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A frameshift mutation affecting the carboxyl terminus of the simian virus 40 large tumor antigen results in a replication- and transformation-defective virus

(synthetic oligonucleotide/site-specific mutagenesis/alternative reading frame/variant tumor antigen)

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ABSTRACT We have constructed a frameshift mutation in the simian virus 40 early region using a novel method of oligonucleotide-directed mutagenesis. The mutated DNA specifies an 84,000-dalton large tumor antigen that consists of ≈75,000 daltons encoded by the wild-type reading frame and 9,000 daltons, by the alternative reading frame (wild-type large tumor antigen is ≈82,000 daltons). The frameshifted carboxyl terminus of the protein bears a strong similarity to the same region of polyoma virus middle-sized tumor antigen. We have found that the mutant DNA is unable to replicate when introduced into permissive monkey cells and incapable of transforming nonpermissive mouse cells.

Polyoma virus encodes three tumor (T) antigens—a small, a middle-sized, and a large—whereas simian virus 40 (SV40) apparently encodes only a large and a small T antigen (1). The polyoma middle-sized T antigen appears to be necessary and sufficient to bring about the transformation of cells in tissue culture. Recombinant plasmids that contain the promoter proximal part of the polyoma early region but lack the distal part (i.e., are unable to encode large T antigen) are able to transform rat cells in tissue culture with high efficiency (2, 3). In addition, plasmids containing the information to encode only middle-sized T antigen transform rat cells with nearly the same efficiency, and to the same extent (i.e., ability to grow in soft agar), as does wild-type polyoma (4). However, very little is known about the biochemical function(s) of middle-sized T antigen. It is a 50-kilodalton (kDa) phosphoprotein, contains a tightly associated protein kinase activity (5–7), and is localized predominantly in the plasma membrane (8, 9). Deletion mutations that affect the carboxyl terminus of middle-sized T antigen greatly reduce transformation efficiency (10). This region of the protein consists of an unusual string of six consecutive glutamic acid residues followed by an extremely hydrophobic region, which is responsible for the membrane localization of the protein and is also required for the transformation and associated protein kinase activities (11).

Because SV40 does not encode a middle-sized T antigen, the ability of this virus to transform cells must reside with the large or the small T antigen. Mark and Berg (12) pointed out the existence of an apparently unused reading frame located near the 3′ end of the SV40 early region. The amino acid residues encoded by it would be extremely hydrophobic (nearly 60% of the codons specify hydrophobic amino acids). Also, at the start of the region with two alternative reading frames, but in the large T-antigen reading frame, there is a stretch of six codons that encode consecutive acidic amino acids. This sequence arrangement bears an obvious and striking similarity to the sequence found in the polyoma middle-sized T antigen, suggesting that SV40 may contain the information to produce a "middle-sized T antigen." However, no evidence exists indicating that this second reading frame is used, either in lytic infection or in transformed cells (13, 14).

To directly address the possible function of this unassigned reading frame, we have used site-specific *in vitro* mutagenesis to construct a specific frameshift mutation in the SV40 genome. Below we discuss the construction of this mutant by a novel method and describe some of its properties in lytic and transforming infections.

MATERIALS AND METHODS

DNA. Two recombinant plasmids were used as starting material. One, pSVRI, consists of the entire SV40 genome inserted in the *Eco*RI site of pBR322. The other, $pSV_{Bgl\text{-}Bam}$, contains the SV40 early region inserted in the *Bam*HI site of pBR322 (15). Plasmid DNA was routinely grown in *Escherichia coli* HB101 and purified by standard procedures (16). The pentadecamer 5′ G-A-C-A-G-C-C-G-G-A-A-A-A-T-G 3′ was chemically synthesized using amino phosphite triester chemistry essentially as described (17).

