Polyomavirus Large T Mutants Affected in Retinoblastoma Protein Binding Are Defective in Immortalization

ANGELE LAROSE,¹ NICHOLAS DYSON,² MONIQUE SULLIVAN,¹ ED HARLOW,² AND MARCEL BASTIN^{1*}

*Department of Microbiology, University of Sherbrooke, Sherbrooke, Quebec J1H 5N4, Canada,¹
and Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724²*

Received 26 September 1990/Accepted 1 February 1991

To clarify the relationship between the various activities of the polyomavirus large T antigen and the contribution of this oncogene to neoplastic transformation, we constructed a series of mutants with small deletions or single-amino-acid substitutions in two separate regions of the protein. These sequences were targeted because they showed considerable similarity to conserved regions 1 and 2 of adenovirus E1A which are thought to be binding sites for the retinoblastoma gene product (pRB). The pRB-binding properties of the large T mutants were assessed with an *in vitro* coimmunoprecipitation assay. pRB binding was readily detected with wild-type large T, but coprecipitation was completely abolished by as little as a single amino acid substitution (Asp-141→Glu or Glu-146→Asp) in region 2 of the polyomavirus large T antigen. Mutants defective in pRB binding were unable to immortalize primary rat embryo fibroblasts, suggesting that association with pRB is an important component of immortalization mediated by polyomavirus large T. The mutations in region 1 affected pRB binding only marginally, yet some of them severely impaired immortalization, indicating that pRB binding may be essential but not sufficient for immortalization.

The early region of polyomavirus encodes three distinct proteins, the large, middle, and small tumor antigens (T-Ags). The middle T-Ag plays a central role in oncogenic transformation. It can transform established cells in culture (45) but requires cooperation from either small T-Ag or large T-Ag to induce tumors in rats (2) or efficiently transform primary rat embryo fibroblasts (3). The large T-Ag is a multifunctional protein that plays important roles in both productive infection and viral transformation. It is required to initiate each new round of viral DNA replication, to stimulate host DNA synthesis, and to repress early-gene expression during late infection (44). The polyomavirus large T-Ag differs from its simian virus 40 (SV40) counterpart in that it is not sufficient to cause malignant transformation by itself. However, it can confer on primary rat embryo fibroblasts the ability to grow in long-term culture without entering crisis, a process called establishment or immortalization (1, 8, 23, 24, 39). It can reduce the serum requirement of established cell lines and act in concert with other oncogenes to transform primary cells or induce tumors upon inoculation into newborn rats (2, 3, 29, 38).

The mechanism by which polyomavirus large T-Ag complements other oncogenes in transformation is not known. Previous studies have shown that the domain involved in immortalization is localized within the amino-terminal 219 residues (1). It is encoded by the first exon and a small portion from the second exon, which also encodes a part of middle T-Ag in another translational reading frame. At first glance, this region does not have any homology with SV40 T-Ag. However, both T-Ags contain the amino acid sequence D/N-L-X-C-X-E (34), a motif that is found in all viral proteins that are known to date to bind to the product of the retinoblastoma susceptibility gene (pRB) (9, 10, 12, 47).

The retinoblastoma gene (RB-1) was identified through its association with inherited predisposition to retinoblastoma. RB-1 is deleted or mutated with high frequency in retino-

blastomas and has subsequently been found to be similarly altered in a variety of tumors and tumor cell lines (for example, see references 18, 19, 20, and 31). Since it is the inactivation of RB-1 that has been linked to tumor development, the normal function of pRB is believed to lie in the negative regulation of cell growth. It has been proposed that by binding to pRB, the viral proteins may inhibit its regulatory function in a manner that mimics the loss of RB-1 seen in tumor cells (9, 47).

Genetic studies show that the regions of human papillomavirus type 16 (HPV-16) E7 protein, adenovirus type 5 (Ad5) E1A polypeptides, and SV40 large T-Ag that are necessary for interaction with pRB are also required for cellular transformation mediated by these oncogenes (9, 13, 25, 32, 35, 49). In each case, mutation or deletion of the D/N-L-X-C-X-E sequence contained within these regions drastically reduces pRB binding and transforming activity, suggesting that this motif is important for interaction (9, 13, 35, 48). The observation that polyomavirus T-Ag contains a D-L-X-C-X-E sequence raises the possibility that pRB is similarly targeted by the amino terminus of polyomavirus T-Ag.

