Ability of a T-Antigen Transport-Defective Mutant of Simian Virus 40 To Immortalize Primary Cells and To Complement Polyomavirus Middle T in Tumorigenesis

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The oncogenic potential of polyomavirus in newborn rats could not be expressed by a genome encoding only the middle T antigen but required the presence of one of the other two viral early genes, small T or large T. The tumorigenicity defect could also be complemented by other viral or cellular genes that are known to be implicated in immortalization and establishment functions. The simian virus 40 (cT)-3 mutant (R. E. Lanford and J. S. Butel, Cell 37:801–813, 1984), which fails to localize to the nucleus, has the capacity to complement polyomavirus middle T in tumorigenesis and to immortalize primary rat embryo fibroblasts when it was cotransfected in the presence of pSV2-neo. Our data suggested that under the conditions of DNA-mediated tumor induction and cotransfection with a dominant selection marker, the cellular alterations achieved by nonnuclear oncogenes such as polyomavirus small T and simian virus 40 (cT)-3 were sufficient to complement polyomavirus middle T in transformation and tumorigenesis.

A challenging problem in the study of carcinogenesis is to determine how oncogenes transform cells and how their various modes of action can be interrelated. Many of the oncogenes identified to date can be grouped into functional classes on the basis of their effects on cellular phenotype, which suggests that there is only a small number of action mechanisms of the oncogene-encoded proteins (see reference 26 for a review). Some of these proteins mediate distinct and perhaps cooperative transformation-related functions that appear to correlate with their particular localization within the cell (13, 21). In the cytoplasm, they may regulate levels of critical second messenger molecules, whereas in the nucleus they probably modulate the activity of the transcriptional machinery of the cell (26). The requirement for two separate viral oncogenes with distinct functions can be illustrated by polyomavirus-mediated transformation. Polyomavirus encodes three different early proteins: the large, middle, and small T antigens (25). The large T antigen is localized in the nucleus, whereas the middle T antigen is found on the cell surface (11). The middle T antigen is capable of transforming established cell lines by itself but requires the presence of either small T or large T to transform primary cells in vitro and to induce tumors in newborn rats (2, 4). This suggests that transformation by polyomavirus requires the cooperation of two distinct functions: one provided by the membrane-bound middle T antigen, the transforming protein, and the other by the nuclear T antigen or any other nuclear function implicated in immortalization or establishment.

In this work we attempted to complement polyomavirus middle T antigen with the simian virus 40 (SV40) cT mutant (15). Because of a single amino acid change (Lys-128 → Asn) which interrupts a stretch of five positively charged amino acids, the mutant large T antigen is not transported to the nucleus. Transformation of established cell lines by the cT mutant is not significantly reduced under normal culture conditions, but transformation of primary cells does not occur in the absence of detectable levels of nuclear T antigen (16). Our results show that, although the mutant large T antigen is not transported to the nucleus, it can efficiently complement polyomavirus middle T antigen in the induction of tumors in vivo and immortalize primary rat embryo fibroblasts in vitro.

MATERIALS AND METHODS

Plasmids. pSV1 (Fig. 1) carries the SV40 early region in the BamHI site of pBR322 and encodes both large and small T antigens. pSV2 (Fig. 1) encodes the SV40 large T antigen only. The sequences coding for large T antigen were provided by deletion mutant Δ2005. Because of a deletion between 0.54 and 0.59 map units, this mutant does not produce the small T antigen (22). pBSV(cT)-3 (Fig. 1) is an intact copy of the SV40(cT)-3 genome cloned into pBR322 at the BamHI site (15). SV40(cT)-3 has a point mutation at nucleotide 4434 that abolishes the transport of T antigen to the nucleus (15). pSV2cT-3 is a recombinant encoding only SV40(cT)-3 large T antigen. pMT3 (Fig. 1) is a recombinant which expresses only the polyomavirus middle T antigen (2). pMSV1 (Fig. 1) is a plasmid encoding both polyomavirus middle T antigen and the SV40 early proteins. pMSV2 is a similar construct encoding both middle T and the SV40 large T antigens. pMSVcT-3 encodes both middle T antigen and the SV40(cT)-3 genome. pMSV2cT-3 encodes both middle T antigen and the large T antigen from the cT-3 mutant. pSV2-neo is a plasmid which expresses neo, a dominant selection marker (23).

