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A Recombinant Murine Retrovirus for Simian Virus 40 Large T cDNA Transforms Mouse Fibroblasts to Anchorage-Independent Growth

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A recombinant murine retrovirus containing the intact cDNA sequence for the simian virus 40 (SV40) large T antigen (T) was constructed by using the pZIPNeo SV(X)1 vector. ψ2 packaging cells were then transfected, and G418-resistant clones were used to generate helper-free viral stocks. NIH 3T3 mouse fibroblasts infected by the recombinant T cDNA retrovirus were selected for G418 resistance. Such cultures synthesized authentic SV40 T and were transformed to anchorage-independent growth at high efficiency. Therefore, this vector has allowed the study of the transformation properties of T under conditions of neutral drug selection and in the absence of SV40 small t antigen.

The early region of simian virus 40 (SV40) encodes two proteins, large T antigen (T) and small t antigen (t) (19). This viral DNA segment is known to be sufficient for the transformation of rodent fibroblasts in vitro (24). In addition, infection of established fibroblast lines with mutants such as *dl*883, which encode only T, can lead to the permanent transformation of infected cells with efficiencies approaching those which accompany wild-type infection when the target cells are cultivated under selected conditions (1, 2, 5–7, 13, 16, 20–22). Under other infection conditions, such cells were transformed by T^+-t^- viruses, but much less efficiently than is normal. To further study the transforming activity of T in the absence of t synthesis, we constructed a recombinant murine T-only retrovirus by using the pZIPNeo vector developed by Cepko et al. (3). This new retroviral genome contains an intact T cDNA coding unit and the bacterial neomycin resistance (Neo^r) gene. Its construction has permitted us to study the effect of T upon the growth control of entire populations of infected nonpermissive cells without having to select first for cells with a clearly transformed phenotype, previously a common practice in studies of the growth control phenotypes of cells stably infected by SV40. In previous experiments of this type, all of the evidence indicated that the vast majority of SV40-infected cells could not be isolated as stable transformants, even though they displayed anchorage-independent growth within a few days after infection (7, 20). The widely suspected cause for this discrepancy was the low efficiency with which viral sequences integrate and are expressed (13). Thus, the T retrovirus vector approach to the study of transformation was chosen. In particular, it offered the prospect that all infected cells would carry an integrated copy of the T gene. Other workers have previously demonstrated the production of SV40 T and T-t recombinant retroviruses (12, 13). Such agents were found to display highly efficient transforming activity on established fibroblasts when infected cultures were scored directly for transformed behavior (13), thereby demonstrating that inefficient integration was a major reason for the relatively low transforming activity of SV40. These results led us to question whether cells could be infected with a T-only retrovirus, then selected for a retroviral genetic marker which was unrelated to the alteration of cell growth control, and subsequently analyzed for their transformed behavior. This question was posed in the experiments described here, in which a recombinant retrovirus physically capable of encoding T (but not t or a truncated derivative thereof) was used. Analogous experiments have been performed by Jat et al (12).

The physical maps of the progenitor plasmids (pZIPTEX and pZIPTEXA) for the recombinant retroviruses encoding T used in these experiments are outlined in Fig. 1. The pZIPNeo SV(X)1 (pZIP) vector (3) was chosen for this purpose, in part because it contains the necessary *cis*-acting elements for virus production as well as the Neo^r gene and can be isolated as a helper-free infectious virus. A clonal copy of an intact SV40 T cDNA sequence (K. Zinn, unpublished data) was derived from the plasmid pSP6TEX, which when transcribed with bacteriophage SP6 RNA polymerase in vitro (18) gave rise to an RNA molecule which directed the synthesis of full-size immunoreactive T in a reticulocyte-free system (Fig. 2). A *Bam*HI fragment containing the entire T cDNA sequence from pSP6TEX was cloned into the *Bam*HI site of pZIP in both orientations, giving rise to the plasmids pZIPTEX (sense) and pZIPTEXA (antisense) (Fig. 1). These plasmids as well as the pZIP vector alone were transfected onto ψ2 packaging cells (15), and stable G418 (400 μg/ml)-resistant clones were selected. Several were expanded into cell lines. ψ2 cells provide all of the *trans*-acting viral and cellular factors needed for retrovirus production, but they do not package viral RNA transcribed from a resident provirus bearing a defective packaging sequence (15). These G418-resistant clones were in turn screened for the production of infectious retrovirus capable of conferring G418 resistance on recipient NIH 3T3 cells. Most were found to produce

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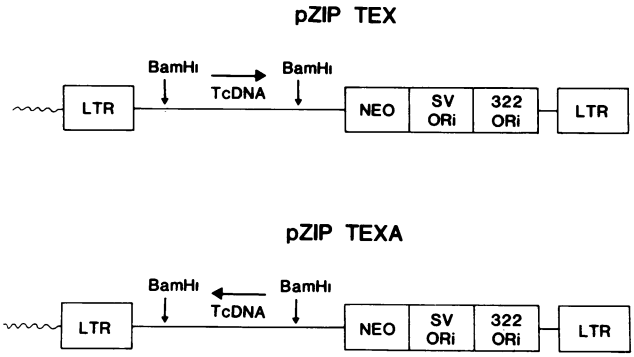

