The Large Tumor Antigen of Simian Virus 40 Encodes at Least Two Distinct Transforming Functions

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Received 10 August 1989/Accepted 21 August 1989

The large tumor antigen (T antigen) of simian virus 40 is necessary and sufficient for the neoplastic transformation of a number of established cell lines. Mutational analysis has revealed that a biochemical activity residing within the amino-terminal 121 amino acids of T antigen is sufficient to induce the transformation of some cell lines, such as C3H10T1/2. The same domain of the molecule also encodes the transactivation function of T antigen and the ability to complex with the retinoblastoma susceptibility gene product. However, the transformation of other lines, such as REF52, requires an additional activity that is affected by mutations in other portions of the molecule.

Simian virus 40 (SV40) induces tumors in animals and transforms a variety of cell types in culture. Evidence from a number of sources has demonstrated that the viral early region which encodes two proteins, the large and small tumor antigens (T antigen and t antigen, respectively), carries the tumorigenic potential of the virus (for a review, see references 5 and 10). In most cases studied, large T antigen alone is capable of full transformation, with small t antigen playing an ancillary role in some cell types or under certain assay conditions (2, 3, 11, 15, 29, 42, 43).

T antigen is a multifunctional protein (6). The single polypeptide chain carries multiple biochemical activities that act alone or in a concerted manner to control various aspects of viral infection and cellular behavior. Figure 1 summarizes current knowledge on mapping the various functional domains of T antigen. The molecular mechanisms by which T antigen induces cellular transformation are not known. However, two cellular proteins, the retinoblastoma susceptibility gene product (p105) and p53, both with suspected roles as tumor suppressors, are known to be complex with T antigen (9, 13, 14, 19, 22–24). Mutants that produce T antigens unable to form a complex with one or the other of these proteins are at least partially defective for transformation (9, 36). In addition, T antigen encodes an activity or activities capable of transactivating several viral and cellular promoters (4, 21, 26). Thus, T antigen acts on multiple cellular targets to induce a variety of changes in cellular properties.

One goal of our laboratories has been to learn which activities of T antigen are involved in contributing to the transformed phenotype. In this report, we discuss the properties of three mutant T antigens that lead us to conclude that at least two, and perhaps more, independent T-antigen-encoded biochemical activities are involved in tumorigenesis.

T-antigen mutants show cell type specificity with respect to transformation. In order to assess the effect of mutations on the transforming ability of T antigen, two different assays were used. In the focus assay, cells transfected with plasmids that express either wild-type or mutant DNA were plated in medium supplemented with 10% fetal bovine serum and maintained 4 to 6 weeks in culture. At this time, the dishes were scored for the presence of foci of dense, multilayered cells overgrowing the monolayer. This assay assesses the ability of T antigen to allow cell proliferation on a monolayer of morphologically normal cells. For mutants dl1137 and dl1137t, a second assay was used. Cells were cotransfected with a T-antigen-expressing plasmid and a plasmid that expresses a gene conferring resistance to the drug G418 and were maintained in a medium supplemented with 10% fetal bovine serum and containing G418. Colonies resistant to the drug were then scored as having either normal or transformed morphologies. These experiments were performed with two different established cell lines, C3H10T1/2 and REF52 (27, 41).

The structures of the mutant T antigens used in this study are shown in Fig. 1. The SV40 deletion mutant dl1137 carries a 31-base-pair deletion (nucleotides 4453 to 4423) in the viral early region and encodes a truncated T antigen consisting of the amino-terminal 121 residues followed by 11 missense residues (35, 40). Thus, the mutant protein is lacking the DNA-binding, ATPase, and adenovirus helping/host range domains present in the wild-type protein. In addition, the nuclear localization signal is missing, although the mutant protein has been shown to be distributed equally between the nucleus and the cytoplasm in transfected cells (40). Mutant dl1137t is a double mutant that also carries a deletion of the small t-antigen gene (38). Mutant 5080 carries an amino acid substitution of Pro-584→Leu that renders it defective for the ability to complex cellular protein p53 (36). 5080 fails to transform REF52 cells but retains the ability to transform the C3H10T1/2 line. In addition, the 5080 T antigen is defective in ATPase activity and oligomerization and is underphosphorylated relative to wild-type T antigen (49). Both were isolated by site-directed mutagenesis, using the heteroduplex mutation procedures (34) with sodium bisulfite as the mutagen. Mutant 5002 carries two amino acid substitutions of Leu-19→Phe and Pro-28→Ser but retains the ability to replicate viral DNA (K. W. C. Peden et al., manuscript in preparation).