Construction of the Frameshift Mutant. Ten micrograms of $pSV_{Bgl\text{-}Bam}$ was digested to completion with *Eco*RI and then treated with 100 units of exonuclease III for 15 min at 32°C. The extent of digestion was monitored by agarose gel electrophoresis of aliquots of DNA (1 μg) following denaturation with glyoxal (18). The conditions described above resulted in the removal of ≈2,500 nucleotides from each end of the DNA. A 0.5-pmol sample of purified DNA and 50 pmol of phosphorylated oligonucleotide were dissolved in 5 μl of 10 mM Tris·HCl (pH 7.5), and 5 μl of 200 mM NaCl/100 mM Tris·HCl, pH 7.5/50 mM $MgCl_2$/2 mM dithiothreitol was added. The solution was heated to 70°C for 5 min and then placed on ice for 45 min. Ten microliters of a solution containing 1 mM dATP/dCTP/dGTP/dTTP, 1 mM ATP, 1 mM dithiothreitol, 0.5 unit of DNA polymerase I, Klenow fragment, and 1,000 units (as defined by supplier, New England BioLabs) of T4 DNA ligase was added and the reaction mixture was incubated at 25°C for 4 hr. A high concentration of ligase and a relatively low concentration of polymerase minimize strand displacement at the site of the hybridized elongated oligonucleotide, which can otherwise reduce recovery of the desired mutant. DNA was purified by phenol

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Abbreviations: T antigen, tumor antigen; SV40, simian virus 40; kDa, kilodalton(s).

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extraction and, after two ethanol precipitations, dissolved in 50 μl of 10 mM Tris·HCl, pH 8.0/1 mM EDTA, and a fraction was used to transform $CaCl_2$-treated *E. coli* HB101 cells (16). Plasmid "minipreps" (16) were prepared from 5-ml cultures of ampicillin-resistant colonies. DNAs were assayed for size and for the presence and, if detected, the location of a new restriction site (*Hpa* II). The nucleotide sequence in the region of a successfully induced mutation was determined by using the method of Maxam and Gilbert (19); a *Bcl* I restriction site (0.19 map units) was used for DNA 5'-end labeling. Mutant alleles were transferred into plasmids containing a complete SV40 genome by first purifying from the mutated plasmid, by polyacrylamide gel electrophoresis, a 433-base-pair fragment extending from the unique *Bcl* I site to a *Pst* I site at 0.27 map units. pSVRI was digested to completion with *Bcl* I and partially with *Pst* I and then incubated with calf intestinal phosphatase; 2 μg of this DNA was mixed with 0.4 μg of the purified mutated fragment, and the sample (20 μl) was incubated with T4 DNA ligase for 4 hr at 16°C. An aliquot of this DNA was then used to transform *E. coli* HB101, and the correct plasmid was identified by restriction enzyme analysis.

Cells and DNA Transfection. NIH 3T3, BSC-1, CV-1, and COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin, and 10% fetal calf serum. The DEAE-dextran method (20) was used to transfect BSC-1, CV-1, or COS-1 cells, while the calcium phosphate method (21) was used for NIH 3T3 cells. Twenty-four hours after transfection, NIH 3T3 cells were plated at 10^5/60-mm Petri dish for low serum selection of dense foci (22). Three weeks later, several clones were picked with steel cloning rings for further analysis. For anchorage-independent selection, 10^5 cells were plated in 0.33% agarose on 60-mm plates previously coated with 0.5% agarose. After 3–4 wk, colonies > 0.2 mm in diameter were picked.

Replication and Production of Virus by the Cloned DNA. DNA replication was measured by Southern blot analysis (23) of DNA extracted by the method of Hirt (24) from transfected cells 4 days after addition of DNA. To assay virus production, cells and medium were collected 10 days after transfection. Intact cells were lysed by freezing and thawing three times, and the resulting lysates were used to infect fresh monolayers of CV-1 cells.

Immunofluorescence and Immunoprecipitation. Cells grown on coverslips were fixed and stained as described (25). Both tumor serum and monoclonal antibodies against either the amino or the carboxyl terminus of SV40 large T antigen (26) were used in this assay. [^{35}S]Methionine-labeled cell extracts were prepared and immunoprecipitated with either normal hamster serum or hamster anti-SV40 tumor serum and proteins were analyzed on 10–20% $NaDodSO_4$/polyacrylamide gradient gels as described (27).

