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Binding of p53 and p105-RB Is Not Sufficient for Oncogenic Transformation by a Hybrid Polyomavirus-Simian Virus 40 Large T Antigen

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To identify regions on the large T antigens of simian virus 40 (SV40) and polyomavirus which are involved in oncogenic transformation, we constructed plasmids encoding hybrid polyomavirus-SV40 large T antigens. The hybrid T antigens were expressed in G418 sulfate-resistant pools of rat F2408 cells, and extracts of such pools were immunoprecipitated with an antibody against p53. Two hybrid T antigens containing SV40 amino acids 337 to 708 bound to p53, whereas another hybrid T antigen containing SV40 amino acids 412 to 708 did not. This suggests that a binding domain on SV40 large T antigen for p53 is contained within amino acids 337 to 708, with amino acids 337 to 411 playing an important role. One of the two hybrids that bound to p53 was chosen for further study. This T antigen contained SV40 large T antigen amino acids 336 to 708 joined to polyomavirus large T antigen amino acids 1 to 521 (PyT_{1-521}-$SVT_{336-708}$). Immunoprecipitation with antibodies directed against the product of the retinoblastoma susceptibility gene, p105-RB, showed that this hybrid bound p105-RB as well as p53. Pools expressing the hybrid PyT_{1-521}-$SVT_{336-708}$ did not grow in soft agar, nor did they form foci on confluent monolayers of nontransformed F2408 cells. The hybrid T antigen was expressed at levels comparable to those seen in retrovirus-infected F2408 cells expressing only SV40 large T antigen, which do show a transformed phenotype. Thus, this level of expression was sufficient for transformation by SV40 large T antigen but not for the hybrid large T antigen. These data, combined with genetic studies from other laboratories, suggest that complex formation with p53 and p105-RB is necessary but not sufficient for the oncogenic potential of papovavirus large T antigens.

Simian virus 40 (SV40) can transform both primary and established cells in culture, and the large T antigen encoded by the viral early region has been implicated in this process. Indeed, vectors expressing only SV40 large T antigen can transform established cells (2, 6, 30, 34). Large T antigen is also capable of immortalizing and transforming cells in primary culture (10, 31, 48). A variety of biochemical functions are performed by the protein, including the ability to specifically bind to the SV40 origin of DNA replication (16, 32) and enzymatic activities such as ATPase (23, 63) and helicase (14, 59). None of these activities has been shown to be required for transformation (1, 24, 33, 41, 50). The SV40 large T antigen has been found in association with the transformation-related proteins p53 (36, 38) and p105-RB, the product of the retinoblastoma (*Rb*) susceptibility locus (15). Recent studies have led to the proposal that, in fact, p53 and p105-RB are products of "anti-oncogenes" with tumor suppressor activity (4, 20, 22, 29). A promising model for oncogenic transformation by SV40 is that the binding of SV40 large T antigen inactivates or alters the function of p53 and p105-RB and prevents them from exerting their normal effects in cells, thereby contributing to the process of transformation (22, 35, 68).

Polyomavirus large T antigen is highly similar to its counterpart from SV40 in both sequence and function (see reference 17). The two large T antigens are approximately 60% similar in amino acid sequence (58). Both are required for the replication of their respective origins in cells (17) and in vitro (37, 45, 60, 70). Polyomavirus large T antigen has DNA-binding (13, 56), ATPase (9), and DNA helicase activities (Wang and Prives, unpublished data) similar to those of SV40 large T antigen. Unlike the SV40 T antigen, polyomavirus large T antigen is not the principal transforming protein of polyomavirus; this activity resides with the middle T antigen (51, 64), a cytoplasmically localized protein which interacts with c-*src* at the plasma membrane (3, 11). Polyomavirus large T antigen is, however, required for immortalization of primary cells (12, 53) and is necessary for middle-T-antigen-mediated transformation of such cells (51). The large T antigen is also necessary for the ability of such transformed cells to grow in low serum concentrations (51) and to maintain the transformed phenotype (52). This activity of polyomavirus large T antigen appears to reside in the amino 40% of the protein (52, 53). Polyomavirus large T antigen also differs from SV40 large T antigen in that it is unable to bind to the cellular protein p53 (40, 67), but it has been shown to bind to p105-RB in vitro (18).

Previous studies have identified the p53 binding region on SV40 large T antigen as residing in the carboxyl end of the protein (42, 44, 57). As it is the amino-terminal end of polyomavirus large T antigen which has been implicated in its ability to immortalize primary cells (52, 53), we constructed hybrid large T antigens consisting of the carboxyl end of SV40 large T antigen joined to the amino end of polyomavirus large T antigen. One of the polyomavirus-SV40 large T antigen hybrids was capable of binding both p53 and p105-RB but was not capable of transforming an established cell line, rat F2408 fibroblasts.

MATERIALS AND METHODS

Plasmids, cells, and antibodies. pSVBam is plasmid pBR322 with the entire SV40 genome inserted into its *Bam*HI site. pSVLT is the plasmid pMK 16 with the entire

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SV40 genome lacking the large T antigen intron (SV40 nucleotides 4917 to 4572) inserted into its *Eco*RI site. pPyBam is the plasmid pAT153 with the entire polyomavirus genome inserted into its *Bam*HI site. pPyLT (73) is the plasmid pAT153 (*Ava*I$^-$) with the entire polyomavirus genome lacking the large T antigen intron (polyomavirus nucleotides 412 to 796) inserted into its *Bam*HI site. pkoneo (65) is a plasmid containing the coding region for aminoglycoside phosphotransferase II under control of the SV40 early-region promoter; expression of this enzyme confers resistance to the antibiotic G418 sulfate.

The F2408 established rat fibroblastic cell line exhibits a low background in standard transformation assays (49) and has been used in studies of polyomavirus-mediated transformation (64). F2408 cells were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (complete medium). Hybridoma lines expressing monoclonal antibodies against both SV40 (PAb 419 and 101) and polyomavirus large T antigens (F4) as well as the cellular protein p53 (PAb 421) were maintained in RPMI medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum. PAb 419 recognizes an epitope on the amino end of SV40 large T antigen (28). PAb 101 recognizes an epitope on the carboxyl end of SV40 large T antigen (8). PAb F4 was raised against the polyomavirus small T antigen and therefore recognizes an epitope at the amino terminus of polyomavirus large T antigen (46). PAb 421 is specific for p53 from a variety of species (28). Monoclonal antibody Rb-PMG3-245 reacts with p105-RB from primate sources (15) and was purchased from Pharmingen (San Diego, Calif.). Polyclonal rabbit serum raised against polyomavirus small T antigen (46) was a kind gift of D. Pallas (Dana-Farber Cancer Institute, Boston, Mass.).

