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# Recombinant Retroviruses Encoding Simian Virus 40 Large T Antigen and Polyomavirus Large and Middle T Antigens

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**We used a murine retrovirus shuttle vector system to construct recombinants capable of constitutively expressing the simian virus 40 (SV40) large T antigen and the polyomavirus large and middle T antigens as well as resistance to G418. Subsequently, these recombinants were used to generate cell lines that produced defective helper-free retroviruses carrying each of the viral oncogenes. These recombinant retroviruses were used to analyze the role of the viral genes in transformation of rat F111 cells. Expression of the polyomavirus middle T antigen alone resulted in cell lines that were highly tumorigenic, whereas expression of the polyomavirus large T resulted in cell lines that were unaltered by the criteria of morphology, anchorage-independent growth, and tumorigenicity. More surprisingly, SV40 large T-expressing cell lines were not tumorigenic despite the fact that they contained elevated levels of cellular p53 and had a high plating efficiency in soft agar. These results suggest that the SV40 large T antigen is not an acute transforming gene like the polyomavirus middle T antigen but is similar to the establishment genes such as *myc* and adenovirus EIa.**

The DNA tumor viruses polyomavirus (Py) and simian virus 40 (SV40) are both capable of transforming both primary and established cell lines in vitro (for a review see reference 70). Extensive work has shown that genes encoded by the early regions of these viruses are both necessary and sufficient for transformation (1, 10, 12, 14, 15, 18, 29, 38, 43, 49, 67; for a review see reference 70). Analysis of the respective roles of these genes has been complicated because the early regions are multiply spliced to specify more than one protein: Py encodes three antigens, the large T (100 kilodaltons [kDa]), the middle T (56 kDa), and the small T (22 kDa) (20, 21, 24, 25, 58); SV40 encodes two antigens, the large T (95 kDa) and the small T (20 kDa) (48, 51, 69). In addition, transformation assays with DNA tumor viruses have generally involved infection or transfection of a cell population and selection of a small fraction of cells with a particular phenotype. For example, this selection could be based on either focus formation, anchorage-independent growth, or growth in low serum, and thus, this type of assay does not necessarily reveal the average phenotype resulting from interaction of the oncogene with the cell. Moreover, the process by which the viral DNA segment integrates into cellular chromosomes can give rise to multiple copies and rearrangements. The resulting variation in levels of viral mRNA and gene expression can significantly affect the range of phenotypes of the transformants. Previous studies of the variation in phenotype observed after transformation by SV40 detected clonal cell lines that ranged over completely, partially, or minimally transformed (50, 56).

We therefore undertook a systematic analysis of the role of the various genes encoded by the early regions of these viruses by isolating cell lines which constitutively express these proteins without prior selection for a particular phenotype. This was done by constructing recombinant retroviruses with the pZipNeoSV(X)1 vector system so that the retroviruses encode resistance to the antibiotic G418 in addition to the inserted gene (7). Thus, recombinant-containing cells can be selected by growth in presence of the drug G418 (12). Here we present the isolation and characterization of cell lines that produce defective helper-free recombinant retroviruses which encode the Py large or middle T antigens or the SV40 large T antigen. Subsequently, recombinant retroviruses prepared from these producers were used to isolate cell lines derived from the established rat cell line F111 (13) by virtue of resistance to G418. The analysis of the role of the Py large T, Py middle T, and SV40 large T protein in transformation, as determined by characterization of the F111-derived cell lines for growth in soft agar, tumorigenicity, and focus formation, is described.

## MATERIALS AND METHODS

**Cells and cell culture.** Rat F111 cells (13) obtained from T. Benjamin, NIH 3T3 cells, and $\Psi_2$ cells (36) were all maintained in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) calf serum and penicillin and streptomycin (complete medium). Cos M6, a subclone of Cos-1 cells (15) isolated by M. Horowitz (19), and CV1 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum.

**Construction of retrovirus recombinant plasmids.** The recombinant plasmids were all constructed by inserting appropriate DNA segments (described in the Results) into the unique *Bam*HI site of pZipNeoSV(X)1 (Fig. 1), using *Bam*HI linkers. The DNA segments for the Py large T, Py middle T, and SV40 early region were derived from pPyLT and pPyMT (provided by A. Cowie) and pBgl-BamSV40 (provided by U. Hansen). Plasmids were propagated either in *Escherichia coli* MC1061 (5) or in its *recA1* derivative, JS4 (a kind gift from John Sedivy). All the manipulations involved in the plasmid constructions were carried out by standard procedures or under the recommended conditions of the supplier (25). Restriction digests were monitored by horizontal agarose gel electrophoresis (36).

**DNA transfections.** Cells which had been passaged 16 to 24

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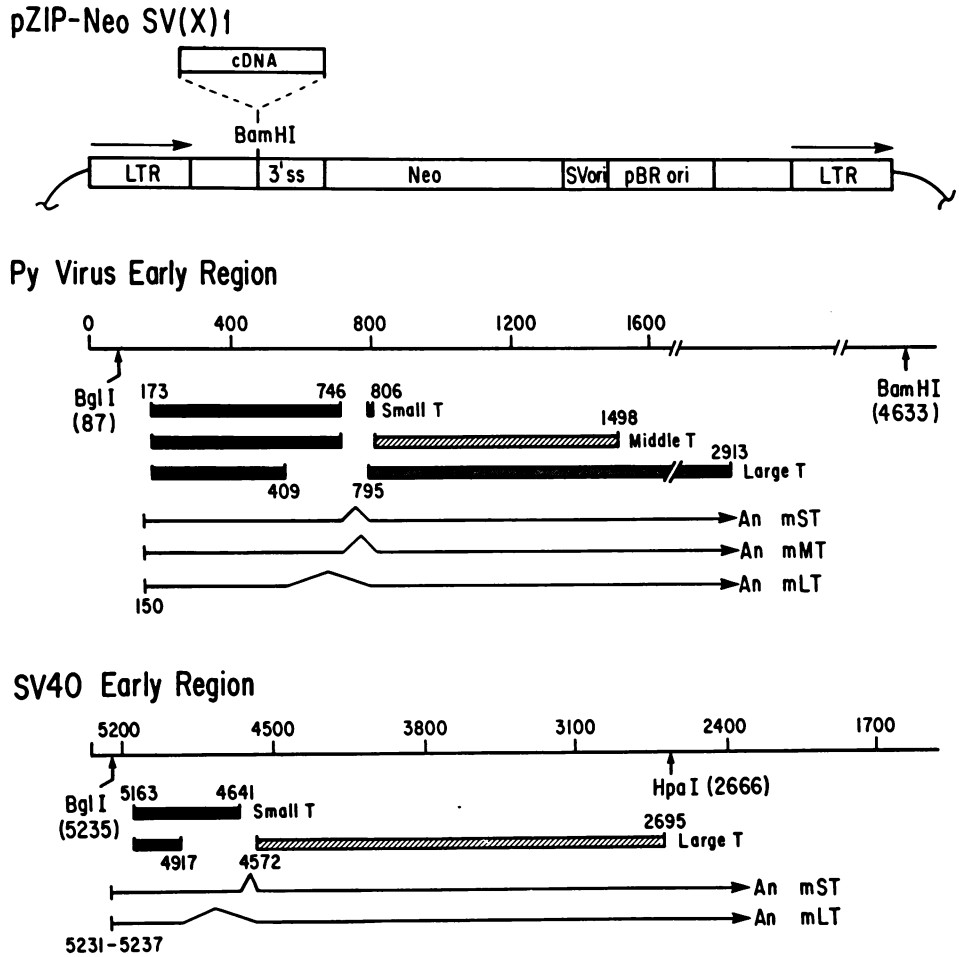



FIG. 1. Structure of the ZipNeo shuttle vector and the Py and SV40 early regions. The structure of pZipNeoSV(X)1 (top) has been described previously (9). Underneath is a schematic of the organization of the Py and SV40 early regions. This shows the different proteins encoded by these regions; the segments of DNA which code for these proteins are also shown. These proteins are translated from differentially spliced mRNAs, the structures of which are also indicated. For a greater in-depth review of the structure of the mRNAs see reference 70. ori, Origen; LTR, long terminal repeat.

h previously were transfected with 10 μg of DNA by the calcium phosphate procedure of Graham and van der Eb (16), as modified by Parker and Stark (46). Four hours later, they were glycerol shocked. Forty-eight hours after glycerol shock, the cells were split 1:10 and 1:20 into complete medium containing 1 mg of G418 per ml (9).