We have previously shown that polyomavirus large T-Ag possesses the ability to bind to pRB polypeptides *in vitro* (10). To clarify the relationship between the various activities of polyomavirus large T-Ag and its contribution to neoplastic transformation, we constructed a series of mutants with small deletions or single-amino-acid substitutions in two separate regions of the protein. These sequences were targeted because they showed considerable similarity to conserved regions 1 and 2 of the pRB binding sites on E1A. We show here that polyomavirus mutants defective in pRB binding are totally deficient in immortalization, indicating that association with pRB is an important component of immortalization mediated by polyomavirus large T-Ag.

MATERIALS AND METHODS

Site-directed mutagenesis. Site-directed mutagenesis was performed with vector M13mp19 and specific oligonucleo-

* Corresponding author.

tides by the method of Zoller and Smith (52). The *Bam*HI-*Hind*III fragment (nucleotides 4632 to 1656) was excised from pPyLT1, the polyomavirus large T-Ag gene (51), and inserted into the cloning vector M13mp19 (33). Specific oligonucleotides were annealed to single-stranded phage DNA, and the second strand was synthesized with deoxynucleotide triphosphates and Klenow polymerase. Heteroduplexes were purified by agarose gel electrophoresis and used to transform *Escherichia coli* JM109. Mutants were detected by dot blot hybridization with the oligonucleotide as a probe and sequenced by the dideoxynucleotide method. Oligonucleotides were synthesized by K. Deugau (Queen's University).

Vectors. The mutations were transferred into p91023LT (8). This vector includes the SV40 enhancer and origin region, the adenovirus major late promoter and tripartite leader, adenovirus VA RNA genes, and part of the mouse dihydrofolate reductase gene. The polyomavirus large T-Ag coding region was inserted downstream of the adenovirus major late promoter, adjoining the dihydrofolate reductase sequences. The large T-Ag mutations were transferred into p91023LT by substitution of the wild-type *Bst*XI fragment (nucleotides 173 to 1701) with those from the mutant DNAs.

A map of pneo-LT1, the vector used in immortalization assays, is shown in Fig. 1. Details of the construction of this vector have been published (30). pneo-LT1 contained the neomycin resistance gene so that transfectants could be placed under G418 selection.

Expression of large T-Ag in COS cells. Plasmids of the p91023LT series were introduced into COS cells as described previously (8) except that Polybrene was used instead of DEAE-dextran (27). After 60 h, the cells were labeled with [³⁵S]methionine (approx. 0.8×10^6 cells per 10-mm dish; 150 μ Ci/ml, 1,098 Ci/mmol; Amersham) in Hanks's balanced salt solution in Dulbecco's modified Eagle's medium (DMEM) for 2 h. The cells were washed with cold phosphate-buffered saline, collected by centrifugation, and lysed by heating at 100°C for 3 min in 500 μ l of solubilization buffer (50 mM triethanolamine-HCl, 100 mM NaCl, 2 mM EDTA, 0.4% sodium dodecyl sulfate [SDS], 2 mM mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride [pH 7.4]). Extracts were cooled down, supplemented with 125 μ l of Triton X-100 (10%) and 100 μ l of iodoacetamide (100 mM), and cleared by centrifugation in an Eppendorf microcentrifuge for 30 min at 4°C. The supernatants were mixed with 2 μ l of anti-T-Ag serum (a gift from B. Schaffhausen), incubated for 30 min at 4°C, mixed with 60 μ l of a 50% (vol/vol) suspension of *Staphylococcus aureus* protein A-Sepharose, and gently agitated for 60 min at 4°C. The precipitates were collected and washed first with cold phosphate-buffered saline, then with 0.5 M LiCl-0.1 M Tris-HCl (pH 6.8), and finally with distilled water. The samples were released from the protein A-Sepharose by boiling for 2 min in 40 μ l of dissociation buffer (125 mM Tris-HCl [pH 6.8], 5% SDS, 20% glycerol, 0.0075% bromophenol blue, 5% mercaptoethanol) and purified further by a second immunoprecipitation. SDS-polyacrylamide (12.5%) gel electrophoresis was followed by fluorography.

pRB binding. The ability of large T-Ag to associate with pRB was tested in an in vitro coimmunoprecipitation assay that has been described in detail previously (12). pRB polypeptides were synthesized in rabbit reticulocyte lysates programmed with RNA prepared by in vitro transcription of the human RB-1 cDNA (47). The pRB polypeptides were mixed with detergent lysates of unlabeled cells expressing large T-Ag. The ability of large T-Ag to form a complex with