Cells, transfections, and growth assays. Primary Fischer rat embryo fibroblasts were transfected in the presence of pSV2-neo by the calcium phosphate-DNA coprecipitation procedure as previously described (1). Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The DNA to be transfected (8 μg) was mixed with high-molecular-weight calf thymus DNA (10 μg) as a carrier. After 20 to 24 h of exposure to DNA, the cells were passaged and plated at 20 to 30% confluence. After 18 h, G418 was added to the medium at a concentration of 400 μg/ml. The medium supplemented with the drug was changed every 5 days. Colonies were first detected after 7 to
FIG. 1. Structure of recombinant plasmids. pSV1 carries the SV40 early region (strain 777) inserted in the BamHI site of pBR322. pBR322 sequences are not shown. pSV2 encodes the SV40 large T antigen only. Because of the deletion (///) between 0.54 and 0.59 map units, the mutant genome does not produce the small T antigen (22). pBSV(cT)-3 carries the SV40(cT)-3 genome. The cT mutant encoding only the large T antigen was designated pSV2cT-3. pMSV1 is a plasmid encoding both polyomavirus middle T (pmt) and the SV40 T antigens. The SV40 genome was cleaved by BamHI (SV40 nucleotide 2533) and inserted into pMT3, the middle T clone (polyomavirus nucleotide 4632). B, BamHI; T, TaqI; H, HindIII; ori, origin. □□, Exons.

10 days in the selective medium, and 2 to 3 weeks later independent colonies were picked, transferred to 15-mm Linbro microplates, and passed at least once in medium containing 400 μg of G418 per ml. Most of the cells that senesced did not sustain more than one or two divisions after passage 1. Some cell lines senesced after passage 3 or occasionally after passage 4. However, once cell lines had been passed five times, they could be considered immortalized. Of 217 lines that reached 5 passages, all could be brought to 10 passages within weeks. Twenty of these lines were picked randomly, and all could be brought to 30 passages. The cell lines that were either immortalized or oncogenically transformed were subcultured at a 1:5 dilution each time they reached confluence and were maintained in G418-free medium. Immortalization efficiencies were evaluated by counting the percentage of G418-resistant colonies sustaining five or more passages 7 to 8 weeks after the first transfer.

In vivo tumorigenesis. Recombinant DNAs were injected subcutaneously (2 μg of linear DNA in 50 μl of phosphate-buffered saline) into the necks of 1-day-old Fischer rats. The animals were examined regularly for tumor development during a 4-month period. Tumor tissues were propagated in culture as described previously (2). Statistical analyses of the results were performed with the χ² test.

RESULTS

In vivo tumorigenesis. Tumor induction by injecting DNA into newborn rodents provides an in vivo equivalent to a transformation assay but appears to be a more stringent and rigorous criterion of oncogenic transformation (5). To determine the ability of recombinant plasmids to induce tumors in newborn rats, we inoculated 2 μg of DNA into the necks of 1-day-old Fischer rats and observed the animals for tumor development over a 4-month period. The plasmid pPB21, carrying the wild-type polyomavirus genome, induced tumors in about half of the rats injected (Table 1). The plasmid pMT3, carrying the middle T gene alone, was completely inactive. This shows that functions other than those expressed by the polyomavirus middle T antigen are required for tumor induction in newborn rats. In a previous study we found that the tumorigenicity defect of middle T antigen could be complemented by either small T or large T antigen (4). The role of small T antigen in transformation is still poorly understood. However, several studies have implicated large T antigen in immortalization and establishment (6, 20). Thus, one could expect other immortalizing genes of viral as well as cellular origin to complement polyomavirus middle T in the tumorigenicity assay. Middle T was indeed complemented (Table 1), although with low efficiencies, by SV40 large T (P = 0.01), adenovirus early region 1A (P = 0.15), and the myc oncogene (P = 0.05). When inoculated separately into newborn rats, none of the plasmids carrying the establishment genes gave rise to tumors (data not shown). Furthermore, we observed virtually the same results when polyomavirus middle T and the establishment genes were co-injected as separate linear plasmids or linked in the form of a hybrid plasmid (data not shown). The recombinant pMSV2-b, encoding the N-terminal fragment of large T antigen, was also active, although at a very low efficiency.