**Retrovirus infections.** Retrovirus infections of both NIH 3T3 and rat F111 cells were done in 10-cm dishes at 37°C for 2 h in 2 ml containing retrovirus and 8 μg of Polybrene (Aldrich Chemical Co., Inc., Milwaukee, Wis.) per ml, after which 8 ml of complete medium was added. Forty-eight hours after infection, the cells were passaged, and the G418-resistant cells were isolated by selection in complete medium containing 1 mg of G418 per ml. The cultures were refed every 4 days. After 10 to 14 days when the colonies were clearly visible, a number of them were selected and isolated onto microtiter dishes. Clones that grew were expanded into cell lines. When infections were done for a focus assay, the cells were passaged at 48 h and grown in Dulbecco modified Eagle medium supplemented with 5% (vol/vol) calf serum and penicillin and streptomycin. These cultures were refed every 4 days for 2 to 3 weeks when they were examined for foci, photographed, and stained with 1% (wt/vol) methylene blue in 50% (vol/vol) ethanol or injected into nude mice for analysis of tumor formation.

**Analysis of proviral DNA.** High-molecular-weight cellular DNA was isolated as previously described (62). For genomic blot analysis, 10 μg of DNA was digested with the restriction endonucleases indicated in the figure legends and fractionated on a 1 to 1.4% agarose gel. Transfer of the fractionated DNA fragments onto nitrocellulose and subsequent processing of these filters including probing with nick-translated DNA fragments (55) was done by standard procedures (36).

**Analysis of protein expression.** Cells were labeled with [$^{35}$S]methionine under the conditions indicated in the figure legends. The extracts were prepared and immunoprecipitated by the following procedures: (i) the method of Kessler et al. (28) as described in Cepko et al. (6) for SV40 large T antigen, and (ii) the method of Ito et al. (25) for Py large T and middle T antigens. The in vitro kinase procedure for the Py middle T antigen was performed by the method of Courtneidge and Smith (8). The immunoprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (31). The gels were treated with En$^3$Hance (New England Nuclear Corp./Dupont, Boston, Mass.), dried, and fluorographed.

**Anchorage-independent growth.** The ability of the various cell lines to grow in an agar suspension was assayed by the method of Macpherson and Montagnier (35) except that SeaPlaque agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) was used instead of Bacto-Agar (Difco Laboratories, Detroit, Mich.). Twenty-one days after seeding, the cultures were examined, and colonies were counted and photographed. The cultures were then stained overnight with *p*-iodonitro tetrazolium violet (Sigma Chemical Co., St. Louis, Mo.), and the agar was scooped out and placed between sheets of Whatman 3MM paper and left to dry. The dried agar was then mounted directly for photography.

**Analysis of tumorigenicity in nude mice.** Tumorigenicity of the cell lines was determined by a subcutaneous injection of $10^5$ to $10^6$ cells in 0.1 ml of phosphate-buffered saline into 4- to 6-week-old male BALB/c/ANCr-Nu mice obtained from the National Cancer Institute-Frederick Cancer Research Facility. The mice were irradiated with 500 rads approximately 24 h previously to eliminate the natural killer cells. The cells for injection were prepared as previously described (32). Nude mice were examined for tumor formation every week for at least 8 to 12 weeks.

## RESULTS

**Construction of recombinant retroviruses.** Retrovirus recombinants were constructed by inserting appropriate DNA fragments into the unique *Bam*HI site of the pZipNeoSV(X)1 shuttle vector (Fig. 1). The viral genes were inserted in the sense orientation with respect to retroviral transcription.

**Recombinants that encode SV40 large T antigen.** Introns should be excised precisely from genomic DNA segments when they are inserted into retroviruses and transmitted as viruses (7, 63, 66). This procedure was used to isolate a recombinant retrovirus encoding only the SV40 large T antigen. The genomic *Bgl*I-*Hpa*I (nucleotides 5235 to 2666; Fig. 1) segment from pBgl-BamSV40 was inserted into the pZipNeoSV(X)1 vector with *Bam*HI linkers. This fragment lacks both the early promoter and the polyadenylation site.

**Recombinants that encode Py large and middle T antigens.** Treisman et al. (71) and Rassoulzadegan et al. (52) have used recombinant DNA techniques and cDNA cloning to construct plasmids containing variants of Py that express either the large, middle, or small T antigens. Retrovirus recombinants were constructed by insertion of the *Bgl*I-*Bam*HI fragment (nucleotides 87 to 4633; Fig. 1) derived from the appropriate cDNA plasmid into the *Bam*HI site of the pZipNeoSV(X)1 shuttle vector. These DNA fragments retain both the early promoter and polyadenylation sites of Py.

**Isolation of stable $\Psi_2$ producer lines.** Cell lines that stably produce the recombinant retroviruses were isolated by introducing plasmids into the $\Psi_2$ packaging cell line by transfection. The $\Psi_2$ cell line contains integrated copies of a defective Moloney murine leukemia virus provirus. These integrated copies provide all the functions necessary in *trans* for encapsidation of the recombinant genomic RNA, but, owing to the absence of the packaging site, RNA from the proviral DNA is not encapsidated (37). Forty-eight hours after transfection, the $\Psi_2$ cells were subjected to G418 selection. For each recombinant virus, at least five G418$^r$ colonies were isolated and analyzed. Virus supernatant from each of these cell lines was used to infect both NIH 3T3 and rat F111 cells, to determine the G418$^r$ titers. The results in Table 1 show the titer of the best producer cell line for each recombinant virus except for Py large T, where the titers of two equivalent producers are shown. These titers range from $10^4$ to over $10^6$ G418$^r$ CFU/ml.

TABLE 1. G418 resistance titers of the best producer cell[a] lines

| Virus | Insert | G418 resistance titer (CFU/ml) | |
|---|---|---|---|
| | | NIH 3T3 | Rat F111 |
| SV-X(1) | Backbone | $10^6$ | $3 \times 10^5$ |
| LT-4,5 | Py large T | $10^5$ | $6 \times 10^4$ |
| MT-5 | Py middle T | $8 \times 10^3$ | $6 \times 10^3$ |
| SV40-6 | SV40 large T only | $2 \times 10^4$ | $2 \times 10^4$ |

[a] For each recombinant retrovirus, at least five G418$^r$ $\Psi_2$ colonies were isolated and expanded into cell lines. Virus supernatant derived from each of these producer cell lines was used to infect NIH 3T3 cells to determine the G418$^r$ titers. The G418 resistance titer of the best producer cell lines is shown. Subsequently, virus supernatant from these producer cell lines was used to infect rat F111 cells, and the G418$^r$ titer was determined.

**Characterization of cell lines derived from F111 cells.** The transformation activity of the various DNA tumor virus oncogenes was investigated by analyzing cell lines derived after retrovirus infection of rat F111 cells (13). These cells are highly contact inhibited and have been extensively used to study transformation by papovaviruses. Virus stocks from each of the producer cell lines were used to isolate independent cell lines by infection of F111 cells and selection for G418 resistance. For each virus, at least two or three independent colonies were selected, expanded, and characterized.

**Cell morphology.** The morphology of representative cell lines is shown in Fig. 2. In addition to the parental F111 cells, panel A shows FSV2a2, a cell line derived from infection with backbone ZipNeoSV(X)1 virus alone, and PyF, a cell line isolated by transformation of F111 cells with Py. (The PyF cell line was a gift from T. Benjamin.) Panel B shows a typical cell line, isolated after infection with retroviruses encoding SV40 large T (FSV4063), Py large T (FLT41), and Py middle T (F2MT52). Surprisingly, only cells derived from infection with Py middle T had a morphology which was significantly different from the parental F111 cells. These cells grew in a disordered fashion like the Py-transformed F111 cells, PyF. The cell lines derived after infection with Py and SV40 large T had morphologies indistinguishable from the parental F111 cells.

The morphology of the NIH 3T3 cells infected with this set of recombinant retroviruses was also determined. Again, infected cells were selected for G418$^r$, and individual colonies were expanded into cell lines. Three such lines were generated for each recombinant retrovirus. As for F111 cells, only the NIH 3T3 cells infected with a retrovirus expressing Py middle T had a morphology distinguishable from the parental cells. These cells were both refractible and elongated and reassembled the F2MT52 cell line shown in panel B of Fig. 2.