RESULTS

***In Vitro* Construction of a Specific Frameshift Mutation.** The scheme that we devised to bring about a frameshift mutation (−1) in the SV40 early region is illustrated in Fig. 1. In addition to creating a new *Hpa* II site, the desired single base-pair deletion destroys a *Bst*NI site. Both of these predicted changes, and no others, were observed (results not shown). DNA sequence analysis confirmed that nucleotide 2,899 [SV40 nucleotide numbering system of Buchman *et al.* (28)] had been deleted (Fig. 2), and no additional changes were detected. After reconstruction of a complete viral genome, SV40 sequences were freed from the mutant (pSVRIH) and wild-type plasmids (pSVRI) by digestion with *Eco*RI. This DNA was then used in subsequent transfection experiments.

Expression of a Variant T Antigen in Monkey Cells. The frameshift mutant produced a T-antigen-related polypeptide in monkey kidney cells, as determined by indirect immunofluorescence (26). The intensity of staining observed was similar in both wild-type- and mutant-transfected cells, as was the intranuclear localization of the T antigen. Transfection with wild-type DNA gave rise to approximately twice as many T-antigen-positive cells as did transfection with the mutant DNA (Table 1). Similar results have been obtained with both polyclonal antitumor serum and a monoclonal antibody directed against the amino terminus of T antigen (Pab 416; ref. 25). However, when monoclonal antibodies with specificity for the carboxyl terminus of T antigen were used (Pab 423 or 405; ref. 25), staining with mutant-transfected cells was negative and similar to that with cells that had not received viral DNA (results not shown).

The structure of the T antigen synthesized in transfected monkey cells was examined more directly by $NaDodSO_4$ gel electrophoresis of immunoprecipitates of extracts prepared from [^{35}S]methionine-labeled transfected cells. Fig. 3, (lanes A and B) displays the proteins precipitated with anti-T antisera. Cells that had been transfected with wild-type DNA synthesized a protein with an apparent molecular mass of 94 kDa as expected (lane A). The apparent molecular mass of the variant T antigen synthesized in response to the frameshift DNA is 82–84 kDa (lane B). This was somewhat surprising, because the predicted amino acid sequence of the frameshift variant indicates that this protein should contain 19 amino acids *more* than the wild-type protein (refs. 12 and 28; see also Fig. 2). However, wild-type

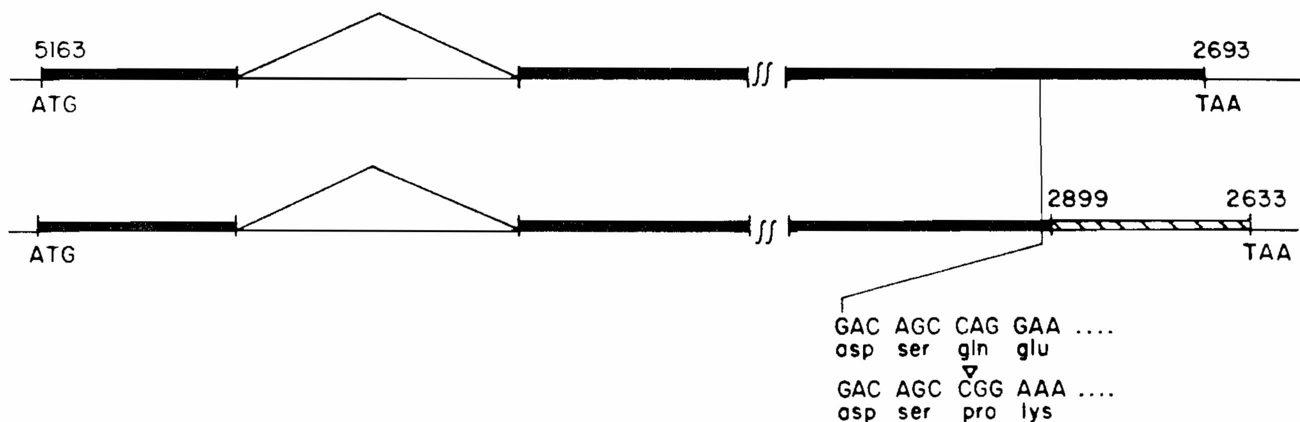

FIG. 1. Schematic diagram of the early region of SV40. ■, Large T antigen and frameshift protein coding sequence; ▧, coding region altered from the wild type by deletion of base pair 2,899.