Constructs expressing hybrid polyomavirus-SV40 large T antigens. pPySV1 was constructed by inserting the *Nde*I-*Bam*HI fragment of pSVLT (SV40 nucleotides 3808 to 2533) into the corresponding region of pPyLT. pPySV2 was constructed by digesting pPySV1 with *Mst*II and *Nde*I, filling the resulting ends with Klenow, and ligating. pPySV3 was constructed by digesting pPySV1 with *Nsi*I, isolating the larger fragment, and ligating it.

Expression of hybrid large T antigens in F2408 cells. Subconfluent 60-mm dishes of rat F2408 cells were cotransfected with 2 μg of the plasmid expressing the appropriate large T antigen and 0.4 μg of pkoneo in the form of a calcium phosphate precipitate (25) for 16 h. Cells were washed with complete medium and allowed to recover for 24 h. Cells were then selected in complete medium containing 800 μg of G418 sulfate per ml, being fed every 5 days. After 2 weeks, approximately 100 to 200 colonies were pooled and passaged as a pool.

Immunoprecipitations. Confluent 150-mm dishes of the appropriate G418 sulfate-resistant pools were lysed in 3 ml of 0.5% sodium deoxycholate–2% Nonidet P-40–0.2% sodium dodecyl sulfate (SDS)–50 mM NaCl–25 mM Tris hydrochloride (pH 7.5) containing the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), aprotinin (50 μg/ml), and leupeptin (50 μM) for 15 min on ice. Lysates were spun at 20,000 × *g* for 15 min, and the supernatant was saved. Protein level was determined by the bichinchonic acid protein assay (Pierce Chemical Company, Rockford, Ill.), and appropriate amounts of protein were incubated on ice for 1 h with either 200 μl of hybridoma supernatants or 5 μg of Rb-PMG3-245 or a nonspecific immunoglobulin G1 (IgG1) control. A solution of 50 μl of 50% protein A-Sepharose in phosphate-buffered saline was mixed with 10 μg of goat anti-mouse IgG, and the mixture was rocked at 4°C, also for 1 h. The Sepharose beads were pelleted, washed twice with 200 μl of lysis buffer, and suspended in 50 μl of lysis buffer. This was added to the antibody-cell lysate mixture and rocked at 4°C for 2 h. The beads were again pelleted, washed three times with 0.5% sodium deoxycholate–0.2% Nonidet P-40–50 mM NaCl–25 mM Tris hydrochloride (pH 7.5). Pellets were suspended with 50 μl of 1× SDS sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol in 62.5 mM Tris hydrochloride [pH 6.8]), and then samples were treated at 95°C for 5 min and spun for 5 min in a microcentrifuge. Samples were loaded on 10% SDS–polyacrylamide gels and electrophoresed at 150 V constant voltage for 3 h. Samples were transferred to nitrocellulose paper overnight. The paper was probed with either undiluted hybridoma supernatant or a 1:2,000 dilution of rabbit anti-polyomavirus small T antigen polyclonal serum in 1% nonfat dry milk (Carnation Company, Los Angeles, Calif.) in phosphate-buffered saline. Second antibody was a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG. Signal was developed by using 4-chloro-1-naphthol.

Growth on confluent monolayers of F2408 cells. Confluent dishes of F2408 cells were split 1:4 into an appropriate number of 60-mm dishes the day before the assay. Then, 500 or 1,000 cells were plated either on fresh 60-mm dishes or on the dishes containing rat F2408 cells. These were fed every 5 days with fresh complete medium. At the appropriate time, cells were stained with 1% crystal violet in 20% ethanol, and foci larger than 0.5 mm in diameter were scored as positive without the aid of a microscope.

Growth in soft agar. A total of 2,000 cells were plated on 60-mm dishes in 0.33% agar in complete medium on a bed of 0.5% agar in complete medium. Cells were fed every 7 days with 0.33% agar in complete medium. Colony formation was scored after 35 days by examining the plates against a dark background without magnification.

Retroviral infection of F2408 cells. Ψ_2 lines constitutively producing the TEXS6 (encoding the SV40 large T antigen and G418 sulfate resistance) and the ZIPNEO (encoding G418 sulfate resistance alone) viruses (6) were a kind gift of I. Bikel (Dana-Farber Cancer Institute, Boston, Mass.). Titers of these viruses on F2408 cells were 5.7×10^5 CFU/ml for TEXS6 and 1.0×10^6 CFU/ml for ZIPNEO. Plates of these producer lines (75% confluent, 60-mm plates) were fed with 4 ml of fresh DMEM containing 10% calf serum. After 16 to 24 h, this medium was removed and centrifuged at 400 × *g* to pellet cells and debris. The old medium was removed from 100-mm plates of F2408 cells, and 2 ml of the virus-containing supernatant supplemented with 8 μg of Polybrene (30) was added. Plates were rocked at 37°C for 2 h, and then 8 ml of DMEM containing 10% fetal calf serum was added. When these virally infected cells were confluent, they were split 1:10 into complete medium containing G418 sulfate (800 μg/ml). Selection was complete in 8 to 10 days, and approximately 100 to 200 colonies were pooled and passaged as a pool.

RESULTS

$PyT_{1\text{-}727}$-$SVT_{337\text{-}708}$ and $PyT_{1\text{-}521}$-$SVT_{336\text{-}708}$, bind p53, whereas $PyT_{1\text{-}452}$-$SVT_{412\text{-}708}$ does not. By using preexisting restriction enzyme cleavage sites, plasmids encoding hybrid large T antigens were constructed. These hybrid T antigens consisted of an amino-terminal fragment of polyomavirus large T antigen joined to a carboxyl-terminal fragment of SV40 large T antigen. These T antigens are expressed from