**DNA analysis.** The structure and number of the recombinant genomes that had been transmitted to the F111 cell lines via infection were determined by Southern blot analysis (36). Genomic DNA was prepared from each cell line and digested with either *Bam*HI or *Xba*I. Since the *Bam*HI site was the site of insertion of the viral segments, digestion with this enzyme released the insert segment and permitted evaluation of its intactness. The integrity of the total provirus was analyzed by digestion with *Xba*I which cleaves once in the long terminal repeats of the vector. Analysis of the *Bam*HI digest with a probe specific for the neomycin gene should indicate the number of proviral inserts since one *Bam*HI site must be contributed by the flanking cellular sequences.

**(i) SV40 early region-derived cell lines.** The Southern blot

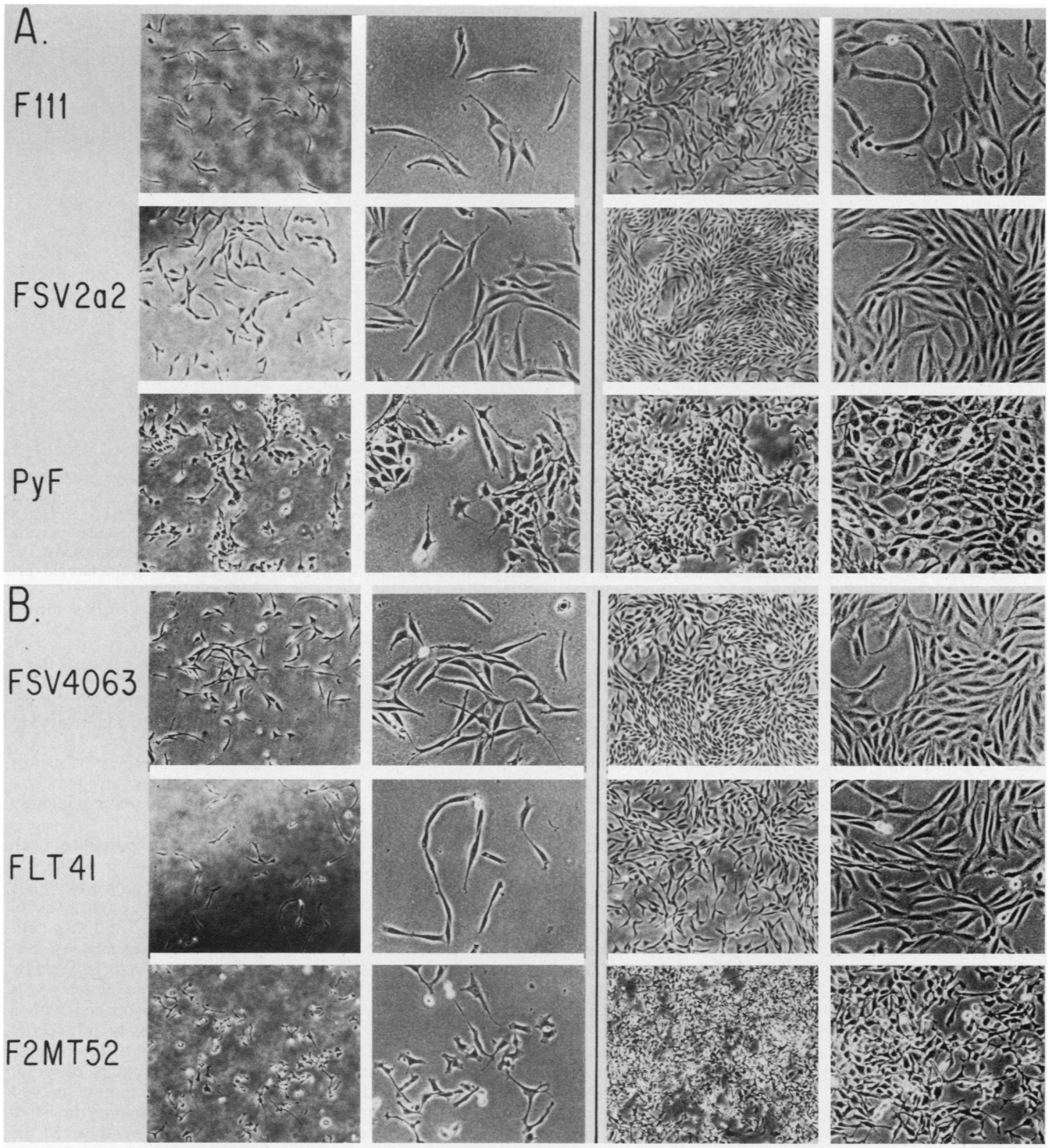



FIG. 2. Cell morphology. Cells derived from the indicated cell lines were photographed with Kodak Tri-X pan film (150400/27°) at 40- and 100-fold magnification at both low (left) and high (right) density.

analysis of two cell lines, FSV4063 and F2SV4061, which were derived from independent infections with virus from the SV40-6 producer cell line, is shown in Fig. 3. Panels A and B show the results of digestion with *Bam*HI, and panel C corresponds to digestion with *Xba*I. Comparison of lengths of the SV40 early region in pZipSV40 (denoted a) with that in FSV4063 and F2SV4061 (denoted b) revealed that the former is 350 base pairs longer. This difference could not be detected in Southern blots of *Xba*I digestions (panel C). Further analysis involving rescue of the provirus from both FSV4063 and F2SV4061 cell lines by the Cos cell fusion protocol (P. Jat, data not shown) demonstrated that the decrease in the length of the SV40 early region corresponded to removal of the intron for the large-T mRNA (69). Analysis

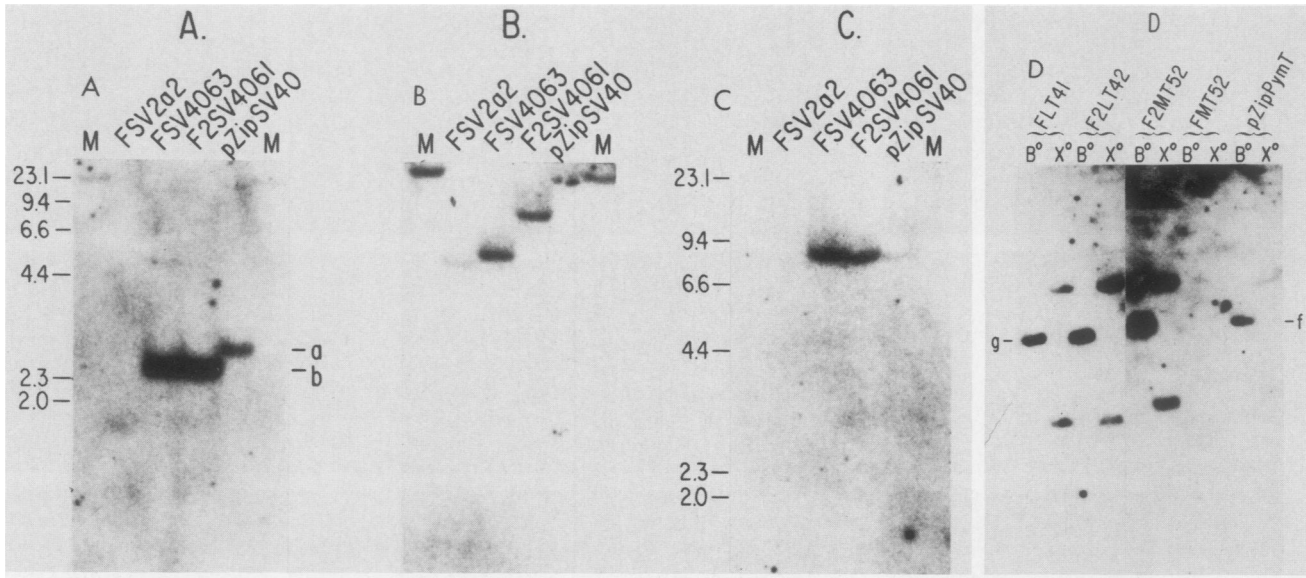



FIG. 3. Analysis of proviral DNA. Cellular DNA prepared from the indicated cell lines was digested with either *Bam*HI (panels A and B and lanes B° in panel D) or *Xba*I (panel C and lanes X° in panel D). Lanes pZipSV40 and pZipPymT correspond to digestion of recombinant plasmid DNA with the appropriate restriction enzyme. Lanes M are *Hin*dIII-digested bacteriophage λ DNA. After transfer of the fractionated DNA fragments to nitrocellulose filters, they were analyzed by hybridization to $^{32}$P-labeled purified fragments corresponding to the SV40-specific *Bam*HI fragment of pZipSV40 (A and C) and the Py-specific *Bam*HI fragment of pZipPyLT (D). After autoradiography, the hybridized probe was removed from the blot shown in panel A by washing twice at 68°C for 1 h in 1 liter of 2.5 mM Tris hydrochloride (pH 8.0–0.1 mM EDTA–1 mM sodium $PP_i$–0.05× Denhardt solution, and the filter was rehybridized to a $^{32}$P-labeled neomycin-gene-specific fragment; this is shown in panel B. Numbers to left of panels A and C show molecular size (kilodaltons); lower-case letters are described in the text.

of the virus produced by the SV40-6 cell line as well as six other independent $\Psi_2$ producer cell lines by infection of NIH 3T3 cells followed by the Cos cell fusion rescue protocol failed to identify a recombinant population in which the small-T intron was excised. Analysis by digestion with *Bam*HI, which cleaves in flanking cellular sequences as well as in the proviral DNA, and hybridization with a neomycin-specific probe showed that both cell lines contain only one provirus located at different sites in the genome (panel B). This total analysis thus demonstrated that the original SV40-6 producer line transmitted intact G418-resistant proviruses harboring only large-T-mRNA-specific sequences.