FIG. 1. (A) Structure of the vector used in immortalization assays and location of conserved regions 1 and 2 in the polyomavirus large T-Ag gene (*pl*t). The structure shown is inserted between the *Pvu*II and *Bam*HI sites of pBR322. The replication defect in the polyomavirus genome (*ori*⁻) was introduced by a 23-bp deletion and addition of an *Xho*I linker at nucleotides 37 to 60 (4). (B) Alignment of amino acid sequences from the large T-Ags of SV40 and polyomavirus (Py) that are homologous to the pRB-binding sequences in E1A. Conserved amino acids are boxed. The positions and predicted amino acid alterations of the mutant T-Ags used in this work are shown.

pRB was determined by coimmunoprecipitation of a characteristic set of pRB polypeptides with a polyomavirus anti-T-Ag serum. In order to eliminate any possible interference in the binding assay by SV40 large T-Ag, which is constitutively expressed in COS-1 cells, the cell lysates were pre-cleared with two monoclonal antibodies that are specific for SV40 T-Ag. Preclearing was carried out by the following procedure. Cell lysates prepared in 1 ml of lysis buffer (12) from three 10-cm plates were incubated on ice for 30 min with 75 μ l of monoclonal antibody PAb416 and 75 μ l of PAb419 (tissue culture supernatants); 10 μ l of rabbit anti-mouse immunoglobulin serum was added, and the lysate was incubated for a further 30 min. The antibodies were removed by incubation with fixed *S. aureus* Cowan I cells. Preclearing was carried out twice before the lysates were assayed for pRB binding. Anti-T-Ag serum (2 μ l) was used as the test antibody, and a cocktail of 2 μ l of normal rabbit serum, 50 μ l of PAb416, and 50 μ l of PAb419 was used as a negative control.

Cells and cultures. All cells were grown at 37°C in DMEM supplemented with 10% fetal calf serum in a 10% CO₂ atmosphere. Immortalization of primary cultures of rat em-

bryo fibroblasts was performed as described previously (1). The large T-Ag gene was linked to the *neo* marker, and the DNA to be transfected (8 μ g) was mixed with high-molecular-weight calf thymus DNA (10 μ g) as the carrier. After 20 h of exposure to DNA, the primary cells were passaged and replated at 20 to 30% confluence. After 18 h, G418 was added to the medium at a concentration of 400 μ g/ml. The medium plus drug was changed every 5 days. Colonies were detected after 7 to 10 days in the selective medium. They were either pooled and grown in 60-mm dishes in the presence of G418 or individually picked and transferred into 15-mm Linbro microplates. Pools were passed at 1:3 dilution every 5 days for five or more passages, whereas individual colonies were subcultured at 1:5 dilution each time they reached confluence. G418 was maintained for the first two or three passages. Immortalization efficiencies were evaluated by counting the percentage of cell lines reaching five or more passages 7 to 8 weeks after the first transfer.

RESULTS

Amino acid sequences in the polyomavirus large T-Ag that are thought to be important for interaction with pRB are shown in Fig. 1. They are designated regions 1 and 2 and are aligned with the corresponding sequences of both E1A and SV40 T-Ag. Both regions 1 and 2 of E1A are known to form actual contact sites with pRB (11a), but for SV40 large T-Ag, only region 2 has been shown to contact pRB (9). Region 2 contains a motif of six amino acids, D/N-L-X-C-X-E (X represents any amino acid), which is found in all proteins that are known, to date, to bind to pRB. In mutant *dl-141*, the whole motif was deleted (nucleotides 978 through 995, Fig. 1). Two other mutants were constructed with minor changes in the sequence. In mutant *141glu*, Asp-141 was replaced by Glu (GAC→GAG), another acidic residue, whereas in *146asp*, Glu-146 was replaced by Asp (GAG→GAC). In both E1A and SV40 T-Ag, the carboxy-terminal half of domain 2 consists of a Ser followed by a string of acidic residues. This motif, which occurs in several nuclear oncogene products (16, 37), constitutes a recognition site for the cellular casein kinase II (28). It is not present in polyomavirus large T-Ag. Nevertheless, small deletions were introduced next to the domain to see how they would affect pRB binding. *dl-152* lacked nucleotides 1011 through 1022 (residues 152 to 155); *dl-167* lacked nucleotides 1056 through 1067 (residues 167 to 170).