FIG. 1. Physical maps of pZIPTEX and pZIPTEXA. pZIPNeo SV(X)1 (pZIP) was cleaved with *Bam*HI and then treated with calf intestinal alkaline phosphatase. pSP6TEX (a plasmid carrying a single full-length copy of the SV40 T cDNA sequence abutting the SP6 promoter sequence [18]) was cleaved with *Bam*HI and the promotorless, but otherwise intact, SV40 T cDNA-containing fragment isolated by electrophoresis through a 1% agarose gel. This fragment was then ligated into the phosphatase-treated pZIP vector, resulting in pZIPTEX (the cDNA sequence in the sense orientation) and pZIPTEXA (the cDNA sequence in the antisense orientation). The methods used in these constructions were described previously (14, 18).

virus. The retrovirus stocks generated by the ψ2 clones chosen for further study displayed titers of $\simeq 10^5$ Neo^r CFU/ml in the case of the pZIP vector and $\simeq 10^4$ Neo^r

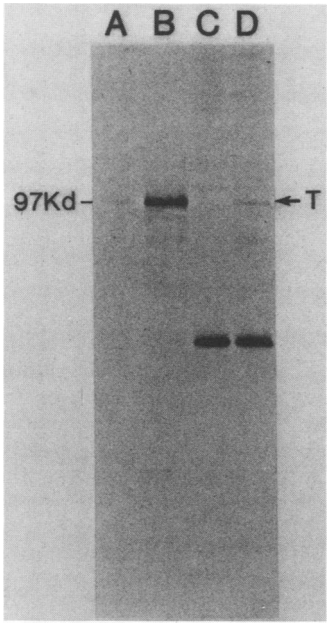

FIG. 2. Coding capacity of a cloned, SV40 T cDNA sequence. An SP6 promoter-driven transcript of a clonal T cDNA sequence was introduced into a micrococcal nuclease-treated rabbit reticulocyte lysate in the presence of [^{35}S]methionine and the products subjected to anti-T immunoprecipitation with PAb419. The sodium dodecyl sulfate-eluted proteins were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (lanes A and B). The nonimmunoprecipitated reaction products were also analyzed in the same gel (lanes C and D). RNA (0.1 μg) was employed in the reactions examined in lanes A and C, and 0.5 μg of RNA was employed in the reactions examined in lanes B and D. The migration position of authentic 97-kilodalton SV40 T is marked on either side of the figure.

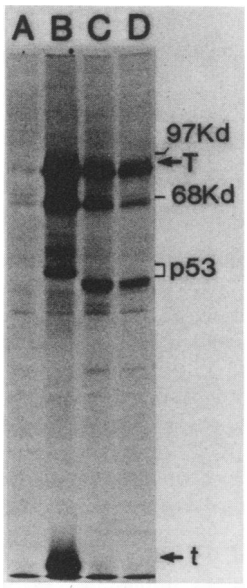

FIG. 3. Immunoprecipitation of T from recombinant retrovirus-infected cells. Subconfluent 60-mm culture dishes (containing $\simeq 10^6$ cells) of each of the recombinant retrovirus-infected G418-resistant NIH 3T3 cell populations were labeled with 100 μCi of [^{35}S]methionine for 3 to 5 h, as described previously (1). An equivalent number of COS-1 cells were labeled in parallel under identical conditions. Nonidet P-40 lysates were prepared and immunoprecipitated with PAb419, as previously described (10). The immunoprecipitates were electrophoresed through a 10% sodium dodecyl sulfate-polyacrylamide gel, which was then stained with Coomassie brilliant blue, destained, and fluorographed. The positions of unlabeled protein markers (phosphorylase A and bovine serum albumin), as well as those of T, t, and p53 are shown. Extracts of the cells were analyzed in the following lanes: A, NZIP2; B, COS-1; C, NTEX3; D, NTEX6.

CFU/ml in the case of the vector containing the T cDNA in either orientation. Retroviral stocks from a pZIP-transfected clone (ψZIP2), a pZIPTEXA clone (ψTEXA2), and two pZIPTEX clones (ψTEX3 and ψTEX6) were used to infect NIH 3T3 cells. G418-resistant cells appeared in all cases, and from these cultures all of the drug-resistant cells were pooled and used in further analyses. Some virus-infected G418-resistant NIH 3T3 clonal cell lines were also isolated. The cell lines expressing T, NTEX3 and NTEX6, assumed a shape on plastic which was distinct from that of control retrovirus-infected or uninfected NIH 3T3. Compared with controls, the T-containing cells were more fusiform and grew to higher cell density.