**(ii) Py large- and middle-T-derived cell lines.** Two cell lines derived by infection with virus from LT-4 $\Psi_2$ producer cell lines (FLT41 and F2LT42) and the MT-5 $\Psi_2$ producer cell line (FMT52 and F2MT52) were analyzed. Although the FMT52 cell line was isolated as a G418-resistant colony, it did not have the characteristic morphology of other lines isolated by infection with retrovirus encoding Py middle T and was therefore included in this analysis. This cell line did not contain Py middle T or neomycin-specific DNA sequences (Fig. 3D). Digestion of DNA prepared from the other cell line, F2MT52, with *Bam*HI (panel D), yielded a fragment (denoted f, 4.5 kilobases) which was identical in size to the original insert (compare F2MT52 DNA with pZip Py middle T DNA). Similarly, Py large-T-derived cell lines yielded fragments (denoted g, 4.2 kilobases) which were the same size as the original insert. Digestion of these DNAs with *Xba*I gave a more complicated pattern than the other recombinants since the inserts have internal *Xba*I sites. Although there are actually two internal *Xba*I sites, they are only 45 base pairs apart and hence behave as a single restriction site in this analysis. The results of *Xba*I digestions confirmed the conclusion from the *Bam*HI digestions that the inserts were intact (Fig. 3, panel D). Hybridization of the filter with a neomycin-specific probe substantiated the results that the inserts were intact, single copy, and located at different sites.

**Analysis of protein expression.** To examine the synthesis of proteins from the transduced genes, cell lines were metabolically labeled with [$^{35}$S]methionine and immunoprecipitated with appropriate antibodies.

**(i) SV40 early region cell lines.** Extracts prepared from cell lines labeled with [$^{35}$S]methionine for 4 h were immunoprecipitated with three different monoclonal antibodies specific for the SV40 large T antigen (pAb412 and pAb416) and the cellular p53 antigen (pAb421) (17). The latter antibody cross-reacts with p53 from a variety of species. Extracts from Cos M6 cells, a subclone of Cos cells isolated by Horowitz et al. (19), were included as positive controls. Cos cells are CV1 cells which have been transformed with a replication-deficient SV40 DNA and express high levels of the T antigens (15). To permit comparisons of the relative level of T-antigen synthesis, equal amounts of acid-precipitable total radioactivity were immunoprecipitated with excess antibody. The two cell lines containing the SV40 large T provirus (FSV4063 and F2SV4061) synthesized the large T polypeptide at about one-fifth the level of the Cos M6 cells (Fig. 4A). All three cell lines yielded about the same levels of the p53 protein. This cellular protein has been shown to be stabilized by formation of a complex with SV40 large T antigen (33, 34, 40). Thus, antibodies to either large T antigen or p53 antigen immunoprecipitate a fraction of both polypeptides. Neither control cell line F111 nor FSV2a2 yielded a band comigrating with authentic SV40 large-T polypeptide. As expected, a low level of p53 antigen was detected in these control cell lines after immunoprecipitation with the pAb421 antiserum. Thus, these results

TABLE 2. Tumorigenicity of the cell lines[a]

| Cell line | No. of tumors/no. of injections[b] |
|---|---|
| F111 | 0/2 |
| SV2a2 | 0/5 |
| Py LT | |
| FLT41 | 0/2 |
| F2LT42 | 0/3 |
| FLT51 | 0/2 |
| F2LT52 | 0/4 |
| Py MT | |
| F2MT52 | 4/4 (27 days) |
| F3MT51 | 2/3 (28 days) |
| SV40LT | |
| FSV4063 | 0/3 |
| F2SV4061 | 0/3 |
| PyF | 3/3 (18 days) |

[a] Tumorigenicity of the cell lines was determined by a subcutaneous infection of $3 \times 10^5$ to $5 \times 10^5$ cells in 0.1 ml of phosphate-buffered saline into 28- to 35-day-old nude mice (BALB/c/AnCr-Nu) irradiated with 500 rads 24 h previously. The nude mice were observed for tumor formation every week for 8 weeks at which time the experiment was terminated.

[b] At 8 weeks unless otherwise shown.

confirm the DNA analysis that the proviral DNA in these two cell lines specifies the large-T polypeptide.

**(ii) Py large-T and middle-T cell lines.** Extracts were prepared from cell lines derived by infection with retrovirus recombinants containing either Py large-T (FLT51, F2LT52, and FLT41) or middle-T (FMT52, F2MT52, and F3MT51) sequences. The cells were labeled for 18 h with [$^{35}$S]methionine. Extracts were immunoprecipitated with either Py antitumor serum or normal rat serum which was the negative control. The cell lines FSV2a2 and PyF were included as negative and positive controls, respectively. The large-T polypeptide was readily detectable in all three cell lines derived from infection with the large-T recombinant retrovirus as a 100-kDa protein (Fig. 4B). This protein comigrates with the large-T protein from lytically infected cells (data not shown). Although we were unable to detect the middle-T protein by this protocol in any of the cell lines derived from infection with the middle-T recombinant retrovirus as well as the PyF control (Fig. 4B), subsequent analyses by Joan Brugge have shown that they all express the middle T antigen. It has been shown previously by Courtneidge and Smith (8) that incubation of Py middle-T immunoprecipitates with [$\gamma$-$^{32}$P]ATP results in phosphorylation of the middle-T protein. Since this constitutes a very sensitive assay for the presence of middle-T protein, this assay was applied to extracts from the middle-T retrovirus cell lines and PyF cell line. It yielded a prominent band of $^{32}$P-labeled middle-T protein (Fig. 4C); the control cell lines were appropriately negative. Thus, we conclude that both the Py large-T and middle-T retrovirus-derived cell lines express the correct protein.

**Growth characteristics. (i) Tumorigenicity.** The retrovirus-derived cell lines were assayed for tumorigenicity by subcutaneous injections of $3 \times 10^5$ to $5 \times 10^5$ cells into irradiated BALB/c/AnCr-Nu mice. These animals are deficient in T-cell-mediated immunity and will graft tissue from any species. Surprisingly, only the Py middle-T-expressing cell lines were able to generate tumors under these conditions (Table 2). These tumors developed within 4 weeks, while mice challenged with cell lines expressing the other oncogenes remained tumor-free for at least 8 weeks. In particular, neither of the cell lines that expressed the SV40 large T antigen was tumorigenic. Since it is possible that particular cell lines may be atypical of the total spectrum of phenotypes generated by infection with a virus stock, total populations containing independently infected cells were tested for tumorigenicity. Populations of G418$^r$ cells were derived by infection with retrovirus stocks that encode SV40 large T, Py large T, or control backbone virus. In each case, the population was generated from more than 1,000 initial colonies. Injection of populations containing $5 \times 10^6$ cells failed to generate tumors in irradiated nude mice with a latency of less than 6 months. Thus, we conclude that only the Py middle-T retroviruses were tumorigenic after infection of F111 cells.

Although we have not completed a systematic analysis of the tumorigenicity of the retrovirus recombinants in NIH 3T3-derived cell lines, some assays with the same nude mice recipients were done. We found that in contrast to the F111 cells, populations of NIH 3T3 cells derived after infection with the Py large-T (two of four) and SV40 large-T (three of six) retroviruses resulted in tumors after about 8 to 10 weeks, whereas the equivalent population derived after infection with the lower titer Py middle-T retroviruses was tumorigenic within 2 to 3 weeks. Reassuringly, populations of NIH 3T3 cells after infection with the control backbone pZipNeoSV(X)1 virus (three of three) did not generate tumors.