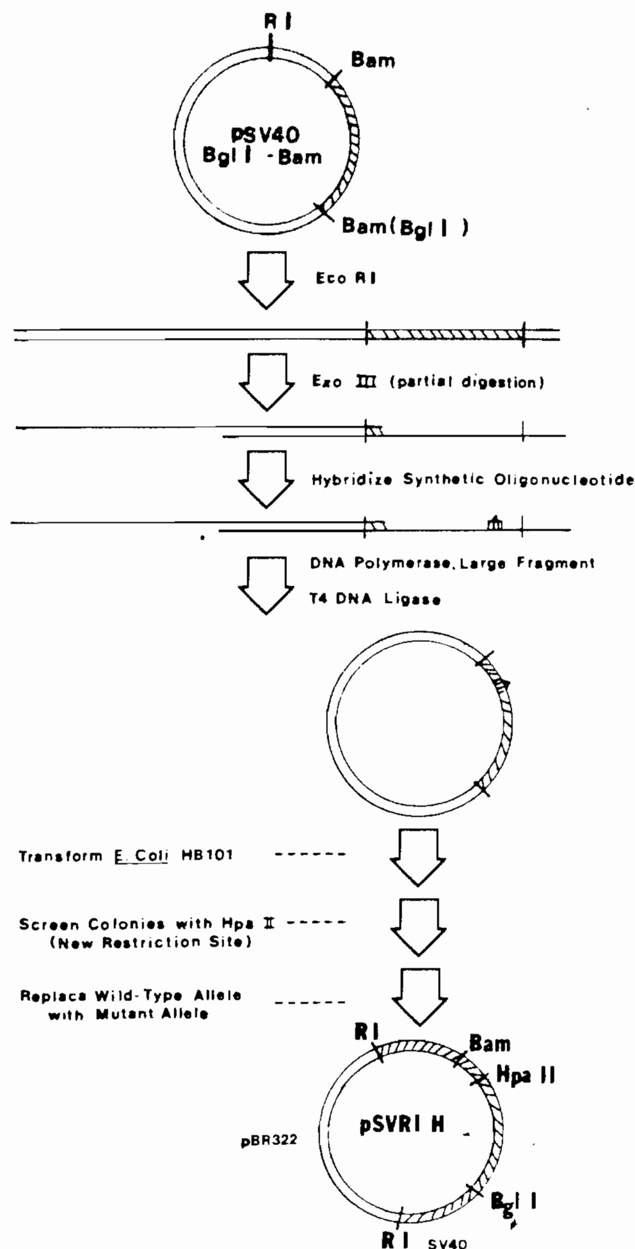

FIG. 2. Protocol for *in vitro* mutagenesis.

T antigen migrates anomalously on $NaDodSO_4$ gels, because its predicted molecular mass is 82 kDa. This anomalous migration is apparently not due to post-translational modification, because T antigens synthesized in various *in vitro* systems also display this same anomolous migration (29, 30). The sequences responsible for this property are located at the carboxyl terminus of the protein (31). Studies with viable mutants containing small in-phase deletions in this region show that the apparent molecular weights of T antigen reduced significantly more than would be predicted from the size of the deletions (32, 33). The dramatic size difference between the wild-type and frameshift T antigens observed here provides strong evidence that the carboxyl terminus of T antigen is indeed responsible for the anomolous migration of the wild-type protein in $NaDodSO_4$ gels. The amounts of T antigen detected by immunoprecipitation in response to both DNAs were comparable and consistent with the immunofluorescence data (Table 1).

