have been very useful for identifying and studying large T antigen functions required for the lytic growth of SV40 (20). In contrast, they have not been as useful for studying the involvement of large T antigen in SV40 transformation.

As Stoker was the first to recognize (17), the functions required for stable transformation by SV40 can, by definition, be divided into two groups. The initiation functions are required to promote efficient integration of SV40 DNA into the cellular genome, which is necessary if the transformed phenotype is to be heritable. The other functions required for transformation, called maintenance functions, confer the transformed phenotype on cells. Several experimenters have demonstrated that when nonpermissive cells are infected with *tsA* virus, the frequency

of stable transformation (as tested by, for example, a dense focus or agar suspension assay) is much greater at the permissive temperature than at the restrictive temperature (5, 19). Since efficient stable transformation requires the action of both initiation and maintenance functions, the observed temperature sensitivity of *tsA* mutants in such assays could be due to a temperature-sensitive initiation function or maintenance function or both.

In attempts to decide among these possible explanations, experimenters have transformed cells with *tsA* mutants at the low temperature, shifted them to the restrictive temperature, and examined their phenotypes. If *tsA* mutants are temperature sensitive for the maintenance of transformation, these transformed cells should revert to a normal phenotype when shifted to the restrictive temperature. However, such experiments have given the puzzling result that, at high temperatures, some *tsA* transformants (the N transformants) do revert to a normal phenotype, whereas others (the A transformants) do not (20). So far, this A/N paradox has not been satisfactorily explained. Because of this paradox, shift-up experiments have shed little light on the question whether the *tsA* transformation defect is in an initiation or a maintenance function.

The temperature sensitivity of *tsA* mutants has also been studied by abortive transformation assays. When SV40 infects nonpermissive cells, a relatively large fraction of the cells (typically a few percent) transiently exhibit a transformed phenotype, e.g., the transient loss of anchorage dependence. In most of these cells, however, the expression of the transformed phenotype is ephemeral, lasting for a few generations, and

FIG. 1. Restriction enzyme map of the *dl884/tsA58* double mutant. We lightly digested DNAs from the mutants *dl884* and *tsA58* with the restriction enzyme *HindIII* to yield linear molecules cut at one of the six *HindIII* sites. We then digested these linear molecules with *BamHI* and gel purified the *BamHI-HindIII*-A fragment of the mutant *dl884* and the *BamHI-HindIII*-B fragment of *tsA58*. We ligated these gel-purified fragments together to yield a double mutant that contained both the *dl884* deletion and the *tsA58* temperature-sensitive mutation.

only a small fraction of these abortively transformed cells becomes stably transformed (17). It was Stoker and Dulbecco who first realized that abortively transformed cells are cells in which the SV40 maintenance functions are being expressed and that abortive transformation, as contrasted with stable transformation, does not require the expression of the SV40 initiation functions (18). Thus, by assaying the ability of a mutant to transform abortively, one can test for the expression of maintenance functions independent of the expression of the initiation functions.

In 1979, Fluck and Benjamin published the results of experiments in which they clearly showed that at 39.5°C, virions of two widely used *tsA* mutants, *tsA30* and *tsA58*, transiently induce the loss of anchorage dependence in rat cells with an efficiency comparable to that of wild-type SV40 (2). These results led the authors to suggest that the two *tsA* mutants might not be temperature sensitive for the maintenance of transformation. However, because they could not rule out the possibility that the high level of abortive transformation observed might be due to the leakiness of the mutants, they could make no firm conclusion on this point.

There are at least three possible explanations

of why the mutant *tsA58* abortively transforms with wild-type efficiency, even at the restrictive temperature. First, the abortive transformation might be due to the leakiness of the large T antigen temperature-sensitive mutation. Second, the large T protein made by *tsA58* might be temperature sensitive for the initiation, but not for the maintenance, of transformation. This would explain why *tsA58* is defective for stable transformation but not for abortive transformation. Finally, the large T protein of *tsA58* might be temperature sensitive for both initiating and maintaining transformation, but the normal small t protein made by *tsA58* might express maintenance functions that could complement the temperature-sensitive large T protein functions.

To test these possibilities, we constructed a *dl884/tsA58* double mutant by ligating together the mutated segments of the two mutants, *tsA58* and *dl884* (Fig. 1). This double mutant has the same temperature sensitivity for viral replication as its *tsA58* parent but does not make a detectable small t protein, a quality inherited from its *dl884* parent. It is important to note that since the *dl884* deletion is completely contained in the region of the SV40 genome unique to small t, the large T proteins of *tsA58* and the *dl884/tsA58* double mutant should be identical.