**(ii) Anchorage-independent growth.** The retrovirus-derived cell lines were assayed for anchorage-independent growth by plating either $10^3$ or $10^4$ cells in 0.31% SeaPlaque agar with 10% calf serum (35). The results indicated that neither the parental F111 cells nor the FSV2a2 cells, the cell line derived by infection of F111 cells with a recombinant retrovirus that encodes only the neomycin gene, grew in soft agar (Fig. 5A; Table 3). The PyF cell line, which expresses Py small, middle, and a truncated large T antigen, grew extremely well; nearly all cells plated grew and produced macroscopic colonies (Jat, data not shown). By contrast, the cell lines that expressed only the Py middle T antigen (F2MT52 and F3MT51) did not grow efficiently in agar suspension (Fig. 5A). F2MT52 produced about 20 large macroscopic colonies upon plating of $10^3$ cells, whereas F3MT51 grew more poorly and produced less than 10 large colonies after $10^4$ cells were plated. When a cell line (NMT5L1) derived from a tumor, the result of a subcutaneous injection of the Py middle-T-expressing cell line F2MT52, was plated in soft agar, it had a much higher plating efficiency and grew significantly better than the parental F2MT52 cell line (Fig. 5A, compare plating of $10^3$ cells from F2MT52 and NMT5L1). However, the cell line NF3MT5B derived from another tumor, the result of a subcutaneous injection of the other Py middle-T-expressing cell line, F3MT51, was not enhanced in its anchorage-independent growth relative to its parental cell line F5MT51 (Fig. 5A, compare F3MT51 and NF3MT5B; also see Fig. 5B). We conclude from these results that F111 cells expressing Py middle T do not grow efficiently in agar suspension but that variant cells can be selected that have this property.

The SV40 large T-containing cell lines (FSV4063 and F2SV4061) were more uniform in their growth in soft agar. Their plating efficiency was high, 16 and 24%, respectively (Table 3), but they produced only microcolonies (Fig. 5B) which were much smaller than the few large colonies obtained with Py middle-T-antigen cell lines. The Py large-T-expressing cell lines exhibited variable growth. In some experiments they yielded microcolonies, both lower in number and smaller in size than the SV40 large-T cell lines. In

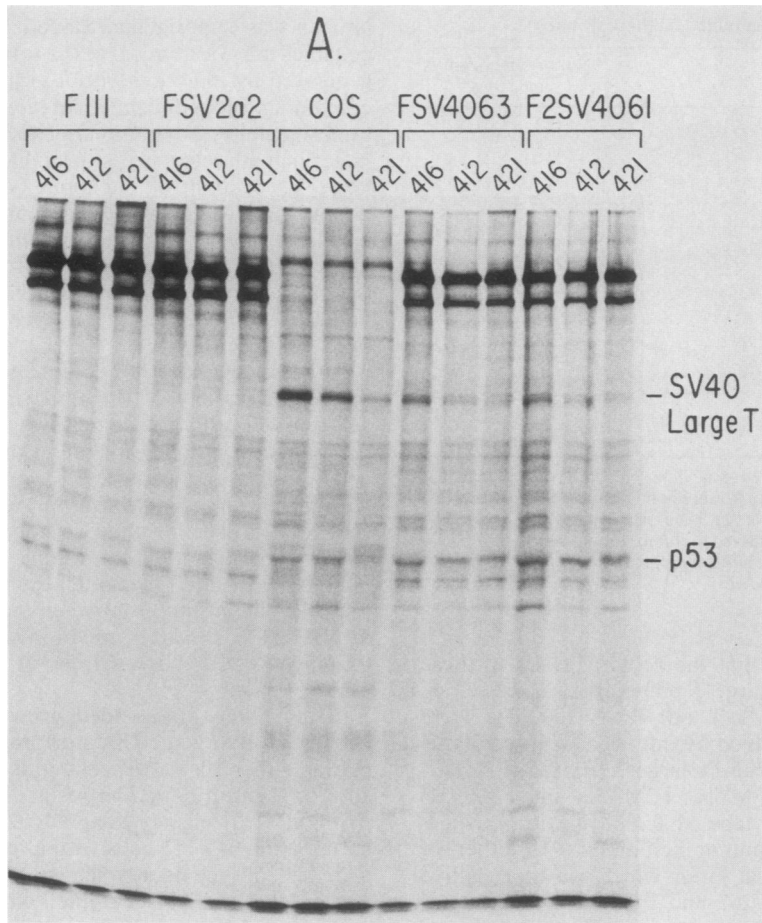
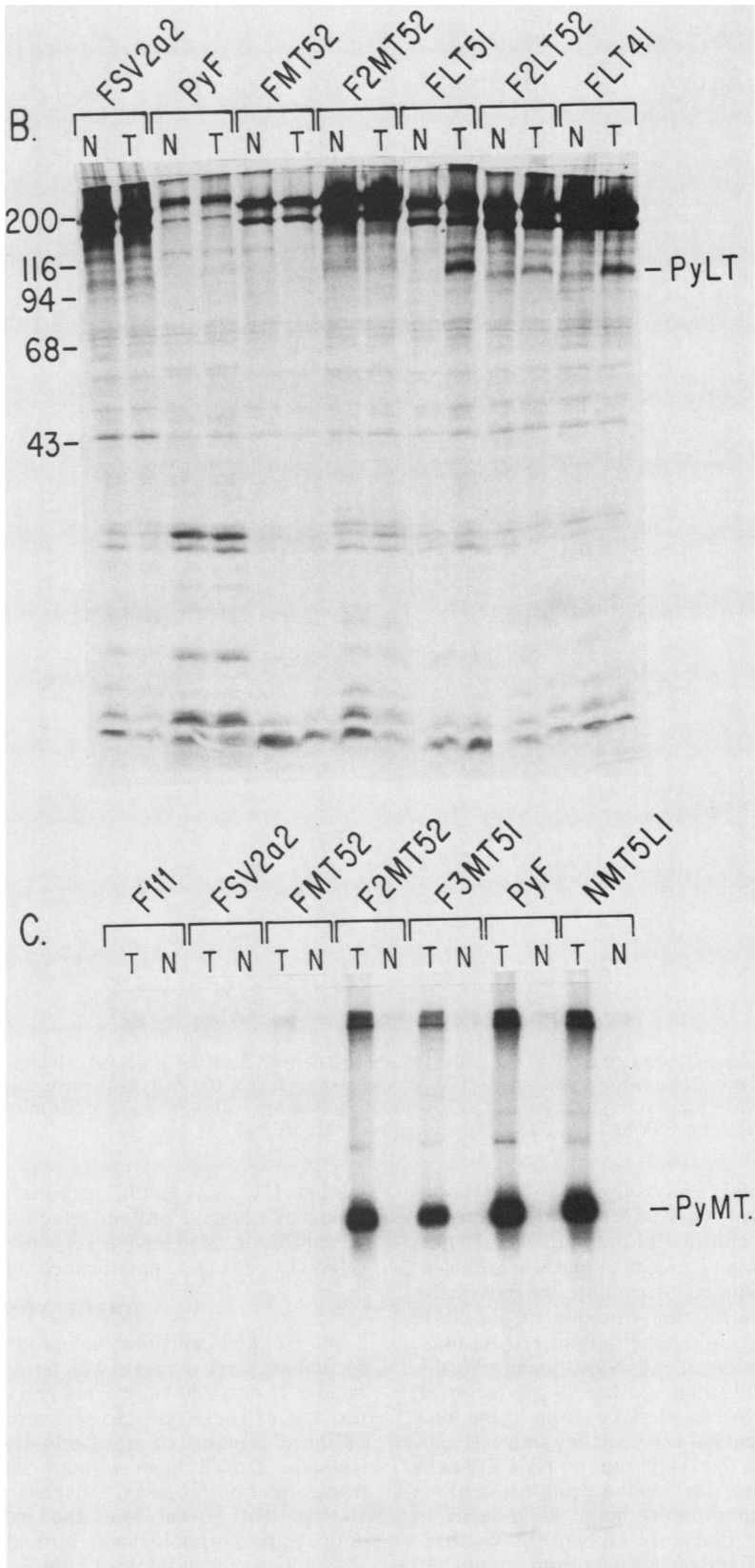



FIG. 4. Analysis of protein expression. The indicated cell lines were labeled with 100 to 200 μCi of [$^{35}$S]methionine for either 4 h (A) and 18 h (B). Extracts were prepared and immunoprecipitated as described in the Materials and Methods section. Immunoprecipitations were done with monoclonal antibodies specific for: the SV40 large T antigen (pAb412 and pAb416) or the cellular p53 antigen (pAb421). In panels B and C, immunoprecipitations were done with rabbit-anti-Py tumor serum (lanes T) or normal rat serum (lanes N). Numbers on the left in panel B correspond to $^{14}$C-labeled size markers (Betthesda Research Laboratories, Gaithersburg, Md.) of the indicated molecular size in kilodaltons. The correspondence between bands on the gel and the different proteins is indicated.

other experiments, there was very little growth compared with the control F111 or FSV2a2 cells.