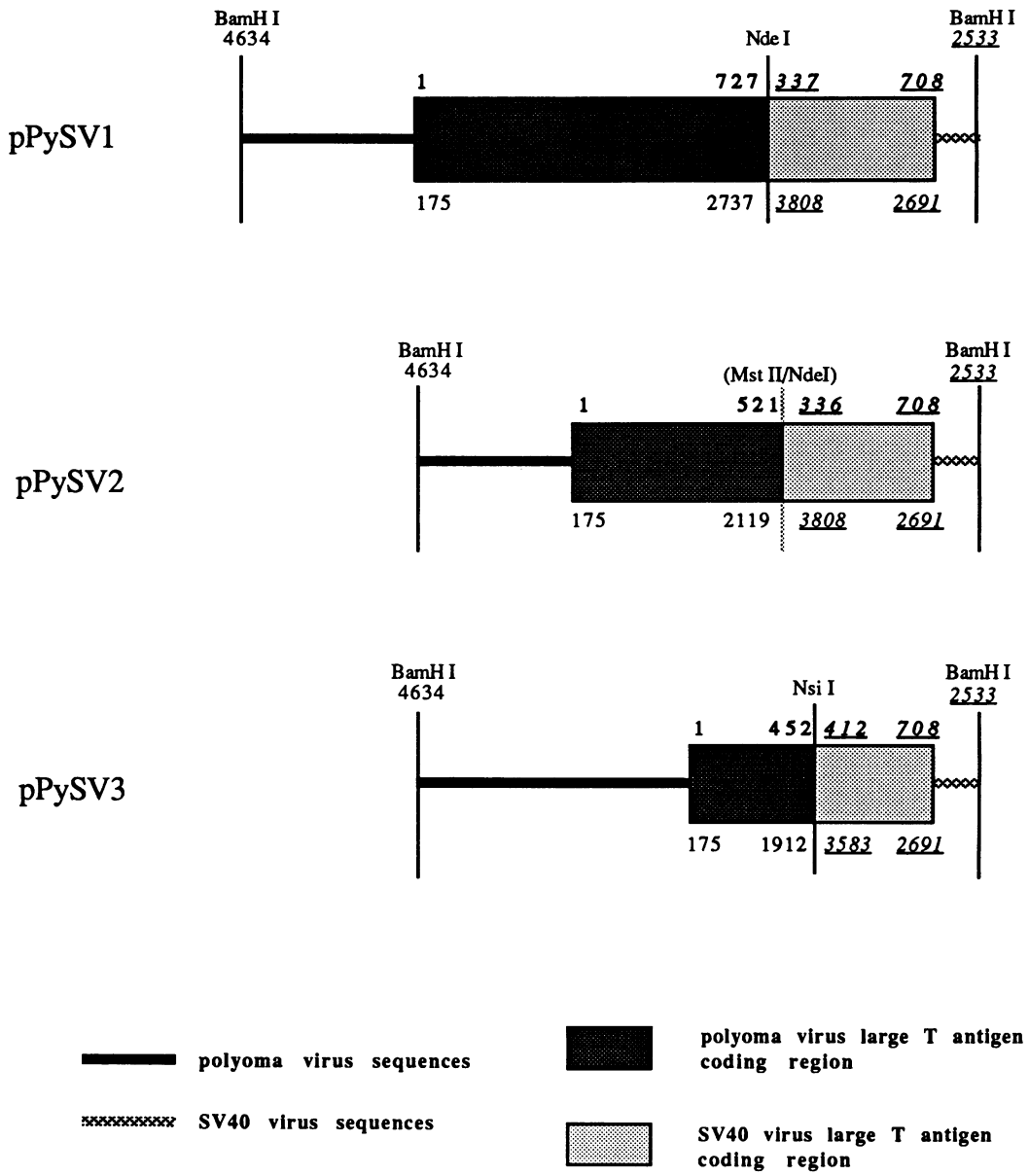

FIG. 1. Hybrid polyomavirus-SV40 large T antigens. Schematic illustrations of the three polyomavirus-SV40 hybrid large T antigens constructed in this study. Polyomavirus nucleotide and amino acid numbers are shown in roman type; SV40 nucleotide and amino acid numbers are italicized and underlined.

the native polyomavirus early promoter but have the SV40 large T antigen 3'-end processing signals. The plasmid pPySV1 encodes a hybrid large T antigen containing the first 727 amino acids of polyomavirus large T antigen joined to amino acids 337 to 708 of SV40 large T antigen (PyT_{1-727}-$SVT_{337-708}$, Fig. 1). The *Mst*II-*Nde*I fragment of pPySV1 was deleted to create plasmid pPySV2, which encodes a hybrid containing amino acids 1 to 521 of polyomavirus T antigen joined to amino acids 336 to 708 of SV40 T antigen (PyT_{1-521}-$SVT_{336-708}$, Fig. 1). The *Nsi*I fragment of pPySV1 was deleted, creating pPySV3, which encodes a hybrid large T antigen containing the first 451 amino acids of polyomavirus T antigen joined to amino acids 412 to 708 of SV40 T antigen (PyT_{1-452}-$SVT_{412-708}$, Fig. 1).

We wanted to constitutively express these hybrid proteins in cells without prior selection for a particular phenotype and then examine the cells for complex formation between the T antigens and p53 and p105-RB, as well as for their level of transformation. Thus, we cotransfected plasmids expressing large T antigens with a plasmid that encodes resistance to G418 sulfate, pkoneo, and performed a selection. From 100 to 200 different colonies were pooled and analyzed as pools. Such an approach allows study of a large number of colonies at the same time rather than restricting analysis to a small number of individual cell lines. This approach has been used by others in studies of both SV40 large T antigen (6) and polyomavirus large T antigen (12). Samples of extracts of such pools were run on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and probed for the presence of a T antigen. PyT_{1-452}-$SVT_{412-708}$ was expressed at a level