REF52 or C3H10T1/2 cells were plated at a density of 5 × 10⁵ cells per 10-cm-diameter dish 24 h before transfection. The cells were fed with fresh medium 4 h before transfection. CaPO₄ precipitates (18) were applied directly to the medium. After incubation for 4 h, the cells were washed...
TABLE 1. Transformation of C3H10T1/2 and REF52 Cells by SV40 Mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Relative Focus-forming ability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C3H10T1/2</td>
</tr>
<tr>
<td>Mock</td>
<td>0.00</td>
</tr>
<tr>
<td>Wild type</td>
<td>1.00</td>
</tr>
<tr>
<td>d1137</td>
<td>0.17</td>
</tr>
<tr>
<td>d1137t</td>
<td>0.07</td>
</tr>
<tr>
<td>5002</td>
<td>0.10</td>
</tr>
<tr>
<td>5080</td>
<td>0.15</td>
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</table>

Wild-type T antigen transformed both cell types with approximately the same efficiency, as measured by both assays. Mutants d1137, 5002, and 5080 exhibited a cell type specificity with respect to transformation (Table 1). Small t antigen is not absolutely required for focus formation on C3H10T1/2 cells, since d1137t was positive in this assay. The G418 assays for d1137 and d1137t gave the same results as the focus assay (data not shown). All of these mutants induced the morphological transformation of the C3H10T1/2 line, although at a consistently reduced frequency relative to that of the wild type. On the other hand, none of these mutants was capable of transforming the REF52 cell line, as determined by the focus assay. One possible explanation for the cell type specificity for transformation exhibited by these mutants is that the altered T antigens are rapidly degraded in REF52 cells but not in C3H10T1/2 cells. To test this possibility, we measured the half-life of the d1137-encoded T antigen. The mutant protein was as stable as wild-type T antigen in both cell types (data not shown).

We conclude that these mutants are altered in an activity required for the transformation of REF52 cells. The same activity or an additional activity is required for transformation of C3H10T1/2 cells at the wild-type frequency. However, since all three of these mutants induced the morphological transformation of C3H10T1/2 cells, we infer that they retain an activity or activities sufficient to transform this cell type. And since d1137 expresses a truncated protein encoding only the amino-terminal 121 residues of large T antigen, we conclude that the biochemical activity or activities of T antigen that reside within these residues are sufficient to transform C3H10T1/2 cells.

T-antigen-p53 complex formation and transformation. Independent C3H10T1/2 lines expressing the wild-type and mutant T antigens were radiolabeled with [35S]methionine, and the T antigen was immunoprecipitated with a polyclonal anti-T-antigen serum. Wild-type T antigen as well as the T antigens encoded by the 5080 and 5002 mutants migrated with an apparent molecular weight of 94,000 on sodium dodecyl sulfate-polyacrylamide gels (Fig. 2). The d1137-encoded T antigen migrated at a molecular weight of approximately 20,000 (Fig. 3). The transformation-related nuclear phosphoprotein p53 was coprecipitated with T antigen in cell lines transformed with the wild type or the mutant 5002. However, mutants d1137 and 5080 lost the ability to complex with p53. We conclude that T-antigen-p53 complex formation is not necessary for the transformation of the C3H10T1/2 line, as measured by the focus assay. Since 5002 retained the ability to complex with p53 but did not transform REF52 cells, we conclude that T-antigen-p53 complex

FIG. 1. Functional domains of SV40 T antigen. Large T antigen is represented as a linear array of amino acids. The various shades represent the different domains or regions of the molecule. NL, Nuclear localization signal; FB, sequences thought to be important for complexing the retinoblastoma susceptibility gene product, Ad, adenovirus.
formation is not sufficient for the transformation of REF52 cells.