Because we wished to use cloned mutant DNA in all our studies, we thought that it was important to confirm the results of earlier experiments involving virion infections. Table 1 shows the results of assays that tested the ability of cloned *tsA58* DNA to induce the formation of dense foci on monolayers of C3H10T-1/2 mouse cells (10). From these studies, we conclude that at 40.5°C, *tsA58* DNA induces focus formation with only about 5% of the efficiency of wild-type SV40 DNA. This result is consistent with those obtained with *tsA* virions (5, 19) and indicates that cloned *tsA58* DNA is temperature sensitive in at least one of the functions required for initiating or maintaining stable transformation.

Next, we tested the ability of *tsA58* DNA to transiently induce anchorage independence in rat F111 cells. These experiments (Table 2) show that cloned *tsA58* DNA is as efficient as wild-type DNA in abortively transforming rat cells at 40.5°C, the same temperature at which *tsA58* DNA was greatly impaired in its ability to transform stably in a focus assay. These abortive transformation results are similar to those obtained by Fluck and Benjamin with *tsA58* virions (2). Because abortively transformed F111 cells appear as grape-like clusters, it is easy to count the number of cells in each transformed colony. Thus, although the number of cells we observed in abortively transformed colonies was low (usually three to eight cells),

TABLE 1. Focus formation in cells infected with cloned viral DNA^a

Type of DNA ^b	33°C		40.5°C	
	Foci per µg of DNA	Relative transformation frequency ^c	Foci per µg of DNA	Relative transformation frequency
Salmon sperm	0	0	0	0
<i>ptsA58</i>	27	150	3.3	4.7
pWT	18	100	70	100

^a We seeded 35-mm plates with 2×10^5 10T1/2 mouse cells and infected them the same day with cloned DNA by a modification (21) of the calcium phosphate technique (4). About 16 h after infection, we changed the medium (Dulbecco modified Eagle medium–10% fetal calf serum) and transferred the cells to the appropriate temperature for the assay. About 48 h after infection we trypsinized the cultures and replated the cells onto 60-mm dishes. Thereafter, we changed the medium every fourth day. Foci were counted without staining at 3 weeks for the 40.5°C assay and at 5 weeks for the 33°C assay.

^b pWT, Cloned wild-type DNA; *ptsA58*, cloned *tsA58* DNA.

^c [(Foci per µg of mutant DNA)/(foci per µg of wild-type DNA)] $\times$ 100.

these assays are quite reproducible, and we routinely measured a 6- to 20-fold difference between the number of abortively transformed colonies in cases of wild-type versus mock infection. In addition, abortively transformed colonies that resulted from *tsA58* infections were indistinguishable both in morphology and in size distribution from colonies arising from wild-type SV40 infections. The abortively transformed colonies were small because we waited for two

cell divisions to take place after infection before suspending the cells in agar (see below).

We then tested the ability of the *dl884/tsA58* double mutant to transform abortively by infecting rat cells with cloned DNAs of this mutant and its two parents. These experiments (Table 3) show that although the DNAs of both *dl884* and *tsA58* abortively transformed with an efficiency comparable to that of wild-type DNA at 40.5°C, no abortive transformation could be detected at this temperature with the *dl884/tsA58* double mutant. At 33°C, the permissive temperature, all three mutants abortively transformed as well as the wild type. Thus, the ability of *tsA58* to transform abortively is not due to leakiness

TABLE 2. Abortive transformation induced by *tsA58* mutant DNA at 40.5°C^a

Type of DNA ^b	Viral DNA per dish (µg)	Abortive transformation	
		% ^c	Relative efficiency ^d
Salmon sperm	0	0.1	
<i>ptsA58</i>	6	0.6	100
pWT	6	0.6	100

^a We seeded 35-mm plates with 3×10^5 rat F111 cells (a kind gift from Tom Benjamin), and about 2 h later we infected them with DNA by the modified calcium phosphate technique. We used salmon sperm carrier DNA when necessary to make the total amount of DNA (viral plus carrier) about 6 µg per dish. These infections were carried out at 37°C. At 16 h after infection, we changed the medium on the cultures and transferred the plates to 40.5°C. The next day, we trypsinized the cultures and plated one-third of each into 0.34% agar–Dulbecco modified Eagle medium–17% calf serum on 100-mm dishes and incubated them at 40.5°C. Eight days later, we counted colonies of abortively transformed cells.

^b Abbreviations as in Table 1, footnote b.

^c Percentage of cells that grew in agar to give colonies with more than two cells. Each entry is the average of the results of duplicate infections. Approximately 5,000 cells were scored for each entry.