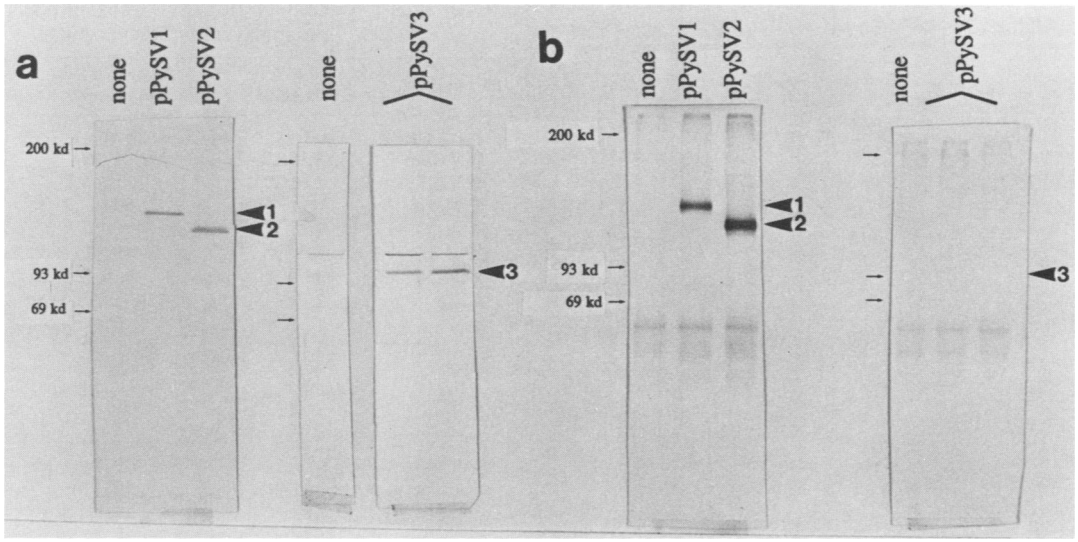

FIG. 2. PyT_{1-727}-$SVT_{337-708}$ and PyT_{1-521}-$SVT_{336-708}$ bind p53, but PyT_{1-452}-$SVT_{412-708}$ does not. (a) Cell extracts (50 μl) expressing the indicated plasmids were run on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with a 1:2,000 dilution of rabbit anti-polyomavirus small T antigen serum. (b) The same extracts (900 μl) were immunoprecipitated with PAb 421, run on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and probed with a 1:2,000 dilution of rabbit anti-polyomavirus small T antigen serum. Lanes labeled "none" are pools generated by transfection with vector DNA pAT153. The two lanes labeled pPySV3 represent two independently derived pools transfected with the plasmid pPySV3. (a) The upper band seen in the lanes labeled pPySV3 is not related to T antigen expression, as it was also seen in a pool generated by transfection with vector DNA. (a and b) Positions of marker proteins, whose molecular masses in kilodaltons (kd) are indicated on the left. Arrows to the right of each panel indicate the position of each hybrid large T antigen.

comparable to PyT_{1-727}-$SVT_{337-708}$ and PyT_{1-521}-$SVT_{336-708}$ (Fig. 2a). The remaining extracts were then immunoprecipitated with PAb 421 and similarly electrophoresed, transferred, and probed. PAb 421 (28) is a monoclonal antibody directed against the extreme carboxyl terminus of the cellular protein p53 (66) that does not cross-react with either SV40 (28) or polyomavirus (67) large T antigen. Whereas PyT_{1-727}-$SVT_{337-708}$ and PyT_{1-521}-$SVT_{336-708}$ were coimmunoprecipitated with p53 by PAb 421, PyT_{1-452}-$SVT_{412-708}$ was not (Fig. 2b). These results indicate that SV40 amino acids 337 to 708 contain the p53 binding site and amino acids 337 to 411 are important for such binding.

Hybrid nature of PyT_{1-521}-$SVT_{336-708}$ confirmed. Since PyT_{1-521}-$SVT_{336-708}$ was expressed at a slightly higher level than PyT_{1-727}-$SVT_{337-708}$ (Fig. 2a), we chose to study it in more detail. The plasmid pPySV2 as well as plasmids containing the entire SV40 genome (pSVBam) and the entire polyomavirus genome (pPyBam) were expressed in rat F2408 cells after cotransfection with pkoneo and G418 sulfate selection. Extracts of each of these pools were immunoprecipitated with a variety of monoclonal antibodies directed against both SV40 and polyomavirus large T antigens, and then these immunoprecipitates were immunoblotted to detect the various T antigens. Since such blots show steady-state levels of the T antigens, they demonstrate the actual levels in cells and demonstrate that the proteins are, in fact, stable. The specificity of these various antibodies is shown in Fig. 3. PAbs 419 and 101, which have epitopes at the amino and carboxyl ends, respectively, of SV40 large T antigen, both immunoprecipitated SV40 large T antigen but not polyomavirus large T antigen. PAb F4, which reacts with an epitope at the amino terminus of polyomavirus large T antigen, immunoprecipitated polyomavirus large T antigen but not SV40 large T antigen. Consistent with its hybrid nature, PyT_{1-521}-$SVT_{336-708}$ was immunoprecipitated by PAb 101 and F4 but not by PAb 419.

The most appropriate plasmids for comparison were pSVLT and pPyLT, which encode SV40 large T antigen alone and polyomavirus large T antigen alone, respectively. However, expression of these plasmids in F2408 cells resulted in barely detectable levels of expression (data not shown). Indeed, when pSVLT-transfected pools were examined in transformation assays, the cells appeared to be phenotypically normal (Table 1). Previous studies in which SV40 large T antigen was expressed alone from its native promoter as a virus showed a low efficiency of transformation, and therefore the approach was to use retroviruses expressing SV40 large T antigen for comparison, leading to a higher transformation frequency (2). Thus, expression of either SV40 or polyomavirus large T antigen cDNAs from their respective native promoters in F2408 cells cannot be used as an adequate control for the experiments presented here. Our further studies therefore compared expression of the hybrid with the large T antigens encoded by pSVBam and pPyBam, plasmids which contain the complete SV40 and polyomavirus genomes, respectively, as well as with the SV40 large T antigen expressed by a recombinant retrovirus, TEXS6 (see below).

As was just noted, transfection of F2408 cells with the plasmid pPyLT, which encodes only polyomavirus large T antigen, resulted in barely detectable levels of T antigen (data not shown). In contrast to cells transfected with pPyLT, cells transfected with pPyBam, which encodes all three polyomavirus T antigens, showed readily detectable levels of polyomavirus large T antigen upon transfection and subsequent selection (Fig. 3, pPyBam). This may be related to the correlation between introns and higher expression levels described in previous reports (5, 7, 26, 27). Surprisingly, upon transfection of cells with pPySV2 (lacking the intron), the hybrid polyomavirus-SV40 large T antigen was expressed at levels comparable to those of polyomavirus large T antigen in cells transfected with pPyBam (containing