**Amino-terminal domain of T antigen encodes a transactivation function.** Large T antigen carries an activity capable of transactivating cellular and viral promoters (4, 21, 26). In order to determine whether the amino-terminal domain of large T antigen was capable of transactivating viral promoters, we tested the ability of the d11137-encoded T antigen to transactivate the adenovirus E2 promoter. The possibility of small t-antigen-mediated transactivation (25) was ruled out by using a d11137-expressing plasmid that carried a second mutation eliminating small t-antigen expression (38). C3H10T1/2 cells were transfected with 10 μg of d11137 DNA and an equal amount of P2cat plasmid, which contains the chloramphenicol acetyltransferase (CAT) gene linked to the adenovirus 5 E2 promoter (52). Cell extracts were prepared 48 h posttransfection, and CAT assays were performed as described elsewhere (17). The results are shown in Fig. 4. d11137 efficiently transactivated the E2 promoter, being comparable in activity to the E1a protein. We conclude that the amino-terminal domain encodes an activity capable of transactivation.

**Multiple transforming functions of large T antigen.** A number of laboratories have undertaken a genetic analysis of this protein in an attempt to correlate activities with transformation. These investigations have given somewhat conflicting results. An activity sufficient to induce the immortalization of primary cells has been reported to reside within the amino-terminal portion of the molecule (1, 8, 46). This activity cooperates with the Ha-ras cellular oncogene to transform primary cells (31). The same region of the molecule has been shown to be sufficient to induce the morphological transformation of some established cell lines, including C3H10T1/2 (7, 39, 44-48). On the other hand, other studies have indicated that an activity altered by mutations within the central portion of the molecule is necessary for the transformation of some primary and established cell lines (28, 36, 37, 39, 40, 50, 51).

The studies presented here indicate that T antigen encodes at least two distinct activities capable of influencing transformation. One of these activities resides in the amino-terminal T-antigen fragment encoded by d11137. We hypothesize that this activity is responsible for the immortalizing activity of T antigen as well as its ability to cooperate with the ras oncogene and to transform certain established cell lines. Since the d11137-encoded protein complexes with the retinoblastoma susceptibility gene product (Peden et al., manuscript in preparation), the transforming activity of this

**FIG. 2.** T antigens synthesized by transformed cell lines. C3H10T1/2 cells transformed with wild-type or mutant T antigen were labeled with [35S]methionine for 3 h, and extracts were prepared as described in the text. T antigens were immunoprecipitated with polyclonal hamster anti-T-antigen serum and displayed on sodium dodecyl sulfate-polyacrylamide gels. T antigen from productively infected monkey cells (lane 1), parental (untransformed) C3H10T1/2 cells (lane 2), cells transformed with wild-type T antigen (lane 3), 3002 (lane 4), and 5080 (lane 5) are shown.

**FIG. 3.** T antigen present in d11137-transformed C3H10T1/2 cells. Results for parental (untransformed) C3H10T1/2 cells (lane 1), cells transformed with wild-type T antigen (lane 2), and three independent lines transformed with d11137 (lanes 3 to 5) are shown.

**FIG. 4.** Transactivation of adenovirus E2 promoter by d11137-encoded T antigen. C3H10T1/2 cells on 10-cm-diameter dishes were transfected with 10 μg of P2CAT plasmid alone (lane 1) or cotransfected with 10 μg of P2CAT and 10 μg of pE1a (lane 2), 10 μg of d11137 (lane 3), or 10 μg of pSV-B3 encoding wild-type SV40 T antigen (lane 4). Extracts were prepared 48 h posttransfection, and one-fifth of the extract was used in a 2-h CAT assay in each case.
domain may involve complex formation with the Rb gene product. The transactivation function of T antigen also resides within this domain, and thus this activity may also play a role in transformation.

Other established lines, such as REF52, require a second activity which is affected by mutations in the central portion of the molecule, such as the classic tsA lesions, and by 5080. This portion of T antigen is known to be required for complex formation with the cellular protein p53 (32, 40, 51). Since p53 itself acts as a dominant oncogene (12, 20, 33), a tumor suppressor (13), or both and has been implicated in SV40-induced transformation (30), it is tempting to speculate that the ability to complex p53 is the second transforming function of T antigen. However, the results reported here indicate that this may not be the case for the REF52 cell line. Mutant 5002-encoded T antigen complexed with p53, yet was defective for the transformation of REF52 cells. Thus, more than two of the multiple activities of T antigen may be involved in conferring the transformed phenotype.

This work was supported by Public Health Service grant CA40586 from the National Institutes of Health and in part by Biomedical Research Support grant 2S07RR07084-23.

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