The role of region 1 is less understood. In Ad5 E1A, the sequence is sufficient for complex formation, although it makes a weaker contact with pRB than region 2 (14, 49). Its function in SV40 T-Ag and HPV-E7 is still unclear, and it does not appear to be essential for binding (15, 35). To see whether mutations in the corresponding region of polyomavirus large T-Ag would affect pRB binding, we constructed mutant *dl-13* (Fig. 1), with a deletion of residues 13 to 17 (nucleotides 210 through 224). Two other mutations were created in the region by replacing Leu-13 by Val (13val) and Leu-16 by Val (16val) (G to C transitions at nucleotides 210 and 219, respectively; Fig. 1).

pRB binding. To determine the ability of mutant T-Ags to associate with pRB, it was necessary to express equal amounts of the different proteins. The mutations were transferred into p91023LT, a vector expressing the polyomavirus large T-Ag. In this vector, transcription of large T-Ag is driven by the adenovirus major late promoter. The vector also contains the adenovirus VA genes to increase translation of the mRNA and the SV40 origin of replication to allow

FIG. 2. Analysis of large T-Ags by immunoprecipitation. COS cells: Mutant T-Ags were transiently expressed in COS-1 cells transfected with plasmids of the p91023LT series. REF: Expression from immortalized rat embryo fibroblast (REF) cell lines. Five independent clones were pooled after the fifth passage, and approx. 5×10^6 cells were labeled with [35 S]methionine for 90 min. Immunoprecipitates were separated on a 12.5% polyacrylamide gel, and labeled proteins were detected by fluorography. Sizes are shown on the left (in kilodaltons).

template amplification when introduced into COS cells expressing the SV40 large T-Ag. Plasmid DNAs carrying the various mutations were analyzed for their ability to express polyomavirus large T-Ag after transfection into COS cells by labeling total cellular proteins with [35 S]methionine for 90 min prior to extraction. The large T-Ags were then immunoprecipitated by a polyclonal anti-T-Ag serum and fractionated on SDS-polyacrylamide gels (Fig. 2). The SV40 T-Ag present in COS cells was not immunoprecipitated by the polyomavirus anti-T-Ag serum used. The constructs carrying mutations in region 2 synthesized equal amounts of the protein. *dl-13* and *16val*, two region 1 mutants, were expressed at slightly lower levels, suggesting that their stability may be affected. Another mutant, *dl-167*, produced a protein with an apparent increase in molecular weight. The reason for this is unclear. On the basis of DNA sequence analysis of the mutated fragment as well as detailed mapping of the entire vector, the possibility of any fortuitous addition of DNA in the large T-Ag coding sequences had to be ruled out (data not shown). Furthermore, like the other mutants, *dl-167* retained the ability to initiate viral DNA replication (data not shown), suggesting that the protein had not sustained major structural rearrangements. The aberrant electrophoretic mobility may be caused by some retardation in migration due the removal of three contiguous Arg residues from the polypeptide.

The ability of the large T-Ags to associate with pRB was determined by coimmunoprecipitation of the labeled pRB polypeptides with an antibody specific for the viral protein but not with control antibody. As already reported (10), pRB binding was readily detected with wild-type large T-Ag. However, no coprecipitation was observed with *dl-141*, *141glu*, or *146asp* (Fig. 3). Thus, pRB binding was completely abolished by as little as a single amino acid substitution (Asp-141→Glu or Glu-146→Asp) in region 2 of the polyomavirus large T-Ag. It was slightly affected by the deletion in *dl-167* but not by that in *dl-152*. Mutants *13val*, *16val*, and *dl-13*, affected in region 1, interacted with pRB, although somewhat less efficiently than the wild-type T-Ag.

Immortalization assays. To assess the capacity of mutant

FIG. 3. Coimmunoprecipitation of mutant T-Ags with pRB. Radioactively labeled RB-1 polypeptides were synthesized *in vitro* and incubated with COS cell extracts expressing mutant T-Ags. Immunoprecipitations were performed with a polyomavirus anti-T-Ag serum (T) or with nonimmune serum (C) as a negative control. Immune complexes were separated on a 10% polyacrylamide gel, and labeled proteins were detected by fluorography. Sizes are indicated in kilodaltons.

T-Ags to immortalize cells, the mutations were transferred into a vector carrying the polyomavirus large T-Ag gene linked to pSV2neo. The polyomavirus large T-Ag gene lacked an active origin of replication because it was found previously that transfection of constructs with the ability to trigger replication at the viral origin could be toxic to the cells (3, 29). Plasmids were introduced into primary rat embryo fibroblasts by calcium phosphate transfection, and transfectants were placed under G418 selection. Two types of assays were used. In the first one, G418-resistant colonies were pooled from several dishes and approximately 10^5 cells were plated in a 60-mm petri dish. The cells were passaged every 5 days at a 1:3 dilution regardless of their confluence and maintained in G418. Under these conditions, cells obtained by transfection with pSV2neo (*neo* alone) were lost after two to three passages, whereas cells transfected with both *neo* and the polyomavirus large T-Ag gene could sustain several passages and eventually gave rise to cell lines capable of growing indefinitely in culture. Figure 4 shows representative dishes from such a transfection. All three mutants with either a deletion (*dl*-141) or a substitution (141glu, 146asp) in the D-L-X-C-X-E motif failed to immor-

FIG. 4. Immortalization of primary rat embryo fibroblasts. From 20 to 30 G418-resistant colonies were pooled from several dishes, and approx. 10^5 cells were plated in a 60-mm petri dish. The cells were passaged every 5 days at a 1:3 dilution. After five passages, immortalized cultures were allowed to grow to confluence. Cells were then fixed and stained.