To determine the properties of the SV40 large T mutant that fails to localize to the nucleus, we used the PARA

TABLE 1. Tumorigenicity of recombinant plasmids

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>Coding capacity</th>
<th>Incidence of tumors in newborn rats b</th>
</tr>
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<tbody>
<tr>
<td>pPB21</td>
<td>Pyd middle T, small T, large T</td>
<td>18/37 48.6</td>
</tr>
<tr>
<td>pMT3</td>
<td>Py middle T</td>
<td>0/58 0</td>
</tr>
<tr>
<td>pME1A</td>
<td>Py middle T + Ad2 E1A</td>
<td>2/23 8.7</td>
</tr>
<tr>
<td>pMc-myc</td>
<td>Py middle T + c-myc</td>
<td>4/40 10.0</td>
</tr>
<tr>
<td>pMSV1</td>
<td>Py middle T + SV40 large T</td>
<td>12/26 46.2</td>
</tr>
<tr>
<td>pMSV2</td>
<td>Py middle T + SV40 large T</td>
<td>3/19 15.8</td>
</tr>
<tr>
<td>pMSV2-b</td>
<td>Py middle T + SV40 large T</td>
<td>1/19 5.3</td>
</tr>
<tr>
<td>pMBSVcT-3</td>
<td>Py middle T + SV40 large T (N terminal, 311 amino acids)</td>
<td>3/19 15.8</td>
</tr>
<tr>
<td>pMSV2c-T3</td>
<td>Py middle T + SV40 large T (cT mutant)</td>
<td>20/41 48.8</td>
</tr>
<tr>
<td>pMBSVcT-3</td>
<td>Py middle T + SV40 large T (cT mutant)</td>
<td>13/45 28.9</td>
</tr>
</tbody>
</table>

* pMT3 was cleaved by HindIII. Plasmids of the MSV series were cleaved by ClaI or SalI (single cut in the pBR322 sequence).
* One-day-old Fischer rats were inoculated subcutaneously in the neck with 2 μg of linear DNA in 50 μl of phosphate-buffered saline.
* Number of rats with tumors/number of rats injected.
* Py, Polyomavirus.
* The E1A region of adenovirus 2 (Ad2) (0 to 5.0% of the genome) was cloned in pBR322 and linked to pMT3. The plasmid was linearized by EcoRI before injection into newborn rats.
* pMT3 was cleaved by BamHI plus EcoRI (nucleotides 4632 and 1500), and the fragment carrying middle T was inserted between the BamHI and EcoRI sites of pSV-c-myc (13). The plasmid was linearized by EcoRI before injection into newborn rats.
* Plasmid derived from pMSV2 by insertion of an Xhol linker of 8 base pairs in the HpaI site at nucleotide 3733.
* The difference between pMSV2 (3/19) and pMSV2c-T3 (13/45) is not significant.
mutant isolated by Lanford and Butel (15). The mutant was as effective as wild-type SV40 in complementing middle T in the tumorigenicity assay (Table 1). The genome used in this experiment, pBSV(cT)-3, is an intact genome capable of encoding the large T as well as the small T antigen. In view of the ability of the polyomavirus small T antigen to complement middle T in tumorigenesis, we suspected that the activity of the SV40(cT)-3 genome could be due to the presence of the small T antigen. To inactivate the small-T-antigen-coding region, the structure of pBSV(cT)-3 was modified by filling the gaps at the TaqI site at nucleotide 4739 (Fig. 1). Because the TaqI site is located in the large-T-antigen intron, the addition of two nucleotides in this site had no effect on the structure of the large T antigen but produced a frameshift in the small-T-antigen-coding sequence after Phe-141 that resulted in a termination signal 23 codons downstream. Surprisingly, inactivation of the small T-antigen-coding region did not abolish the activity of the SV40(cT)-3 mutant (Table 1). pMSV2cT-3 was less active than pMBSVcT-3 (P = 0.09), the construct encoding both large T and small T antigens, but so was pMSV2, the plasmid encoding the wild-type SV40 large T antigen (P = 0.06). These results indicate that the SV40 small T antigen contributed to the tumorigenic potential of both pMSV1 and pMBSVcT-3. They also showed that the cT mutation did not affect significantly the ability of the SV40 large T protein to complement polyomavirus middle T in tumorigenesis.