**Focus formation.** The ability of the recombinant viruses to induce focus formation on F111 cells was also assayed. The results shown in Fig. 6A indicate that none of the retroviruses, even those that transduce the Py middle T antigen, induced focus formation on F111 cells. However, when dishes which had been infected with the Py middle-T retrovirus were examined under the microscope, patches of cells with the characteristic Py middle-T-antigen morphology were clearly visible. Identical results were obtained if the original pZip Py middle-T construct was introduced as DNA via calcium phosphate transfection into F111 cells. By contrast, the same recombinant retrovirus encoding the Py middle T antigen readily induces focus formation on Rat-1 and NIH 3T3 cells (Jat, data not shown). The inability of the Py middle-T retrovirus to induce foci on F111 cells could be overcome by complementation with the SV40 large T antigen (Fig. 6B) but not by the Py large T (Fig. 6C). The number of foci obtained in the experiment shown in Fig. 6B corresponded very closely to the number of G418-resistant colonies obtained on F111 cells with that particular dilution of virus and thus indicates that the SV40 large T antigen alone is sufficient to complement the Py middle T antigen for focus formation on F111 cells. Since the lack of complementation between the Py large T and Py middle T for focus formation was very surprising, we investigated it further by introducing either wild-type or mutant Py DNAs into the Py large-T-expressing F111 cell line (F2LT42) by cotransfection with *Eco-gpt* as a selectable marker (42). These results showed that intact Py DNA readily transforms both Py large-T-expressing F111 cells and F111 cells, i.e., induces lots of foci. However, recombinants that encode only the Py middle T antigen gave morphologically transformed *gpt*$^+$ colonies but very few foci. By contrast, BC1051, a mutant that can express both the middle and small T antigens (G. Magnusson, personal communication), induced clear foci, although the size and number were less than that obtained with wild-type Py DNA. Thus, these preliminary results suggest that focus formation by Py virus on F111 cells is likely to require expression of the small as well as the large and middle T antigens.

We also assayed focus formation by these recombinant retroviruses on NIH 3T3 cells. The Py middle-T retrovirus induced foci very efficiently: nearly a 1:1 ratio for G418$^r$ colony and focus formation. By contrast, the Py large-T retrovirus did not induce foci. However, the SV40 large-T-only retrovirus did induce foci but at a low frequency when


B.
FSV2a2
PyF
FMT52
F2MT52
FLT5I
F2LT52
FLT4I
N T N T N T N T N T N T N T
200-
116-
94-
68-
43-
-PyLT
C.
F111
FSV2a2
FMT52
F2MT52
F3MT5I
PyF
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-PyMT.

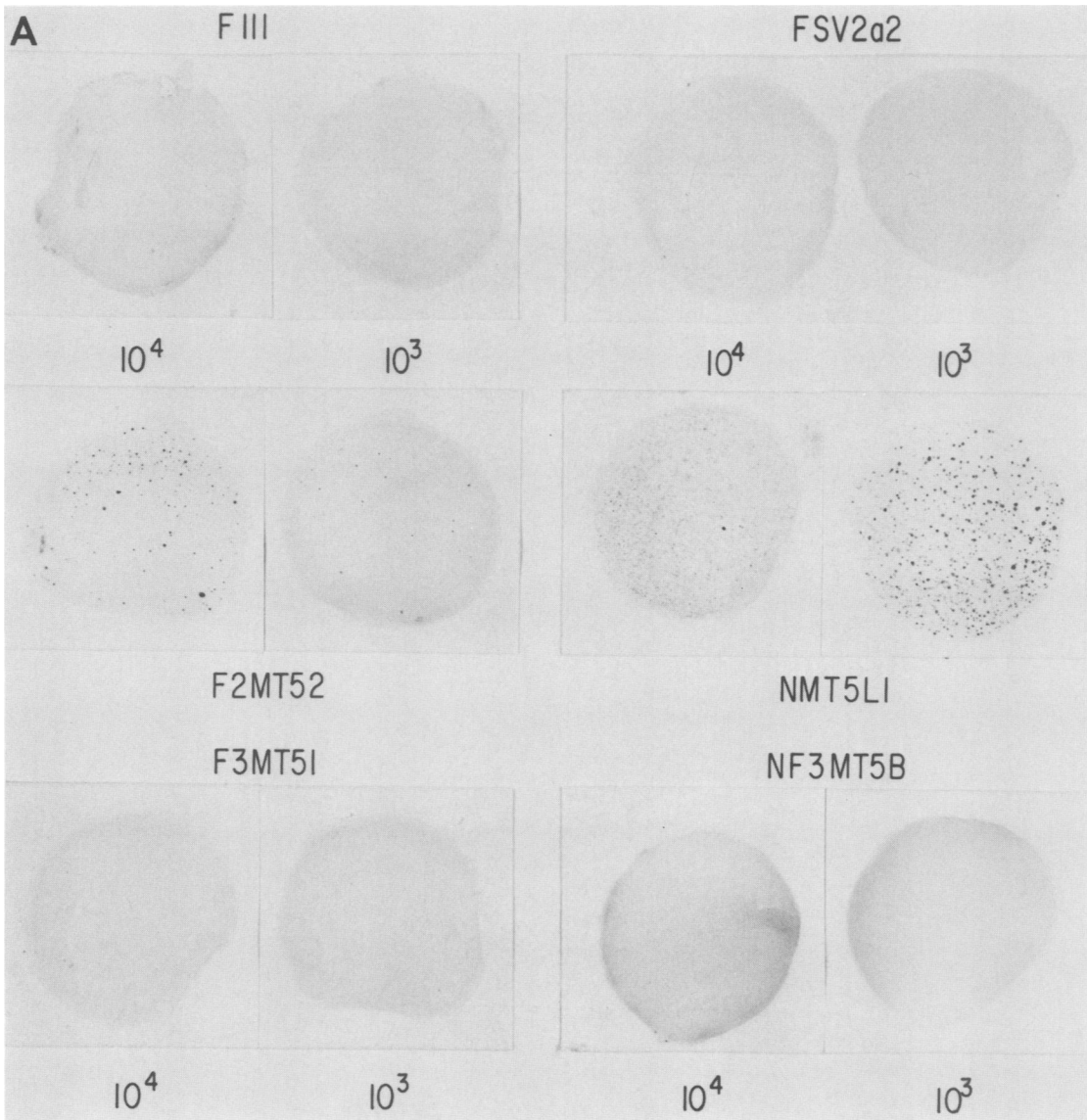
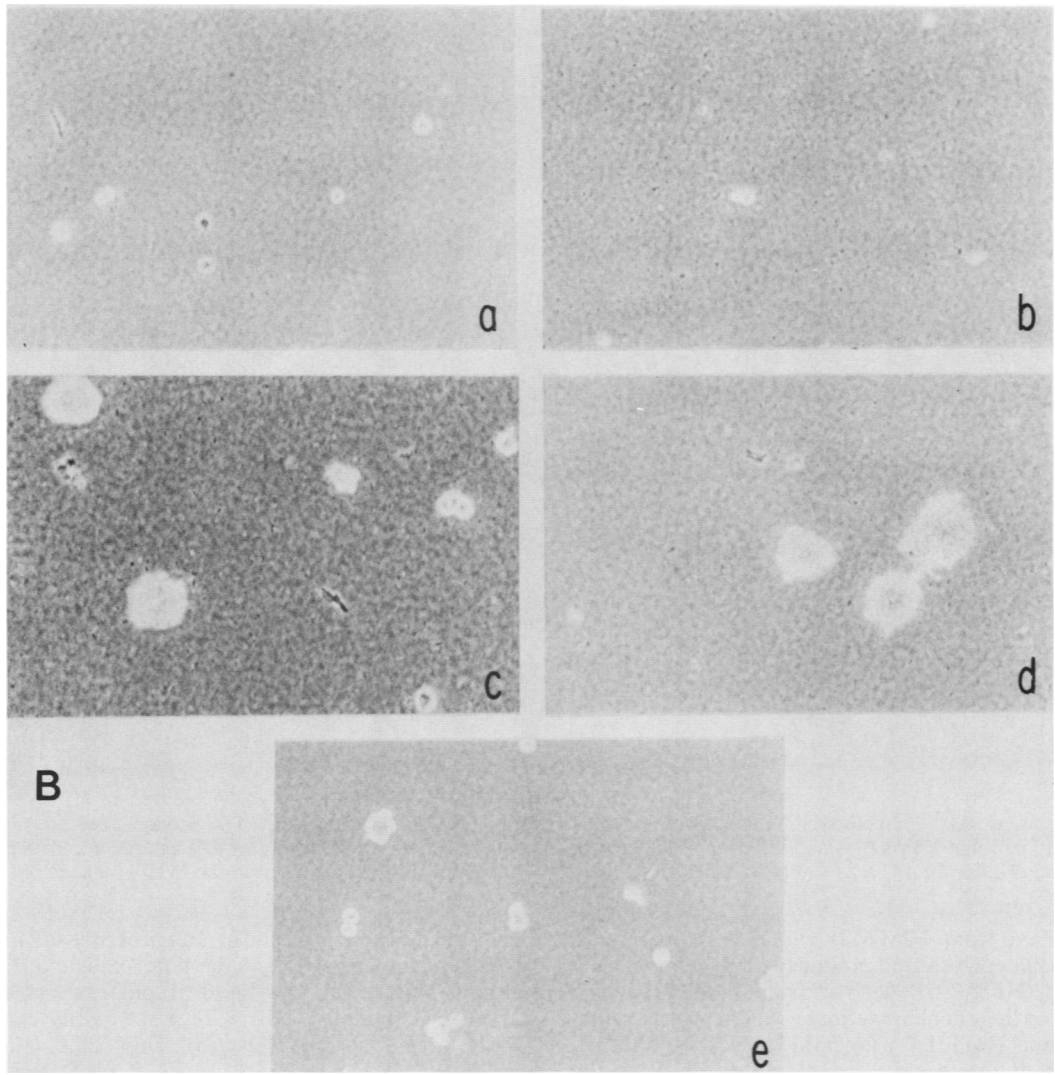