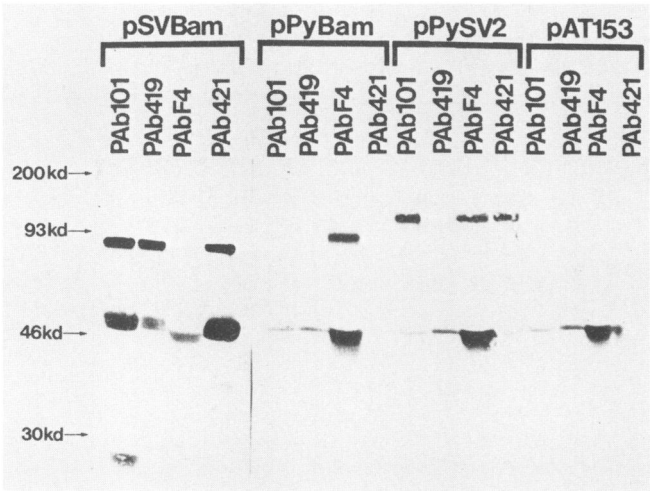

FIG. 3. Hybrid nature of PyT_{1-521}-$SVT_{336-708}$ confirmed by immunoprecipitation with monoclonal antibodies. Cell lysate protein (125 μg) from each G418 sulfate-resistant pool of F2408 cells expressing the indicated plasmid was immunoprecipitated with PAb 419 (epitope is contained between amino acids 1 and 82 of SV40 large T antigen), PAb 101 (epitope is contained within amino acids 522 to 610 of SV40 large T antigen), PAb F4 (epitope is contained within amino acids 1 to 79 of polyomavirus large T antigen), or PAb 421 (epitope is at the extreme carboxyl terminus of p53). Samples were run on 10% polyacrylamide gels, transferred to nitrocellulose, and probed either with undiluted PAb 101 (pSVBam) or with a 1:2,000 dilution of rabbit anti-polyomavirus small T antigen serum (remaining plasmids). Arrows to the left indicate the positions of relevant marker proteins in kilodaltons (kd).

the intron) (Fig. 3). Thus, the presence of both polyomavirus and SV40 sequences in this plasmid, even though it lacks an intron, allows greater levels of expression of the hybrid T antigen than were detected with plasmids lacking introns but encoding either polyomavirus or SV40 large T antigen alone (data not shown). Previous studies with the expression of SV40 late-region transcripts lacking introns have shown that the defect is posttranscriptional (26, 27) and is related to mRNA stability in the nucleus and subsequent transport to the cytoplasm (54). Whether the greater level of expression of the polyomavirus-SV40 large T antigen that was observed here is related to altered transcription of the DNA, translation of the RNA, or stability of the protein remains to be determined.

PyT_{1-521}-$SVT_{336-708}$ binds to both p53 and p105-RB. Extracts of G418 sulfate-resistant pools were immunoprecipitated with PAb 421, electrophoresed on 10% SDS–polyacrylamide gels, transferred to nitrocellulose, and probed for the presence of large T antigen in the immunoprecipitates. Both SV40 large T antigen and PyT_{1-521}-$SVT_{336-708}$ were immunoprecipitated by PAb 421, whereas polyomavirus large T antigen was not (Fig. 3). The amounts of T antigen that were immunoprecipitated by PAb 421 relative to the amounts immunoprecipitated by PAb 101 for both SV40 T antigen and the hybrid T antigen were similar. This suggests that the same relative proportion of each T antigen was bound to p53.

Immunoprecipitation with the antibody Rb-PMG3-245 (15), directed against p105-RB, the protein encoded by the retinoblastoma susceptibility gene, can be used to demonstrate complex formation between large T antigens and p105-RB (15, 21). In order to detect such complex formation, it was necessary to immunoprecipitate six times as much extract as was used in the already mentioned immunoprecipitations (750 versus 125 μg). Even with this large amount of extract, we have been unable to demonstrate complex formation between SV40 large T antigen and p105-RB in rat F2408 cells (data not shown). Polyomavirus large T antigen, however, was immunoprecipitated by the anti-p105-RB antibody (Fig. 4, pPyBam), as was PyT_{1-521}-$SVT_{336-708}$ (Fig. 4, pPySV2). Clearly, only a very small amount of the total T antigen expressed in these cells, as determined by parallel immunoprecipitation with PAb F4, was immunoprecipitated by the Rb-PMG3-245 antibody (anti-p105-RB).

TABLE 1. Growth of G418 sulfate-resistant pools on confluent monolayers of F2408 cells

Plasmid[a]	No. of foci on F2408 cells[b]	No. of colonies on plastic[b]	Relative plating efficiency[c]
Exp I			
pSVBam	158 ± 19	216 ± 30	0.73
pSVLT	2 ± 1	173 ± 16	<0.02
pPyBam	ND[d]	ND	ND
pPyLT	2 ± 1	224 ± 20	<0.01
pPySV2	3 ± 3	288 ± 11	<0.01
pAT153	ND	ND	ND
None	2 ± 1	158 ± 34	<0.02
Exp II			
pSVBam	523 ± 43	580 ± 72	0.90
pSVLT	ND	ND	ND
pPyBam	195 ± 76	472 ± 80	0.41
pPyLT	ND	ND	ND
pPySV2	2 ± 2	545 ± 39	<0.01
pAT153	9 ± 2	657 ± 78	<0.02
None	2 ± 1	712 ± 120	<0.01
Exp III			
pSVBam	315 ± 45	366 ± 17	0.86
pSVLT	ND	ND	ND
pPyBam	202 ± 49	324 ± 12	0.62
pPyLT	ND	ND	ND
pPySV2	1 ± 1	492 ± 58	<0.01
pAT153	4 ± 1	569 ± 68	<0.01
None	0	588 ± 117	<0.01

[a] pSVBam encodes the entire SV40 genome, pSVLT encodes SV40 large T antigen alone, pPyBam encodes the entire polyomavirus genome, pPyLT encodes polyomavirus large T antigen alone, pPySV2 encodes the hybrid T antigen PyT_{1-521}-$SVT_{336-708}$, and pAT153 is the vector backbone. Either 500 (experiment I) or 1,000 (experiments II and III) cells were plated per 60-mm dish.

[b] Average number of foci or colonies of ≥0.5 mm in diameter per 60-mm dish ± standard deviation (n = 5), scored at 10 days after plating.

[c] Relative plating efficiency was calculated as average number of foci on F2408 cells/average number of colonies on plastic.

[d] ND, Not determined.

PyT_{1-521}-$SVT_{336-708}$ does not transform rat F2408 cells. Two assays were performed to assess the transformation state of the G418 sulfate-resistant pools: the ability to form foci on confluent monolayers of normal cells and the ability to grow in soft agar. For both assays, data are presented here from analyses of G418 sulfate-resistant pools (Table 1 and Fig. 5). Identical results were obtained from analyses of individually picked clones (data not shown). Pools expressing the whole SV40 genome formed foci on confluent monolayers of nontransformed F2408 cells with an efficiency of 86 to 90% (Table 1, pSVBam), whereas pools expressing the whole polyomavirus genome formed foci at a lower efficiency (41 to 62%, Table 1, pPyBam) and formed these foci at a slower rate (data not shown). Pools expressing the hybrid T antigen PyT_{1-521}-$SVT_{336-708}$ did not form observable foci (Table 1, pPySV2), even after 20 days (data not shown).