TABLE 1. Properties of mutant T-Ags

Vector	Mutation	Immortalization efficiency ^a (%)
Wild type	None	28/60, 25/60 (44)
<i>dl</i> -141	Deletion of Asp-141 through Glu-146	0/61 (0)
141glu	Asp-141→Glu	0/96 (0)
146asp	Glu-146→Asp	0/96 (0)
<i>dl</i> -152	Deletion of residues 152–155	18/96 (19)
<i>dl</i> -167	Deletion of residues 167–170	21/96 (22)
<i>dl</i> -8	Deletion of residues 145–174	0/45 (0)
<i>dl</i> -149	Deletion of residues 149–176	0/59 (0)
<i>dl</i> -13	Deletion of Leu-13 through Leu-17	5/96, 0/96 (3)
13val	Leu-13→Val	0/96, 0/30 (0)
16val	Leu-16→Val	26/96, 8/30 (27)

^a Colonies of cells resistant to G418 were picked 3 to 4 weeks after selection and transferred to 15-mm Linbro microplates. The cells were subcultured at a 1:5 dilution each time they reached confluence. They were considered immortalized after the fifth passage. Immortalization efficiencies were evaluated by counting the percentage of cell lines reaching five or more passages 7 weeks after the first transfer [shown as number of plates with immortalized cells/number of plates prepared (percent immortalized)].

talize cells. *dl*-152 and *dl*-167, with deletions in the vicinity of region 2, were also negative. However, when tested in another assay, in which individual colonies were picked and passaged in culture, they were able to immortalize cells at a reduced efficiency (Table 1). All cell lines established by this procedure expressed the large T-Ag. Representative immunoprecipitations are shown in Fig. 2. *dl*-13 and 13val, two of the mutants affected in region 1, were severely impaired in immortalization. However, 16val immortalized cells virtually as efficiently as did wild-type large T-Ag.

In a previous study (30), it was reported that some polyomavirus mutants with deletions affecting both the middle and large T-Ags were deficient in immortalization. In one such mutant, designated *dl*-8, the deletion removed amino acid residues 145 through 174 from the large T-Ag (42), affecting the D-L-X-C-X-E motif in region 2. We sought to determine the ability of this mutant T-Ag to associate with pRB. However, despite several attempts, we were unable to visualize the large T-Ag by immunoprecipitation (not shown). It is possible that the *dl*-8 protein was not recognized by our anti-T-Ag serum or that it was unstable and expressed in amounts too small to be detected. We attempted to restore the biological activity of *dl*-8 by reconstituting an intact D-L-X-C-X-E motif. This was achieved by inserting a tyrosine and a glutamic acid residue between Cys-144 and Glu-175. The new mutant, designated *dl*-149, had, in addition to the D-L-X-C-X-E motif, the next two amino acid residues which are present in the wild-type sequence (Fig. 1). However, despite this sequence, *dl*-149 failed to immortalize primary rat embryo fibroblasts.

DISCUSSION

We show in this work that the ability of the polyomavirus large T-Ag to associate *in vitro* with pRB polypeptides and to immortalize primary rat embryo fibroblasts can be abolished by as little as a single amino acid substitution in the D-L-X-C-X-E sequence of conserved region 2, one of the putative pRB binding sites on large T-Ag. In experiments not described here, we have found that these mutants are fully competent in viral promoter *trans* activation and DNA replication (30; unpublished results), suggesting that despite

their functional defect they retain significant native structure and that their stability is not grossly altered by the mutations. Mutations in conserved region 2 of E1A and the homologous sequences in SV40 large T-Ag impair transformation. Unlike the latter, polyomavirus large T-Ag does not transform cells. However, it can confer upon primary cells the capacity for continued proliferation. Thus, while in vivo complex formation has yet to be demonstrated, it seems that association with pRB is an important component of immortalization by polyomavirus large T-Ag.