FIG. 5. Analysis of anchorage-independent growth. (A) Cells ($10^4$ and $10^3$) derived from the indicated cell lines were seeded in 6-cm dishes in 0.31% SeaPlaque agarose. After 21 days they were stained, dried, and photographed. (B) Cells ($10^3$) were seeded in 6-cm dishes in 0.31% SeaPlaque agarose. After 21 days they were photographed at 100-fold magnification with a phase-contrast microscope. Cells were derived from cell lines F111 (a), FSV2a2 (b), FSV4063 (c), F2SV4061 (d), and NF3MT5B (e).

compared with the G418$^r$ titer (1 focus for 100 to 1,000 G418$^r$ colonies), suggesting that not every infected cell gives rise to a focus. Since this was in contrast to previous work which suggested that the SV40 large-T protein alone was sufficient for focus formation, we wanted to exclude the possibility that the decreased focus formation was due to our SV40 producer cell line (SV40-6) producing mutant retroviruses. We repeated the construction of pZipSV40 using two different viral DNA stocks and isolated other $\Psi_2$ producer cell lines. Recombinant retroviruses derived from these producer cell lines yielded identical results; they induced very few foci in comparison with the G418$^r$ titer on NIH 3T3 cells (Jat, data not shown). Thus, our SV40-6 producer cell line does not transduce a mutant SV40 large T antigen. In addition, we have found that recombinant retroviruses which transduce the large T antigen derived from mutant d10 (26), which produces a cytoplasmic large-T protein, or mutant U19 (A. Smith, personal communication), which produces a large-T protein defective for binding to the origin of replication, also have a very low focus-forming ability on NIH-3T3 cells (Jat, unpublished data).

## DISCUSSION

We isolated cell lines that produce defective helper-free recombinant retroviruses which transduce the Py large T, Py middle T, or SV40 large T antigen as well as encoding G418 resistance. These retroviruses were used to analyze the role of these proteins in transformation of rat F111 cells by isolation of cell lines without selection for any specific transformation property. Analysis of these clonal cell lines showed that: (i) cell lines that express the SV40 large T antigen are morphologically normal, contain elevated levels of cellular p53, and do not induce tumors in nude mice but have a high plating efficiency for formation of microcolonies




in soft agar; (ii) cell lines that express the Py middle T antigen are both morphologically altered and highly tumorigenic; (iii) clonal cell lines expressing the Py large T antigen are unaltered by the criteria of morphology, anchorage-independent growth, and tumorigenicity.

**Py large and middle T antigens.** Previous experiments have demonstrated that the Py middle T antigen alone is capable of completely transforming established fibroblast cell lines (11, 71), whereas the Py large T antigen is required in concert for transforming primary fibroblasts (52, 54, 57). Our results obtained from analysis of cell lines derived after infection with recombinant retroviruses encoding either Py middle or large T antigen are in accordance with these conclusions. G418$^r$ cell lines derived from F111 cells after infection with the Py large-T retrovirus were morphologically normal, did not grow in agar suspension, and were not tumorigenic upon injection into nude mice. The Py large T antigen as transduced by the retrovirus is biologically active since it has been shown to be capable of efficiently establishing rat embryo fibroblasts without crisis (P. S. Jat and P. A. Sharp. submitted for publication).

By contrast, G418$^r$ cell lines isolated after infection with the Py middle-T retrovirus were morphologically transformed and highly tumorigenic in nude mice. Suprisingly, the highly tumorigenic Py middle-T-transformed cell lines did not grow well in soft agar (Fig. 5). One of the two cell lines (F2MT52) had a low plating efficiency but produced a few large macroscopic colonies, whereas the other cell line (F3MT51) did not grow in soft agar. Moreover, passage of these cell lines in nude mice as tumors resulted in cell lines

TABLE 3. Anchorage-independent growth of cell lines expressing SV40 large T antigen

| Cell lines | Total no. of microcolonies | |
|---|---|---|
| | $10^{4a}$ | $10^{3a}$ |
| F111 | 11 | 11 |
| FSV2a2 | 20 | 17 |
| FSV4063 | TMTC[b] | 156 |
| F2SV4061 | TMTC | 244 |

[a] $10^4$ and $10^3$ cells from the indicated cell lines were plated in 0.31% Sea Plaque agarose. Eighteen days after seeding, the colonies were counted.
[b] TMTC, Too many to count.