Pools expressing the whole SV40 genome grew in soft agar

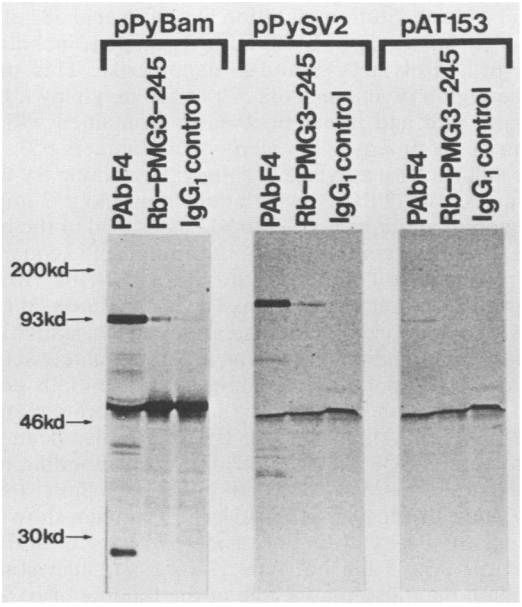

FIG. 4. PyT_{1-521}-$SVT_{336-708}$ binds p105-RB. Cell lysate protein (750 μg) from each G418 sulfate-resistant pool of F2408 cells expressing the indicated plasmid was immunoprecipitated with PAb F4, Rb-PMG3-245, or a control IgG1 antibody. Samples were run on 10% polyacrylamide gels, transferred to nitrocellulose, and probed with a 1:2,000 dilution of rabbit anti-polyomavirus small T antigen serum. Arrows to the left indicate the positions of relevant marker proteins in kilodaltons (kd). The faint band seen in the PAb F4 immunoprecipitate of the pAT153-transfected pool is not related to T antigen, since it was only occasionally observed and was seen in the parental F2408 cell line as well.

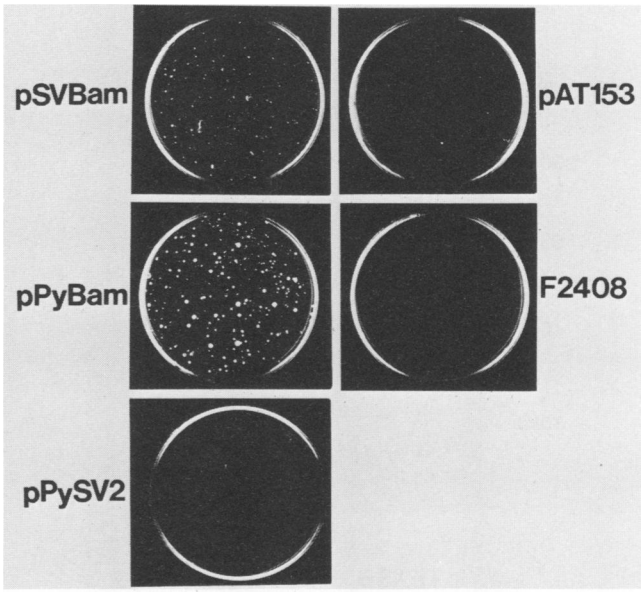

FIG. 5. Growth of G418 sulfate-resistant pools in soft agar. A total of 2,000 cells of each G418 sulfate-resistant pool expressing the indicated plasmid were plated in 0.33% agar on a bed of 0.5% agar in 60-mm dishes. After 35 days, plates were photographed against a dark background without magnification.

at a low efficiency (6 to 13%), and the colonies that formed were small (Fig. 5, pSVBam). In contrast, the pools expressing the whole polyomavirus genome grew more efficiently in soft agar (13 to 20%), and the colonies were much larger than those seen with the whole SV40 genome (Fig. 5, pPyBam). Pools expressing PyT_{1-521}-$SVT_{336-708}$ formed extremely small colonies which were not large enough to be scored (Fig. 5, pPySV2). This low level of inefficient growth is apparently related to the expression of resistance to G418 sulfate, as such growth was also observed in G418 sulfate-resistant pools derived by cotransfection with vector DNA but not with untreated F2408 cells (Fig. 5, pAT153 and F2408). Thus, pools expressing PyT_{1-521}-$SVT_{336-708}$ did not form foci on confluent monolayers of normal F2408 cells, nor did they grow in soft agar.

Level of expression sufficient for transformation by SV40 large T antigen is not sufficient for PyT_{1-521}-$SVT_{336-708}$. Having established that transfection with pSVBam but not pPySV2 produced cells with a transformed phenotype as seen in two different assays, we were concerned that this might be related to differences in the levels of the T antigens that were expressed by either construct. Various amounts of extracts of G418 sulfate-resistant pools expressing either the SV40 early region or the hybrid encoded by pPySV2 were immunoprecipitated with PAb 101. These immunoprecipitates were run on a 10% polyacrylamide gel, transferred to nitrocellulose, and probed with the same antibody, PAb 101 (Fig. 6a). The levels of SV40 large T antigen that were expressed were clearly higher than those of the hybrid T antigen PyT_{1-521}-$SVT_{336-708}$. As the amount of SV40 large T antigen observed in 100 μg of extract was similar to that of the hybrid T antigen in 400 μg of extract, there was approximately fourfold more SV40 large T antigen than PyT_{1-521}-$SVT_{336-708}$ in these pools.

These results raised two possible explanations for the inability of the hybrid T antigen to transform cells. First, its level of expression in these pools was too low to observe transformation. Second, the expression of SV40 small t antigen (encoded by pSVBam) is also necessary. To address these possibilities, pools of G418 sulfate-resistant F2408 cells were generated by infection with TEXS6, a retrovirus expressing only SV40 large T antigen, which was previously shown to transform NIH 3T3 cells (6). Immunoprecipitation of various amounts of extracts of these pools was compared with that of two independently established pools expressing the hybrid T antigen PyT_{1-521}-$SVT_{336-708}$ (Fig. 6b). In this case, the levels of T antigen expression among these pools were comparable. In contrast to what was observed with pools of cells expressing PyT_{1-521}-$SVT_{336-708}$ (Fig. 5), the retrovirus-infected pool expressing SV40 large T antigen formed many more colonies in soft agar (mean ± standard deviation, 153 ± 69; $n = 5$) than the ZIPNEO-infected pool (7 ± 3; $n = 5$) and uninfected F2408 cells (3 ± 3; $n = 5$). Thus, when expressed at comparable levels, expression of SV40 large T antigen in the absence of small t antigen produces cells with a transformed phenotype, whereas expression of the hybrid T antigen does not.