Table 1. Transfection of wild-type and frameshift DNAs into monkey kidney cells

DNA	% cells T-antigen positive after 48 hr	Virus titer, pfu/ml
pSVRI	10.7	7×10^7
pSVRIH	5.3	0

Results are means of three separate experiments. pfu, plaque-forming unit(s).

The Frameshift Mutant Is Nonviable. Several studies have shown that in-phase deletions covering the region downstream of the frameshift are completely viable (32, 34–36), as is one that results in premature chain termination in this region as a result of a frameshift into the third possible reading frame (36). Wild-type and frameshift DNAs were transfected into BSC-1 monkey cells and, 10 days later, lysates were prepared. Aliquots of these lysates were used to infect monolayers of BSC-1 cells, and the titers of virus in the cell lysates were determined by plaque assay. The results show that no plaques were obtained from the frameshift mutant, strongly suggesting that this mutant is totally nonviable (Table 1).

An alternative method for determining viability is to measure T-antigen production by immunoprecipitation after infection. For this, cells were labeled with [^{35}S]methionine 48 hr after treatment with lysates obtained from the transfected cells. The results (Fig. 3, lanes C and D) confirm the conclusions from the plaque assay: no T-antigen synthesis was detected in the cells treated with the lysate obtained from the frameshift mutant-transfected cells. These results establish that the frameshift mutation results in synthesis of a T antigen incapable of giving rise to a productive infection.

The nature of the defect appears to reside in the inability of the variant T antigen to bring about viral DNA replication. The results of Southern blot analysis of viral DNA obtained by the Hirt procedure 4 days after transfection into COS-1 or CV-1 monkey cells are shown in Fig. 4. Although the frameshift DNA was replicated efficiently in COS-1 cells (lane 2), which contain a functional T antigen (37), no replication of this DNA could be detected in CV-1 cells (lane 5). These results establish that the T antigen encoded by the frameshift DNA is defective for DNA replication.

The Frameshift Mutant Is Transformation Defective. To determine whether the frameshift mutation affects the ability

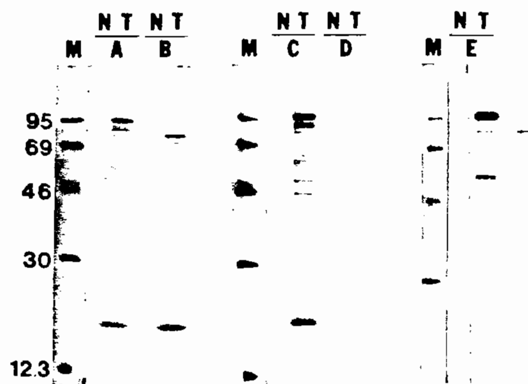

FIG. 3. [^{35}S]Methionine-labeled cell extracts from BSC-1 cells transfected with pSVRI (lane A) or pSVRIH (lane B) immunoprecipitated with normal hamster serum (N) or hamster anti-SV40 tumor serum (T) and analyzed on a 10-20% $NaDodSO_4$/polyacrylamide gel. Lanes C and D, extracts, from BSC-1 cells were made 48 hr after infection with lysates from either pSVRI (lane C) or pSVRIH (lane D) transfection; lane E, NIH 3T3 transformed with both pSVRIH and pSVRIori$^-$ plasmids; lanes M, markers (kDa). Note the 94-kDa large T antigen contributed by pSVRIori$^-$ and the 82- to 84-kDa protein from pSVRIH (arrow).