We have shown that mutations in conserved region 1 of polyomavirus large T-Ag affect pRB binding only marginally. Yet some of the mutants (*dl-13* and *13val*) are severely impaired in immortalization. *13val* appears to be expressed at wild-type levels, at least in COS cells. The stability of *dl-13* may be slightly altered, but so is that of *16val*, which is still active in immortalization. It is therefore unlikely that the defect of region 1 mutants is due to instability. It is possible that with these mutants, the association with pRB is not tight enough to result in its inactivation. Alternatively, binding to pRB may be essential but not sufficient for immortalization and, as already reported for E1A (11, 49), complex formation with other cellular proteins may be required. The E1A proteins are associated with a series of cellular proteins in transformed cells (21, 50). The binding sites for at least two additional proteins (p300 and p107) which are required for transformation have been mapped to conserved regions 1 and 2 of E1A (48, 49). Moreover, p107 has been shown recently to associate with the large T-Ags of SV40 and JC virus (11). The identity of these proteins is presently unknown. Further work will be needed to determine whether they associate with polyomavirus large T-Ag and whether such interaction is required for immortalization.

It has been suggested that association between pRB and either E1A or the SV40 large T-Ag is part of the mechanism by which these viral oncogenes transform cells. Since polyomavirus large T-Ag can form a similar complex with pRB, one wonders why it cannot confer, like its SV40 counterpart, a typical transformed phenotype upon cells as measured by focus formation or anchorage-independent growth. It is conceivable that in vivo polyomavirus large T-Ag binds only to a fraction of pRB and cannot fully inactivate its growth suppressor function. Alternatively, full loss of pRB function may not be enough to evoke fully transformed behavior, and other viral oncogenes may operate through interactions with other cellular oncogenes or tumor suppressors as well. This could be the case with SV40 since, in addition to the pRB-binding sequences in the amino-terminal half of the large T-Ag, other sequences are required for transformation. These sequences, which have been localized between amino acids 325 and 625 (43), overlap the region implicated in binding to the p53 protein (amino acids 272 to 517) (41). Thus, full transformation by SV40 may require two or more separate domains of the SV40 large T-Ag, one associating with pRB and the other with p53. Like pRB, p53 is probably the product of a tumor suppressor gene (17, 22, 36). Interestingly, the correlation between pRB and p53 binding and transformation can also be demonstrated in adenoviruses and various types of HPV. However, these viruses have divided the interactions seen in two different domains of the SV40 T-Ag into two separate proteins. Adenovirus E1A and HPV-E7 proteins bind to pRB (35, 47), while adenovirus E1B and HPV-E6 proteins, which are also required for transformation, bind to p53 (40, 46).

The existence of two separate transforming domains in SV40 large T-Ag may explain an apparent discrepancy

between our results and those in a recent report by Chen and Paucha (5), claiming that some SV40 mutants with deletions affecting the pRB binding site are able to rescue primary rat and mouse embryo fibroblasts from senescence without conferring upon them a typical transformed phenotype. These results would indicate that the ability of SV40 to immortalize cells is independent of its ability to bind to the retinoblastoma gene product. The domain responsible for this activity has not been formally identified, but it is conceivable that it is the p53-binding domain. Consistent with this hypothesis is the observation that the E6 protein from HPV-16, which forms a complex with p53 (46), can immortalize primary rat cells (26). Conversely, deletion of the p53-binding domain from SV40 T-Ag does not completely inactivate its oncogenic potential. The amino-terminal fragment of T-Ag has significant transforming activity (6, 7) and is fully competent in the immortalization of primary rat embryo fibroblasts (1). This suggests that the function specified by either the N-terminal or the carboxy-terminal domain of SV40 T-Ag can be sufficient to elicit a partially transformed phenotype, such as immortalization, but that both domains are required for full transformation.

ACKNOWLEDGMENTS

We thank C. Bergeron, J. Toutant, and P. Guida for technical assistance and B. Schaffhausen for a generous gift of polyomavirus anti-T serum.

N.D. was supported by Amersham International. This work was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada to M.B. and by Public Health Service grant CA 13106 to E.H.