DISCUSSION

p53 binding site on SV40 large T antigen. Genetic studies with deletion mutants have localized the binding site for p53 on SV40 large T antigen to the carboxyl half of the protein (42, 44). Tevethia et al. (62) have shown that a deletion mutant lacking amino acids 627 to 708 still binds p53. Mole et al. (43) constructed a chloramphenicol acetyltransferase fusion protein containing SV40 large T antigen amino acids

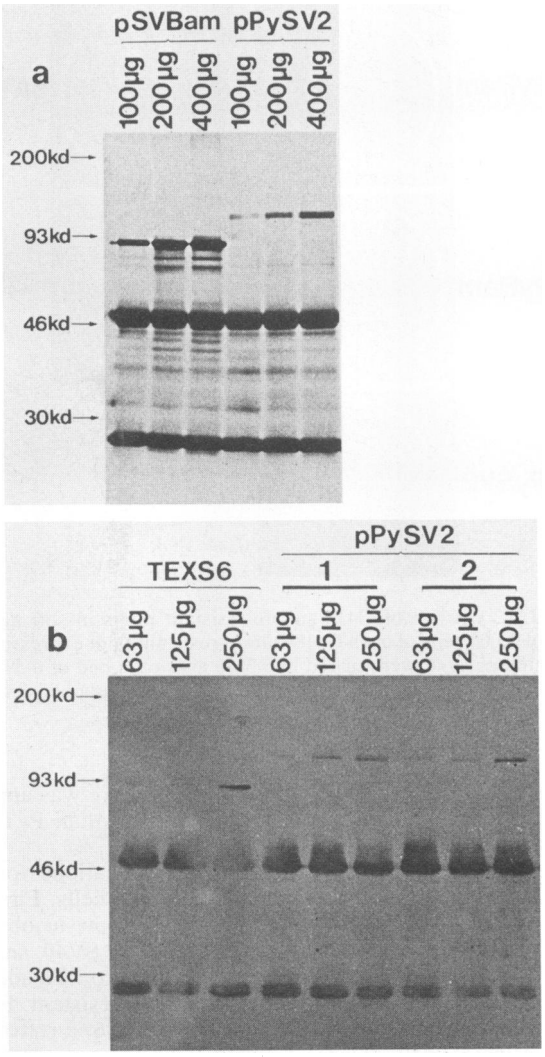

FIG. 6. Comparison of T antigen levels in G418 sulfate-resistant pools expressing SV40 large T antigen and PyT_{1-521}-$SVT_{336-708}$. (a) The indicated amounts of cell lysate protein from G418 sulfate-resistant pools of F2408 cells expressing either pSVBam or pPySV2 were immunoprecipitated with PAb 101. (b) The indicated amounts of cell lysate from G418 sulfate-resistant pools of F2408 cells infected with the TEXS6 retrovirus or two independently derived pools of F2408 cells transfected with pPySV2 were immunoprecipitated with PAb 101. Samples were run on a 10% polyacrylamide gel, transferred to nitrocellulose, and probed with undiluted PAb 101 hybridoma supernatant. Arrows to the left indicate the positions of relevant marker proteins in kilodaltons (kd).

272 to 708 which binds p53. Schmieg and Simmons (57), using controlled proteolysis of purified SV40 large T antigen, identified a fragment consisting of amino acids 131 to 517 which binds p53 in vitro. We have constructed hybrid SV40-polyomavirus large T antigens which contain amino acids 337 to 708 of SV40 large T antigen that are capable of binding p53 (Fig. 2 and 3). Hence, a putative binding region for p53 is amino acids 337 to 517.

Peden et al. (47) have identified base substitution and deletion mutations in the vicinity of amino acids 570 to 588 which abolish p53 binding. Tevethia et al. (62) have characterized a deletion mutant missing amino acids 587 to 589 which does not bind p53. Tack et al. (61) have shown that, in fact, a base substitution mutation at amino acid 584 affects a number of functions of SV40 large T antigen, including the ability to hydrolyze ATP and to oligomerize. This suggests that the region of amino acids 570 to 588 may play a general structural role and is not necessarily contained within the domain of SV40 large T antigen which contacts p53.

Our studies with a hybrid T antigen containing SV40 large T amino acids 412 to 708 which does not bind p53 implicate the region of amino acids 337 to 411 as crucial in the binding of p53. Indeed, a comparison of the amino acid sequences of three p53-binding T antigens (those of SV40, JC virus, and BK virus) with that of polyomavirus large T antigen (Fig. 7) shows that the region of greatest sequence similarity between the p53-binding T antigens and the greatest sequence divergence with polyomavirus large T antigen falls between amino acids 337 and 411. The 58-kilodalton protein encoded by the E1B RNA of adenovirus type 5 has also been shown to bind to p53 (55, 72), whereas the corresponding protein from adenovirus type 12 does not (39, 72). A short stretch of amino acids, 481 to 497, of SV40 large T antigen shows some sequence similarity to amino acids 67 to 84 of E1B from adenovirus type 5 but not type 12 (Fig. 7), suggesting that this region may also play a role in the binding of p53.

p105-RB binding site on SV40 and polyomavirus large T antigens. DeCaprio et al. (15) have identified several base substitution and deletion mutants of SV40 large T antigen which do not bind p105-RB. The substitutions are at amino acids 107, 108, and 112 and at 105 and 114; the deletions are amino acids 107 to 113 and 92 to 114. The regions of the adenovirus E1A protein which bind the *Rb* gene product have recently been identified (69). Comparison of the amino acid sequences of these regions with that of SV40 large T antigen and the E7 protein of human papillomavirus 16 are consistent with the results found with the substitution and deletion mutants, implicating the region of amino acids 99 to 117 in the binding of the *Rb* gene product by SV40 large T antigen.

Comparison of the amino acid sequence of polyomavirus large T antigen with these other DNA tumor virus transforming proteins shows that amino acids 138 to 157 of polyomavirus large T antigens have similar key residues (18). Studies of the binding of hybrid SV40-polyomavirus large T antigens to p105-RB show that hybrids containing polyomavirus large T antigen amino acids 1 to 727 (data not shown) or 1 to 521 (Fig. 4) bind to p105-RB. This suggests that the binding region on polyomavirus large T antigen for p105-RB is contained within its first 521 amino acids.