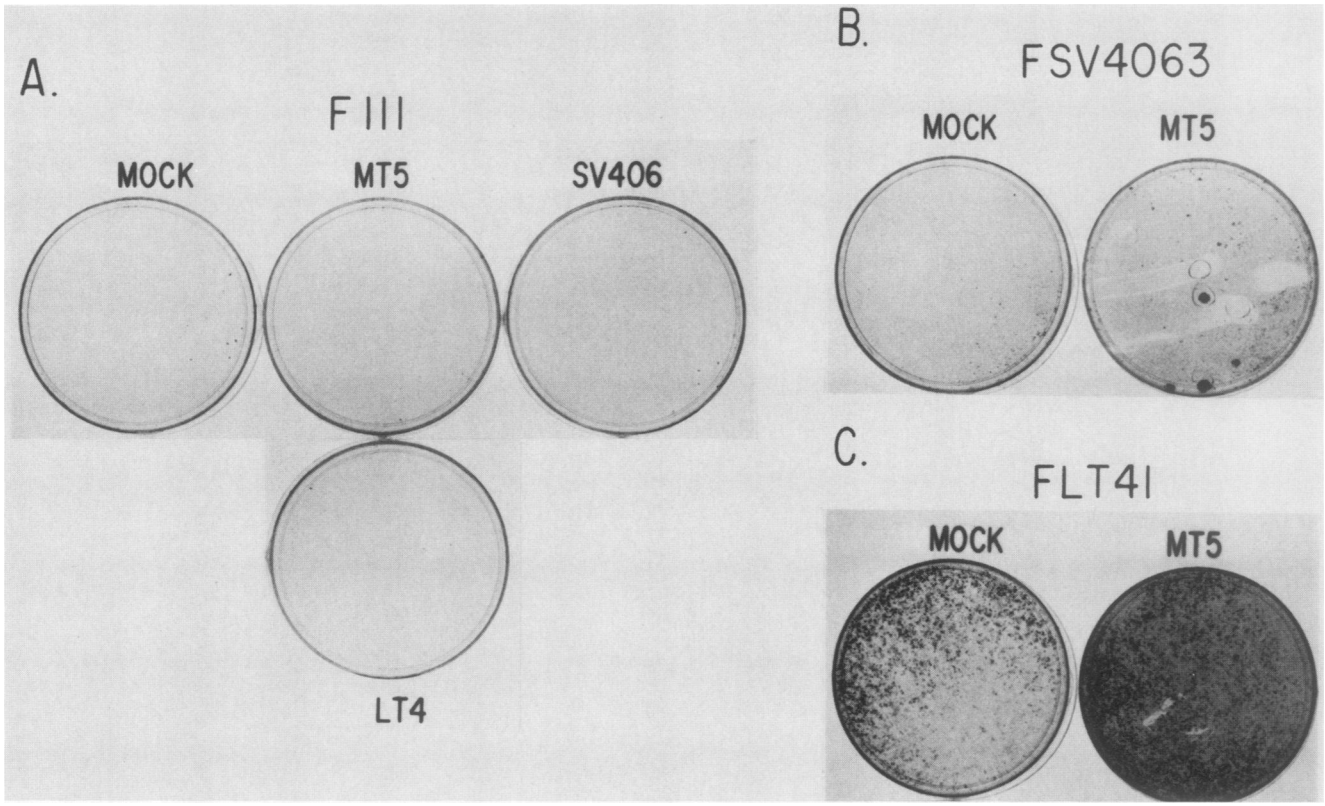



FIG. 6. Focus assay. (A) F111 cells were infected with recombinant retroviruses from the indicated $\Psi_2$ producer cell lines. (B) The SV40 large-T-expressing cell line FSV4063 was either mock infected or infected with retrovirus from the MT5 producer cell line. (C) The Py large-T-expressing cell line FLT41 was similarly either mock infected or infected with retrovirus from the MT5 producer cell line.

(NMT5L1 derived from F2MT52) which grew very well (nearly all cells plated grew and produced large colonies) and other cell lines (NF3MT5B derived from F3MT51) which were unaltered in their anchorage-independent growth (compare F2MT52 and NMT5L1 with F3MT51 and NF3MT5B, Fig. 5). These differences in growth potential were not attributable to differences in the levels of Py middle T antigen (Fig. 4C; J. Brugge, personal communication). Thus, induction of anchorage-independent growth in F111 cells by Py middle T antigen probably involves selection of a variant cell which responds more dramatically to the viral activity. The lack of efficient induction of anchorage-independent growth by Py middle T antigen may be related to the use of F111 cells. Other cell lines have been efficiently transformed to anchorage-independent growth by plasmids which express a Py middle-T cDNA gene (32, 52, 71). However, the important observation with regard to the experiments reported here is that Py middle T antigen efficiently transformed F111 cells to high tumorigenicity.

The Py middle-T retrovirus also did not induce foci on F111 cells. This is probably not due to the inability of Py middle T to morphologically transform F111 cells since infection of these cells and selection of $G418^r$ colonies uniformly yielded colonies which were morphologically transformed. Similar results have been obtained in experiments by McCoy et al. (41; personal communication), in which a recombinant containing an activated Kirsten *ras* gene was unable to induce foci on NIH 3T3 cells, but all colonies obtained after cotransfection with a selectable marker were morphologically transformed and tumorigenic. At a superficial level, this inability of middle-T-expressing cells to develop a focus in the presence of an excess of normal cells suggests that the latter cells exert a dominant effect. This effect can be overcome by infection of Py middle-T-containing cells with a retrovirus expressing the SV40 large T antigen (Fig. 6B). This combination of genes produced large and rapidly growing foci. Surprisingly, combinations of Py large-T- and middle-T-antigen retroviruses did not produce foci on F111 cells (Fig. 6C). This pattern of complementation for foci formation is not unique to F111 cells since similar results were obtained when the Py middle-T retrovirus was used to infect rat embryo fibroblast cell lines established by infection with either SV40 or Py large-T retrovirus (Jat, unpublished data). Since wild-type Py DNA can generate foci after transfection of F111 cells, it is likely that expression of the small T as well as the large and middle T antigens is necessary. The SV40 large T antigen gene apparently encodes the activity supplied by both the Py small and large T antigens.

**SV40 large T antigen.** Initial experiments by many workers indicated that SV40 can readily transform susceptible cells (for a review see reference 70). Subsequently, use of conditional or deletion mutants and cloned DNA fragments demonstrated that both of the T antigens encoded by the early region were necessary and sufficient for transformation (10, 29, 70). The role of the different T antigens was analyzed with either the *dl*54/59 or *tsA* mutants (2, 61; for a review see reference 68). Experiments with the *dl*54/59 mutants have shown that the small T antigen is a mitogenic factor which is helpful for initiation of transformation of quiescent cells but is not essential for either the transformed phenotype or transforming growing cells (22, 39, 60, 65, 70). Experiments

with the *tsA* mutants, which encode a temperature-sensitive large T antigen, have yielded conflicting results (3, 4, 29, 38, 44, 45, 59, 67, 70). Some cell lines transformed by the *tsA* mutants are clearly temperature sensitive (N-type transformants) for the transformed phenotype, whereas others do not exhibit such a distinct temperature sensitivity (A-type transformants; 45, 53, 59, 70). This difference between the type A and N transformants may be attributable to a partial stabilization of the newly synthesized large T antigen in the A-type transformants at the nonpermissive temperature (22). Moreover, in the N-type transformants the large T antigen is only detectable in the G2 phase of the cell cycle, whereas in the A type it can be detected throughout the cell cycle (23). Thus, these results strongly suggest that the large T antigen is important both for initiation and maintenance of the transformed phenotype. These results were extended by Kriegler et al. (30) by the study of an SV40 large-T-only retrovirus. They found that this retrovirus was capable of inducing foci on both NIH 3T3 and Rat-2 cell lines and thus concluded that the SV40 large T antigen alone was sufficient for transformation. However, in all these analyses, SV40 transformants were isolated by selection for either focus formation, anchorage-independent growth, or growth in low serum. Therefore, it was possible that products of a minority of the typical virus-cell interactions were being selected for further study. This problem has previously been partially addressed by Pollack's group who infected mouse 3T3 or rat embryo fibroblasts with SV40 and isolated cell lines with the minimal possible preselection, i.e., random single-cell cloning (50, 56). From these experiments they concluded that infection of cells by SV40 can result in either (i) full transformation in which the cells grow in low serum, are anchorage independent, and are tumorigenic within a short latency period, less than 4 weeks; or (ii) minimal transformation in which the cells express T antigen, grow in low serum, are established, but are not anchorage independent for growth (50, 56). They also found that it was possible to isolate full transformants from the minimal transformants by selection for anchorage-independent growth in methyl cellulose or tumor formation in a BALB/c nude mouse (64).

Our analysis of cell lines derived by infection of either rat F111 cells, mouse NIH 3T3 cells, or primary rat embryo fibroblasts with retrovirus recombinants encoding SV40 large T antigen and selection for resistance to G418 (presented here and elsewhere [Jat and Sharp, submitted]) strongly suggests that the large T antigen alone is not sufficient for full transformation. Although it is possible that the lack of full transformation of F111 cells is due to the level of T-antigen expression, this is probably not the case. These cell lines contained about one-fifth the level of T antigen present in the Cos M6 cell line. Immunoprecipitation analysis of a cell line derived from a tumor obtained after injection of a bulk population of SV40 large-T retrovirus-infected NIH 3T3 cells showed that the expression level of the large T antigen was only 1/10th to 1/20th of that in Cos cells (unpublished data). Moreover, cell lines established from secondary rat embryo fibroblasts by using this SV40 large-T-only retrovirus contained T-antigen levels equivalent to that in Cos cells, yet only one of five such lines was tumorigenic in nude mice (Jat and Sharp, submitted). We conclude that the SV40 large T antigen alone is sufficient for stabilizing cellular p53, promoting efficient formation of microcolonies in soft agar, establishing without crisis primary embryo fibroblasts (Jat and Sharp, submitted), and complementing Py middle T antigen in focus formation on F111 cells. However, it is not an acute transforming gene like the Py middle T antigen (11, 71), EJ c-Ha-*ras*1 (62), or avian *src* (47). It may have transforming activities beyond those commonly associated with other establishment genes such as cellular *myc* (27) and Py large T (presented here and in Jat and Sharp, submitted). It should be kept in mind that both of these oncogenes also yield full transformants on some cell lines, albeit at a lower level than that reported for the SV40 large T antigen. Thus, the occasional full transformants obtained after infection or transfection with the SV40 large-T-antigen gene probably reflects an atypical gene-cell interaction.

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