REFERENCES

- Asselin, C., and M. Bastin. 1985. Sequences from polyomavirus and simian virus 40 large T genes capable of immortalizing primary rat embryo fibroblasts. *J. Virol.* **56**:958-968.
- Asselin, C., C. Glinas, and M. Bastin. 1983. Role of the three polyomavirus early proteins in tumorigenesis. *Mol. Cell. Biol.* **3**:1451-1459.
- Asselin, C., J. Vass-Marengo, and M. Bastin. 1986. Mutation in the polyomavirus genome that activates the properties of large T associated with neoplastic transformation. *J. Virol.* **57**:165-172.
- Bautch, V. L., S. Toda, J. A. Hassell, and D. Hanahan. 1987. Endothelial cell tumors develop in transgenic mice carrying polyoma virus middle T oncogene. *Cell* **51**:529-538.
- Chen, S., and E. Paucha. 1990. Identification of a region of simian virus 40 large T antigen required for cell transformation. *J. Virol.* **64**:3350-3357.
- Clayton, C. D., D. Murphy, M. Lovett, and P. W. J. Rigby. 1982. A fragment of the SV40 large T-antigen gene transforms. *Nature (London)* **299**:59-61.
- Colby, W. W., and T. Shenk. 1982. Fragments of the simian virus 40 transforming gene facilitate transformation of rat embryo cells. *Proc. Natl. Acad. Sci. USA* **79**:5189-5193.
- Cowie, A., J. de Villiers, and R. Kamen. 1986. Immortalization of rat embryo fibroblasts by mutant polyomavirus large T antigens deficient in DNA binding. *Mol. Cell. Biol.* **6**:4344-4352.
- DeCaprio, J. A., J. W. Ludlow, J. Figge, J.-Y. Shew, C.-M. Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston. 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54**:275-283.
- Dyson, N., R. Bernards, S. H. Friend, L. R. Gooding, J. A. Hassell, E. O. Major, J. M. Pipas, T. Vandyke, and E. Harlow. 1990. Large T antigens of many polyomaviruses are able to form complexes with the retinoblastoma protein. *J. Virol.* **64**:1353-1356.
- Dyson, N., K. Buchkovich, P. Whyte, and E. Harlow. 1989. The cellular 107K protein that binds to adenovirus E1A associates with the large T antigens of SV40 and JC virus. *Cell* **58**:249-255.
- Dyson, N., P. Guida, C. McCall, and E. Harlow. Unpublished data.