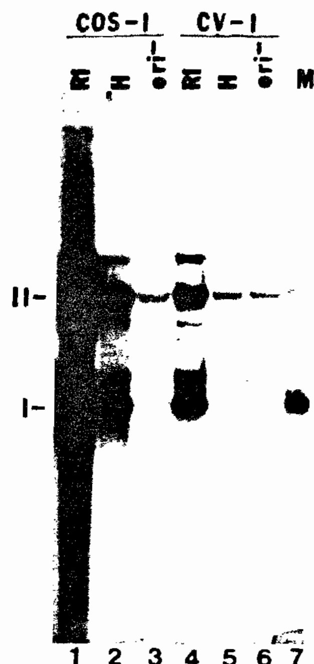

FIG. 4. Analysis of DNA after transfection. Autoradiogram of Southern blots of pSVRI (RI), pSVRIH (H), and pSVRIori⁻ (ori⁻) DNAs transfected by the DEAE-dextran method into COS-1 and CV-1 cells. DNA was harvested 4 days later. The marker lane (M) contains form I, supercoiled, and form II, relaxed circular, SV40 DNA.

of the viral DNA to transform nonpermissive mouse cells, wild-type and mutant DNAs were transfected into NIH 3T3 cells and, 48 hr later, samples of the cells were stained for T antigen detection by indirect immunofluorescence. The results (Table 2) are in contrast to those obtained when transfected monkey cells were similarly examined (Table 1). The percentage of fluorescent cells in the mutant-transfected cells was ≈5% of that of wild-type-transfected cells.

The transforming potential of the DNA was determined by measuring the capacity of the transfected cells to give rise to both dense foci and anchorage independent clones (Table 2). The results establish that the frameshift DNA is much less efficient at bringing about cellular transformation than is wild-type SV40 DNA. The reduction to <1% of that observed for wild type may in fact represent an overestimate of the ability of this mutant to transform cells: although all of the transformed clones obtained gave rise to a high percentage of cells that were initially positive for T-antigen immunofluorescence, subsequent staining of the same clones after passage showed all to be negative. Concomitantly, the ability of these cells to grow to high density or in agarose was lost. The basis of this unusual behavior is currently unknown. We considered the possibility that establishment or maintenance of the transformed state might require the 94-kDa wild-type T antigen but that the frameshift variant protein might facilitate or enhance this process. To test this idea, NIH 3T3 cells were cotransfected with the frameshift mutant DNA plus a SV40 genome that had been liberated from

Table 2. Transfection of wild-type and frameshift DNAs into mouse fibroblasts

DNA	% cells T-antigen positive after 48 hr	Dense foci, no. per 10^6 cells	Anchorage-independent clones, no. per 10^6 cells
pSVRI	0.2	130	75
pSVRIH	0.01	1	1
pSVRIori⁻	0.2	105	70
pSVRIori⁻/pSVRIH	0.21	100	65

Results are means of at least two independent experiments. A total of three dense foci and two anchorage-independent clones was isolated from transfections with pSVRIH.

another plasmid, pSVRIori⁻. This plasmid contains an intact SV40 genome with a small substitution at the origin of DNA replication (15). The consequences of this mutation are that the viral DNA is not replicated in monkey cells and, in mouse cells transformed with this DNA, the only large T-antigen species observed is the 94-kDa protein; i.e., the 100-kDa super T antigen invariably observed in anchorage-independent transformed mouse cells (27) is not produced (15).

The results in Table 2 show that the transformation frequencies obtained are not influenced by the presence of the frameshift DNA. This finding argues that the frameshift protein is not able to increase the efficiency of establishment of transformation by wild type and that the mutation is recessive with respect to the transformation function of T antigen. That transformed cells actually contain and express both DNAs was ascertained by analysis of the [^{35}S] methionine-labeled proteins precipitable with anti-T antisera. An example of such an analysis is contained in Fig. 3 (lane E), which shows that both wild-type and frameshift T antigens were synthesized. Although all of the >20 independent clones analyzed produced both T antigens, in all cases the amount of wild-type protein was much greater than the amount of variant protein. These results are consistent with the hypothesis that the frameshift protein is unstable in mouse cells.