**Viral proteins synthesized in tumor cells.** In a previous study we showed that the tumors obtained by injecting middle T-producing plasmids into newborn rats expressed, in addition to the middle T antigen, the polypeptides encoded by the cooperating gene (e.g., polyomavirus small T antigen, SV40 large T antigen, and adenovirus E1A) (3). Tumors induced by pMSV2 (polyomavirus middle T plus SV40 large T) produced the host-encoded protein designated p53 as well (3). To further characterize these tumors, several representative cell lines were examined by immunofluorescence for the intracellular localization of T antigen. All of the cell lines derived from pMSV2-induced tumors displayed the typical nuclear T-antigen pattern of fluorescence (Fig. 2a). By contrast, all of the tumor cell lines established by pMSVcT-3 displayed a bright cytoplasmic reaction for T antigen (Fig. 2b). No faint or diffuse nuclear reaction could be observed in these cells.

FIG. 2. Intracellular distribution of SV40 large T antigen in tumor and immortalized cell lines. Tumor cell lines induced by injecting pMSV1 (panel a) and pMBSV(cT)-3 (panel b) into newborn rats are shown, as are primary rat embryo fibroblasts immortalized by transfecting pSV2 (panel c) and pBSV(cT)-3 (panel d) in the presence of pSV2-neo. After fixation in acetone-methanol (2:1) for 15 min at -20°C, the cells were stained for large T antigen with a hamster anti-SV40 tumor serum. Magnification, ×130.
antigen binding is the determining factor regulating the intracellular localization and accumulation of p53 in the tumor cells.

**Imortalization of primary rat fibroblasts by SV40(cT)-3.**

The ability of pSV2cT-3 to complement polyomavirus middle T antigen in tumorigenesis raised the intriguing question of the effect of cytoplasmic large T antigen on the cell phenotype. To determine whether the mutant was able to immortalize primary cells, we transfected primary rat embryo fibroblasts with recombinant plasmids in the presence of pSV2-neo so that the transfected cultures could be placed under G418 selection. pSV2cT-3 immortalized as efficiently as did wild-type large T antigen (Table 2). Although most of the cell lines established with these plasmids exhibited a flat, untransformed phenotype in cultures, some cell lines had a morphology characteristic of transformed cells. When these lines were assayed for growth in soft agar, only some of them were able to form colonies (data not shown). This suggests, in agreement with a previous study (4), that as a result of neo selection most of the cell lines exhibited only a partially transformed phenotype. Several representative cell lines were examined by immunofluorescence for the intracellular localization of T antigen. All of the cell lines established by pSV2 displayed a nuclear reaction for T antigen (Fig. 2c). Those established by pBSV(cT)-3 displayed the typical cytoplasmic T-antigen pattern of fluorescence (Fig. 2d). Although these observations did not rule out the possibility that some cT antigen accumulated in the nucleus, they implied that the capacity of pBSV(cT)-3 to immortalize or transform primary rat embryo fibroblasts did not depend upon the ability of a subpopulation of the transfected cells to transport the mutant T antigen to the nucleus.

**DISCUSSION**

The oncogenic potential of polyomavirus in newborn rats cannot be expressed by a genome encoding only the middle T antigen but requires the presence of one of the other two viral early genes, small T or large T. A similar complementary function can also be exerted by other viral or cellular genes such as SV40 large T, adenovirus E1A, and the myc oncogene, i.e., genes that are known to be implicated in immortalization and establishment (6, 10, 18). This is consistent with the widely held hypothesis which suggests that oncogenic transformation requires cooperation between establishment and transforming functions (13, 21). The establishment of primary cells is usually associated with the expression of oncogenes whose products are localized in the nucleus, while malignant transformation of immortalized cells is associated with the expression of cytoplasmic oncoproteins (26). In this work we show that the cytoplasmic middle T oncogene can be complemented by the SV40(cT)-3 mutant which fails to localize to the nucleus. This result is reminiscent of the effect of polyomavirus small T antigen in tumorigenesis. Although small T antigen can cooperate with middle T antigen in the induction of tumors in newborn rats (2, 5), the former cannot be implicated in establishment and immortalizing functions (19). This suggests that all the genes that complement the transforming function of polyomavirus middle T antigen do not necessarily create identical changes in cellular behavior but may, on the contrary, interact with different cellular targets. It is curious, however, that the SV40(cT)-3 large T antigen immortalized primary rat embryo fibroblasts with virtually wild-type efficiency. This supports the notion that, unlike small T antigen, SV40(cT)-3 may complement middle T antigen in tumorigenesis by providing an establishment function.