12. Dyson, N., P. M. Howley, K. Münger, and E. Harlow. 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**:934-936.
13. Edmonds, C., and K. H. Vousden. 1989. A point mutational analysis of human papillomavirus type 16 E7 protein. *J. Virol.* **63**:2650-2656.
14. Egan, C., T. N. Jelsma, J. A. Howe, S. T. Bayley, B. Ferguson, and P. E. Branton. 1988. Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. *Mol. Cell. Biol.* **8**:3955-3959.
15. Ewen, M. E., J. W. Ludlow, E. Marsilio, J. A. DeCaprio, R. C. Millikan, S. H. Cheng, E. Paucha, and D. M. Livingston. 1989. An N-terminal transformation-governing sequence of SV40 large T antigen contributes to the binding of both p110^{Rb} and a second cellular protein, p120. *Cell* **58**:257-267.
16. Figge, J., T. Webster, T. F. Smith, and E. Paucha. 1988. Prediction of similar transforming regions in simian virus 40 large T, adenovirus E1A, and *myc* oncoproteins. *J. Virol.* **62**:1814-1818.
17. Finlay, C. A., P. W. Hinds, and A. J. Levine. 1989. The p53 proto-oncogene can act as a suppressor of transformation. *Cell* **57**:1083-1093.
18. Friend, S. H., R. Bernards, S. Rogelj, R. A. Weinberg, J. M. Rapaport, D. M. Albert, and T. P. Dryja. 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature (London)* **323**:643-646.
19. Fung, Y.-K. T., A. L. Murphree, A. T'Ang, J. Qian, S. H. Hinrichs, and W. F. Benedict. 1987. Structural evidence for the authenticity of the human retinoblastoma susceptibility gene. *Science* **236**:1657-1661.
20. Harbour, J. W., S.-L. Lai, J. Whang-Peng, A. F. Gazdar, J. D. Minna, and F. J. Kaye. 1988. Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. *Science* **241**:353-357.
21. Harlow, E., P. Whyte, B. R. Franza, and C. Schley. 1986. Association of adenovirus early-region 1A proteins with cellular polypeptides. *Mol. Cell. Biol.* **6**:1579-1589.
22. Hick, G. G., and M. Mowat. 1988. Integration of Friend murine leukemia virus into both alleles of the p53 oncogene in an erythroleukemic cell line. *J. Virol.* **62**:4752-4755.
23. Jat, P. S., and P. A. Sharp. 1986. Large T antigens of simian virus 40 and polyomavirus efficiently establish primary fibroblasts. *J. Virol.* **59**:746-750.
24. Jat, P. S., and P. A. Sharp. 1989. Cell lines established by a temperature-sensitive simian virus 40 large-T-antigen gene are growth restricted at the nonpermissive temperature. *Mol. Cell. Biol.* **9**:1672-1681.
25. Kalderon, D., and A. E. Smith. 1984. *In vitro* mutagenesis of a putative DNA binding domain of SV40 large-T. *Virology* **139**:109-137.
26. Kanda, T., S. Watanabe, and K. Yoshike. 1988. immortalization of primary rat cells by human papillomavirus type 16 subgenomic DNA fragments controlled by the SV40 promoter. *Virology* **165**:321-325.
27. Kawai, S., and M. Nishizawa. 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. *Mol. Cell. Biol.* **4**:1172-1174.
28. Kuenzel, E. A., J. A. Mulligan, J. Sommercorn, and E. G. Krebs. 1987. Substrate specificity determinants for casein kinase II as deduced from studies with synthetic peptides. *J. Biol. Chem.* **262**:9136-9140.
29. Land, H., L. F. Parada, and R. A. Weinberg. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature (London)* **304**:596-602.
30. Larose, A., L. St-Onge, and M. Bastin. 1990. Mutations in polyomavirus large T affecting immortalization of primary rat embryo fibroblasts. *Virology* **176**:98-105.
31. Lee, W.-H., R. Bookstein, F. Hong, L.-J. Young, J.-Y. Shew, and E. Y.-H. P. Lee. 1987. Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science* **235**:1394-1399.
32. Lillie, J. W., M. Green, and M. R. Green. 1986. An adenovirus E1a protein region required for transformation and transcriptional repression. *Cell* **46**:1043-1051.
33. Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* **101**:20-78.
34. Moran, E. 1988. A region of SV40 large T antigen can substitute for a transforming domain of the adenovirus E1A product. *Nature (London)* **334**:168-170.
35. Münger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow, and P. M. Howley. 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO J.* **8**:4099-4105.
36. Munroe, D. G., B. Rovinski, A. Bernstein, and S. Benchimol. 1988. Loss of a highly conserved domain of p53 as a result of a gene deletion during Friend virus-induced erythroleukemia. *Oncogene* **2**:621-624.
37. Ralston, R., and J. M. Bishop. 1983. The protein products of the *myc* and *myb* oncogenes and adenovirus E1a are structurally related. *Nature (London)* **306**:803-806.
38. Rassoulzadegan, M., A. Cowie, A. Carr, N. Glaichenhaus, R. Kamen, and F. Cuzin. 1982. The roles of individual polyoma virus early proteins in oncogenic transformation. *Nature (London)* **300**:713-718.
39. Rassoulzadegan, M., Z. Naghashfar, A. Cowie, A. Carr, M. Grisoni, R. Kamen, and F. Cuzin. 1983. Expression of the large T protein of polyoma virus promotes the establishment in culture of "normal" rodent fibroblast cell lines. *Proc. Natl. Acad. Sci. USA* **80**:4354-4358.
40. Sarnow, P., Y. S. Ho, J. Williams, and A. J. Levine. 1982. Adenovirus E1b 58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 Kd cellular protein in transformed cells. *Cell* **28**:387-394.
41. Schmieg, F. I., and D. T. Simmons. 1988. Characterization of the *in vitro* interaction between SV40 T antigen and p53: mapping the p53 binding site. *Virology* **164**:132-140.
42. Smolar, N., and B. E. Griffin. 1981. DNA sequences of polyomavirus early deletion mutants. *J. Virol.* **38**:958-967.
43. Srinivasan, A., K. W. C. Peden, and J. M. Pipas. 1989. The large tumor antigen of simian virus 40 encodes at least two distinct transforming functions. *J. Virol.* **63**:5459-5463.
44. Tooze, J. 1981. *Molecular biology of tumor viruses*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. Treisman, R., U. Novak, J. Favalaro, and R. Kamen. 1981. Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein. *Nature (London)* **292**:595-600.
46. Werness, B. A., A. J. Levine, and P. M. Howley. 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248**:76-79.
47. Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow. 1988. Association between an oncogene and an antioncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature (London)* **334**:124-129.
48. Whyte, P., H. E. Ruley, and E. Harlow. 1988. Two regions of the adenovirus early region 1A proteins are required for transformation. *J. Virol.* **62**:257-265.
49. Whyte, P., N. M. Williamson, and E. Harlow. 1989. Cellular targets for transformation by the adenovirus E1A proteins. *Cell* **56**:67-75.
50. Yee, S.-P., and P. E. Branton. 1985. Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. *Virology* **147**:142-153.
51. Zhu, Z., G. M. Veldman, A. Cowie, A. Carr, B. Schaffhausen, and R. Kamen. 1984. Construction and functional characterization of polyomavirus genomes that separately encode the three early proteins. *J. Virol.* **51**:170-180.
52. Zoller, M. J., and M. Smith. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Methods Enzymol.* **100**:468-500.