^d Percentage of abortive transformation relative to that induced by pWT.

TABLE 3. Abortive transformation induced by *dl884/tsA58* double mutant DNA and the DNA of its parents^a

Expt	Type of DNA ^b	Viral DNA per dish (µg)	% Abortive transformation	
			33°C	40.5°C
1	Salmon sperm	0	0.14	0.04
	<i>pd1884</i>	6	0.85	0.50
	<i>ptsA58</i>	6	1.2	0.75
	<i>pd1884/tsA58</i>	6	1.0	0.06
	pWT	6	1.7	0.80
2	Salmon sperm	0	<0.04	0.1
	<i>pd1884</i>	6	0.2	0.9
	<i>ptsA58</i>	6	0.5	0.6
	<i>pd1884/tsA58</i>	6	0.2	0.1
	pWT	6	0.2	0.8

^a These experiments were carried out as described in Table 2, footnote a, except that at 16 h after infection, the cells were placed at either 33 or 40.5°C as shown. Colonies with more than two cells were scored for experiment 1 at 33°C; colonies with more than three cells were scored for the other assays.

^b Abbreviations as in Table 1, footnote b.

FIG. 2. SV40-specific proteins made by the mutant 372dl. We infected BSC-1 monkey cells with DNAs of the *tsB4/dl884* double mutant helper (lane 1), the helper plus 372dl (lane 2), or wild-type SV40 (lane 3) by a modification (15) of the technique of McCutchan and Pagano (9). We labeled the infected cells with [35 S]methionine (0.4 mCi/ml, 1,000 Ci/mmol) for 2 h, extracted labeled proteins, immunoprecipitated these proteins with anti-SV40-tumor serum (1), and subjected the precipitates to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6 to 15%) (6). Shown here is a fluorogram of that gel. Positions of molecular weight standards (molecular weights in thousands [K]) and large T and small t antigens are also shown.

since the large T antigen of the double mutant should be as leaky as the large T antigen of its parent. Since the double mutant does not abortively transform at the restrictive temperature, the large T proteins of the double mutant and the *tsA58* mutant must be temperature sensitive for the maintenance of transformation. This defect can be complemented by some activity of the small t protein made by *tsA58*, indicating that the small t protein can provide at least one function required for the maintenance of transformation. It is not totally unexpected that the small t protein might be able to express one or more of the large T protein functions since the proteins have 82 amino acids in common at their amino termini.

To determine whether small t antigen alone is sufficient for the maintenance of transformation, we constructed a deletion mutant, 372dl, that lacks the early region sequences between the two *HpaI* restriction enzyme sites at 0.17 and 0.37 map units. We accomplished this by partially digesting cloned wild-type DNA with *HpaI* and by gel purifying linear molecules that had deletions of the desired size. We religated these linear DNA molecules to form circles, transfect-

ed *Escherichia coli*, and selected a colony that contained SV40 molecules deleted between 0.17 and 0.372 map units. To be sure that this mutant, which retained all the sequences necessary to encode small t antigen, could indeed make small t, we excised 372dl from its plasmid and used this DNA to infect BSC-1 cells together with the DNA of an appropriate helper virus. The helper, which is a *tsB4/dl884* double mutant (16), can supply the large T antigen necessary for the replication of 372dl but cannot make a detectable small t antigen. Figure 2 shows that a protein that comigrates with small t antigen can be detected in the mixed infection, 372dl plus helper, but not in cells infected with the helper alone, thus indicating that 372dl can make small t antigen. In addition, we were unable to detect any truncated forms of large T antigen made by this mutant. When we tested cloned DNA of 372dl in an abortive transformation assay, we detected no abortive transformation (Table 4). This result indicates that small t antigen alone is not sufficient to maintain transformation.

Taken together, these experiments lead us to several conclusions. First, *tsA58* is not temperature sensitive for the maintenance of transformation. This insensitivity to temperature must result from the expression of a small t antigen maintenance function since the *dl884/tsA58* double mutant, which cannot make a functional small t antigen, is temperature sensitive for maintenance. Thus, small t antigen is able to perform at least one of the large T maintenance functions and can complement the maintenance defect in the *tsA* large T protein. However, since the mutant 372dl which expresses small t antigen but not large T antigen, does not abortively transform, small t antigen cannot express all the

TABLE 4. Abortive transformation induced by 372dl mutant DNA

Expt	Type of DNA	Viral DNA per dish (μ g)	% Abortive transformation
1 ^a	Salmon sperm	0	<0.01
	p372dl	6	<0.03
	pWT	6	0.4
2 ^b	Salmon sperm	0	0.03
	p372dl	6	0.05
	pWT	6	0.29

^a These experiments were carried out like those shown in Table 2, except that the incubations were at 37°C and the cells were passed to agar 1 day after infection. Colonies of more than three cells were scored.