DISCUSSION

Construction of a Specific Single-Base-Change Mutation by Use of Oligonucleotide-Directed Mutagenesis. The method that we have developed for oligonucleotide-directed *in vitro* mutagenesis is based on those used by others (38–40) but offers several significant advantages. First, the method uses pBR322-derived recombinant plasmids as starting material. This allows for the straightforward mutagenesis of relatively large DNA fragments. The use of large DNA fragments can be especially advantageous if large genes, transcription units, or, in this case, viral genomes are to be reconstructed after oligonucleotide-directed mutagenesis. Second, the steps required to convert the plasmid into a substrate for mutagenesis are straightforward (Fig. 1). The partial exonuclease III digestion can be easily controlled, and the extent of degradation can be readily monitored by denaturing agarose gel electrophoresis. Third, only relatively short stretches of DNA need to be synthesized by DNA polymerase (see Fig. 1). With other techniques, which require that a large single-stranded circular molecule be converted to a covalently closed double-stranded circle, this synthesis can be a limiting step. Finally, the method is quite efficient. We have found that, when reactions are carried out as described in *Materials and Methods*, 5–10% of the clones isolated after transfection contain the desired mutant plasmid.

A Frameshift into the Carboxyl-Terminal Alternative Reading Frame Inactivates Large T Antigen. Our initial hypothesis was that the frameshift large T antigen might function analogously to the polyoma middle-sized T antigen and perhaps facilitate or enhance transformation of nonpermissive mouse cells. However, the results shown here suggest that the opposite may in fact be true: the frameshift protein appears to be grossly defective in its ability to bring about cellular transformation, as measured by standard assays. The molecular basis for this defect is not yet clear. A number of studies have analyzed the effects of deletion mutations in this region on transformation (32-36). Among those studies, the entire region downstream of the frameshift mutation described here has been deleted, although none of the mutations results in a frameshift into the long open reading frame. None of these mutations affect in any detectable way the transforming potential of the resulting T antigens. Such observations suggest that the frame-

shift protein is defective not because required amino acids are not present but rather, because the amino acids encoded as a result of the frameshift are in fact deleterious in some way.

The frameshift mutant is also totally nonviable when the DNA is introduced into CV-1 monkey cells, apparently because it is completely unable to bring about viral DNA replication. This result is somewhat surprising, because the carboxyl terminal deletions studied by others that are transformation positive are also completely viable. However, it is noteworthy that many of these deletions (at least eight different mutations; see refs. 32, 34, and 35) were selected for viability, and all of these have proven to be in phase. Two other viable mutants were not selected for viability, but neither results in a frameshift into the long open reading frame (36). These findings are consistent with the idea that specific amino acids downstream from the site of the frameshift mutation are not required for lytic growth but the sequences encoded by the alternative reading frame are "poisonous" to T-antigen function.

How do these carboxyl-terminal sequences inactivate T antigen? They do not appear to change the nuclear localization of the protein, as judged by indirect immunofluorescence of fixed cells. Thus, the hydrophobic domain at the carboxyl terminus of the protein is not sufficient to cause the protein to become associated with the plasma membrane. It may be that, in mouse cells at least, these new sequences render the protein unstable. This hypothesis is consistent with our observations that only very low levels of this protein can be detected by both immunofluorescent staining of transfected cells (Table 2) and $NaDodSO_4$ gel electrophoresis of immunoprecipitates obtained from lysates of transformed cells (Fig. 3). It is conceivable that this inability of the frameshift T antigen to accumulate to wild-type concentrations in mouse cells is sufficient to explain its defect in transformation. However, protein instability is not sufficient to explain the failure of the frameshift T antigen to function in lytic growth because nearly wild-type levels of the protein are found in transfected monkey cells (Table 1 and Fig. 3).

Our experiments do not rule out a role for the alternative reading frame in either lytic infection or transformation. The reading frame may be expressed independently of T antigen, for example, to give rise to an ≈10-kDa protein. Alternatively, a fusion protein may in fact exist, but the precise site at which the frameshift occurs may be crucial and different from the one that we constructed *in vitro*. Finally, a protein similar to the one analyzed here may in fact play some role in productive infection or transformation, but perhaps we failed to detect this function with the assays and cells that we have used to date. Further experiments may reveal such a function.

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