Our immortalization studies are in prima facie disagreement with previous reports showing that SV40 mutants which fail to localize to the nucleus are defective for transformation of primary cells (8, 16). It has been proposed that transformation by SV40 is regulated by both nuclear and plasma membrane-associated T antigen (16). Immortalized cells could dispense with the nuclear T-antigen function. However, the cT mutant would be transformation negative in primary cells. The model also predicts that the cT antigen would be very ineffective in immortalization. An apparent anomaly in this scheme is the transformation of mouse embryo fibroblasts, because a subpopulation of cells has the capacity to partially transport the cT antigen to the nucleus. Our observations cannot completely exclude the possibility that undetectable yet biologically functional amounts of cT antigen accumulate in the nucleus. However, they do not confirm the claim by Lanford et al. (16) that the capacity of the mutant cT antigen to immortalize is dependent upon the ability of a subpopulation of the transfected cells to transport the antigen to the nucleus. If both the immortalization of primary cells and the immortalization of middle T antigen in tumorigenesis are due to undetectable levels of nuclear cT antigen, one would expect the cT mutant to be less active in these processes than is the wild-type genome. This assumption is based upon previous experiments with polyomavirus showing that the ability of primary cells containing the large T gene to become established in culture is related to the level of large T expression as early as the first passage (1). Furthermore, after transfer of a large T plasmid carrying a tsa mutation, cell lines are established at 33°C with the same efficiency as that of the wild-type large T gene, but their growth is arrested after a shift to 40°C, with a progressive loss in cell viability (20; unpublished results). These results indicate that only cells capable of expressing sufficient amounts of large T antigen can become immortalized and that there is a continuous requirement for a large T-antigen function in the maintenance of immortality. Thus, currently we favor the model whereby SV40(cT)-3 immortalizes primary rat embryo fibroblasts and complements middle T antigen in tumorigenesis by providing a nonnuclear large T-antigen function. The conflict between this model and the observation that the mutant is defective for transformation of primary cells probably reflects differences in the transfection conditions. The effect of neo and gpt selection on the phenotype of cells transfected with various oncogenes is well documented. For example, both ras and polyomavirus middle T, which are usually considered to be late-step or

**TABLE 2. Immortalization efficiencies***

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Coding capacity</th>
<th>No. immortalized/ no. tested</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>pSV2</td>
<td>SV40 large T</td>
<td>73/95</td>
<td>76.8</td>
</tr>
<tr>
<td>pBSV(cT)-3</td>
<td>SV40 large T (cT mutant)</td>
<td>31/40</td>
<td>77.5</td>
</tr>
<tr>
<td></td>
<td>+ SV40 small T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSV2cT-3</td>
<td>SV40 large T (cT mutant)</td>
<td>51/77</td>
<td>66.2</td>
</tr>
</tbody>
</table>

* Colonies of cells resistant to G418 were picked 3 to 4 weeks after selection and transferred into Linbro microplates. The cells were subcultured at a 1:5 dilution each time they reached confluence. Immortalization efficiencies were evaluated by counting the percentage of cell lines reaching five or more passages 7 to 8 weeks after the first transfer. Plasmid DNA (6 µg) was cotransfected with 2 µg of pSV2-neo and 10 µg of calf thymus carrier DNA. Only 1 of 452 colonies established by pSV2-neo, lacking an immortalizing gene, could be brought to five passages.
conversion genes, have been shown to be immortalized when
introduced into cells in the presence of a selection marker (4,
24). Furthermore, cells bearing a single oncogene will not
grow into a large mass unless the normal neighboring cells
are removed by killing with a cytotoxic drug (7, 24). It is
interesting that like the cT mutant, bc1051 (a recombinant
encoding both middle and small T antigens) does not trans-
form primary cells in culture, yet both recombinants are
biologically active when they are cotransfected with the neo
marker (4; this work) or when they are assayed for in vivo
tumorigenicity (2). In our interpretation of these data we
suggest that the response of a cell to an oncogene is strongly
influenced by its environment. Under certain conditions, like
those observed in cotransfection with a dominant selection
marker or in DNA-mediated tumor induction, the cellular
alterations achieved by nonnuclear oncogenes such as small T
antigen and SV40(cT)-3 would be sufficient to complement
polyomavirus middle T in transformation and tumorigenesis.
Other studies have shown that some nuclear oncogenes can
cooperate with one another (12) or even transform sponta-
neously immortalized cell lines (7). Thus, it is conceivable
that different oncogenes affect both immortalization and
transformation to various degrees, depending on the assay
system chosen. This suggests that, although classification
of oncogenes into these two categories may be a useful opera-
tional definition, the two processes may be functionally
related.

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