Implications for the mechanism of oncogenic transformation. The general model that has been proposed for the mechanism of oncogenic transformation by DNA tumor viruses is that the transforming proteins of these viruses bind to and inactivate the functions of the products of tumor suppressor genes, namely p53 and p105-RB (22, 35, 68). Experiments have been presented here demonstrating that a hybrid polyomavirus-SV40 large T antigen which binds to both of these proteins (Fig. 2 to 4) still does not transform established rat cells (Fig. 5 and Table 1). The preliminary studies with G418 sulfate-resistant pools of transfected F2408 cells suggested two possibilities. First, the level of SV40 large T antigen in the pools expressing the entire SV40 early region was greater than that seen with the hybrid large T antigen (Fig. 6a). Second, the cells transfected with the SV40 early region were presumably also expressing small t antigen, whereas those expressing the hybrid T antigen were not; a role for small t antigen in transformation by SV40 large T antigen has been suggested, particularly under conditions

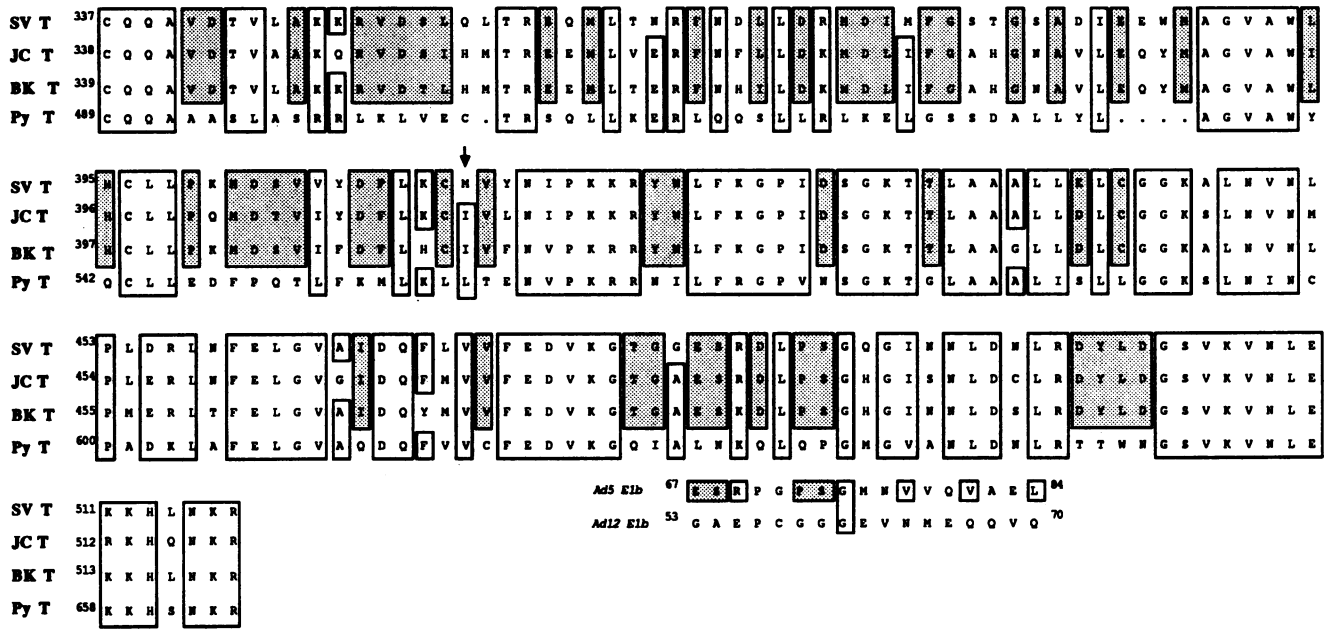

FIG. 7. Sequence comparison of the putative p53 binding region of papovavirus large T antigens. The amino acid sequence of SV40 large T antigen (SV T) from residues 337 to 517 is shown aligned to the corresponding region of two other p53-binding papovavirus large T antigens, those of JC virus (JC T) and BK virus (BK T), as well as the corresponding region of polyomavirus large T antigen (Py T), which does not bind p53. Regions of amino acid identity or conservation are boxed; those shared only by the p53-binding T antigens are shaded. The vertical arrow indicates SV40 large T antigen amino acid 412. Also indicated is a short stretch of amino acid similarity between these T antigens and the 58-kilodalton protein of the E1B region of adenovirus type 5 (Ad5), which also binds p53. The corresponding protein from adenovirus type 12 (Ad12) does not bind p53 and is also shown for comparison.

in which the level of expression of the large T antigen is limiting (2). To rule out this latter possibility, transfected pools of cells expressing the hybrid large T antigen were compared with pools of cells which had been infected with a retrovirus which encoded only large T antigen and not small t antigen. Expression of a level of SV40 large T antigen comparable to that seen with the hybrid large T antigen (Fig. 6b) in the absence of small t antigen expression caused transformation of rat F2408 cells. Thus, neither the presence of small t antigen nor the level of protein expression is an adequate explanation for the failure of the hybrid large T antigen to transform cells.

Clearly, simple binding of p53 and p105-RB by a large T antigen as detected by immunoprecipitation is not sufficient for oncogenic transformation. Several potential explanations may serve as the basis for further experimentation. First, the hybrid large T antigen may not be quantitatively binding and inactivating all of the relevant p53 or p105-RB in these cells. Second, the binding site for p105-RB in the hybrid protein is derived from polyomavirus large T antigen. Since polyomavirus large T antigen alone was reported not to transform cells, in contrast to SV40 large T antigen, there may be functional differences between the p105-RB binding sites on these two T antigens. Third, there may be important interactions between p53 or p105-RB and other cellular proteins which are disrupted by the binding of these tumor suppressor proteins by a transforming large T antigen such as SV40 but not by the hybrid large T antigen. Finally, something other than interactions with either p53 or p105-RB may be involved. For example, the binding of another cellular protein, p107, has been implicated in transformation by SV40 large T antigen (19, 21). Mutants with mutations in the amino-terminal domain of SV40 T antigen which do not bind p105-RB also do not bind p107 (19, 21). Since it has yet to be demonstrated whether polyomavirus large T antigen also binds p107, one explanation for the failure of the hybrid protein to transform may be its inability to bind to p107 or some other cellular factor, such as the adenovirus E1A-binding protein p300. The binding of p300 by E1A has been implicated in transformation by adenovirus (69).

Genetic studies have shown that mutants of SV40 large T antigen which do not bind either p53 or p105-RB have a reduced ability to transform cells; such binding is necessary for the oncogenic potential of SV40 large T antigen. The studies reported here show that a polyomavirus-SV40 hybrid large T antigen which is capable of binding to both p53 and p105-RB does not transform established rat cells. Further attempts to reconstitute the transforming ability of SV40 large T antigen through the use of polyomavirus-SV40 hybrids will be important to delineate which interactions of T antigen are sufficient for its oncogenic potential.

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