^b The same conditions were used as for experiment 1 except that the cells were passed to agar 2 days after infection. Colonies with more than two cells were scored.

functions required for maintenance. Therefore, at least two functions are required for the maintenance of transformation by SV40.

In a stable transformation assay at the restrictive temperature, the mutant *tsA58* transformed with an efficiency that is only about 5% that of wild type. Since this assay tests both maintenance and initiation functions and since *tsA58* is not temperature sensitive for maintenance, we can infer that *tsA58* is temperature sensitive for the initiation of transformation. The temperature-sensitive initiation defect in *tsA58* reduces its ability to initiate stable transformation by about 20-fold relative to the wild type, suggesting that an active SV40 initiation function can increase the probability of successful integration at least 20-fold over the low level of fortuitous integration. Finally, since *tsA58* is temperature sensitive for the initiation of transformation and since *d1884/tsA58* and *tsA58* have identical large T proteins, we can infer that the *d1884/tsA58* mutant is also temperature sensitive for initiation.

These results lead to a natural explanation of the A/N paradox. The *tsA* transformants that do not revert to a normal phenotype at the restrictive temperature would be those in which a functional small t protein is expressed at levels sufficient to complement the *ts* defect in large T antigen. Because the small t protein is required for efficient transformation of resting cells (8, 13), we would expect that most *tsA* transformants obtained by infecting resting cells at the permissive temperature would express a functional small t antigen. If our explanation of the A/N paradox is correct, these transformants should be predominantly temperature insensitive (A type). It has in fact been observed that the infection of resting cells with *tsA* mutants preferentially gives rise to temperature-insensitive transformants (12). Our explanation is also consistent with the finding that rat cells infected with *d154-59/tsA* double mutants always generate temperature-sensitive (N-type) transformants (13). However, it has been shown that double mutant-infected Chinese hamster lung cells can give rise to both A-type and N-type transformants (7). This result raises the possibility that some cells might be able to supply the small t antigen maintenance function(s). The idea that cellular functions could be involved in the maintenance of transformation is, of course, not new (3).

It has been widely assumed that *tsA* mutants are temperature sensitive for the initiation of transformation. Our results show that this assumption is justified, at least in the case of *tsA58*. On the other hand, it has frequently been assumed that *tsA* mutants are also temperature sensitive for the maintenance of transformation.

Our experiments demonstrate that for one popular *tsA* mutant, *tsA58*, this assumption is erroneous. Thus, experiments in which this assumption was made probably should be reevaluated. Such experiments could more properly be done with a double mutant such as the *d1884/tsA58* mutant which we have described here.

While this manuscript was in preparation, Rubin et al. reported that a t^+T^- virus is unable to transform mouse and hamster cells abortively (11), a result that is consistent with our findings with cloned 372*dl* DNA (Table 4). However, in the experiments of Rubin et al. and also in those of Fluck and Benjamin (2), virions of the mutant *d1884* were shown to be defective in abortively transforming cells to anchorage independence. These results appear to be at odds with our findings that cloned *d1884* DNA abortively transforms rat cells as efficiently as wild-type SV40. Fortunately, the reason for this apparent discrepancy is easily understood. It has been recognized for some time that whereas t^- mutants such as *d1884* stably transform rapidly growing cells as efficiently as wild-type SV40, they are defective in their ability to transform resting cells stably (8, 13). Thus, small t antigen can provide a function that facilitates the stable transformation of resting cells. We have observed a similar effect in abortive transformation. When we suspended *pd1884*-infected cells in agar as early as 16 h postinfection, the efficiency of abortive transformation was greatly reduced relative to that induced by wild-type DNA (data not shown). This reduced efficiency is similar to that observed by Rubin et al. and Fluck and Benjamin, who suspended their cells in agar 16 and 5 h, respectively, after infecting cells with *d1884* virions. However, as Table 3 shows, this facilitator defect in *pd1884* can be overcome by allowing infected cells to grow for about two generations before performing the agar assay. It is important to note that since all our experiments were carried out under conditions in which *pd1884* abortively transforms with wild-type efficiency, the small t antigen maintenance function that we describe in this communication is not the small t facilitator function previously identified.

We thank Lyn Pierce for her excellent technical assistance.

This material is based upon work supported in part by Public Health Service grant CA-24924 from the National Cancer Institute and in part by the National Science Foundation under grant no. PCM-8110366.

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