To test for the synthesis of T in the various infected G418-resistant cultures, samples of the above-noted cells and an equivalent number of COS-1 (9) control cells were metabolically labeled with [^{35}S]methionine. Then detergent lysates of each were prepared, and T was immunoprecipitated from each with a monoclonal antibody (PAb419) which recognizes both T and t, as described in the legend to Fig. 3. The two populations of NIH 3T3 cells infected with the vector containing T in the sense concentration, NTEX3 and NTEX6 (lanes C and D), synthesized full-size T which comigrated with T from the COS-1 control (lane B). Moreover, approximately equivalent amounts of T were immunoprecipitated from the three extracts which were in turn generated from equal numbers of the two murine transformants and COS-1. In contrast, NIH 3T3 cells infected by the

vector alone (lane A) or by the antisense T retrovirus (not shown) did not synthesize T. In addition, the cell phosphoprotein p53, known to bind tightly to T in vivo (17), was present in PAb419 immunoprecipitates of the T-containing cell extracts but not in those of the non-T producer cultures (data not shown). A form of p53 was identified in the primate cell immunoprecipitate (lane B) that was slower migrating than forms in the T-containing murine extracts, as noted earlier by others (11).

Growth in semisolid medium is one assay for transformation of fibroblasts by SV40. To assess whether the T-containing NIH cells were transformed, we plated 2×10^4 cells of the various pooled G418-resistant populations noted above in soft agar in 35-mm dishes as described previously (1, 20) and measured their colony-forming ability. The T-producing cell lines, NTEX3 and NTEX6, formed colonies at high efficiency under these conditions (Table 1). In contrast, NZIP2 (a ZIP vector-infected line), NZIPC1 (another G418-resistant NIH 3T3 cell line infected with ZIP control virus), and NTEXA2 cells (a line infected with the antisense retrovirus) all failed to grow efficiently under these conditions. Typical soft-agar colonies of NTEX6 are shown in Fig. 4A, and a comparable field of NTEXA2 is shown in Fig. 4B.

The data indicate that we had constructed recombinant murine retroviruses whose infection can lead to NIH 3T3 stocks which express an SV40 T cDNA sequence as efficiently as COS-1 cells express their resident T genome. Moreover, these viruses can efficiently transform NIH 3T3 fibroblasts to anchorage-independent growth in the absence of selective pressure for transformation, and SV40 t is not required for this effect. Jat et al. (12) have also recently reported the construction of a recombinant retrovirus encoding and expressing SV40 T. In their experiments, two F111 (rat)-derived retrovirus-infected lines expressing SV40 T were able to grow in soft agar at 16 and 24% efficiency, respectively. However, these lines failed to form tumors in nude mice. In contrast, they found that three of six mouse NIH 3T3-derived lines expressing SV40 T were tumorigenic in nude mice. Our results, demonstrating very efficient growth in soft agar of the mouse NIH 3T3-derived lines expressing SV40 T, are consistent with these findings. The differences in the efficiency of soft agar growth and tumorigenicity between the F111 and NIH 3T3-derived lines may reflect differences in the susceptibility to transformation of these two lines by SV40 T. Conversely, these differences may reflect differences in the ability of the Moloney murine leukemia virus-derived retroviral vector to express T in rat F111 versus mouse NIH 3T3 cells.

A number of laboratories have previously reported a synergistic effect between SV40 t and T for the growth of established lines of rodent cells in soft agar during abortive and stable transformation tests (1, 2, 4, 7, 16, 20, 23). Included in these data were results obtained with NIH 3T3 cells. The results presented here suggest that t is not essential in these cells for efficient, stable, anchorage-independent growth resulting from the action of T synthesized in quantities equivalent to those observed in COS-1 cells. Thus, t may act in the abortive transformation of NIH 3T3 and other such rodent lines by directly or indirectly altering the efficiency of the T transformation maintenance function. By contrast, t would appear not to affect the absolute ability of T to trigger those events which are minimally required for anchorage-independent growth when present in the quantities noted here. Given this conclusion, we are currently investigating the kinetic and quantitative requirements for SV40 T in the transformation of established murine lines which do and do not express t, with the goal of better defining the nature of the interaction between the two SV40 early gene products.

TABLE 1. Colony-forming ability of cells in soft agar[a]

Cell line	Cells forming colonies (%)
NZIP2	8
NZIPC1	<1
NTEX3	83
NTEX6	89
NTEXA2	<1

[a] 2×10^4 cells of each cell line were plated in 1 ml of soft agar medium (final agar concentration, 0.34%) in duplicate 35-mm culture dishes, as previously described (19). Cultures were fed with fresh top agar every 2 days. At 2 weeks, the percentage of cells that had formed colonies of ≥16 cells was determined from an analysis of at least 300 cells.

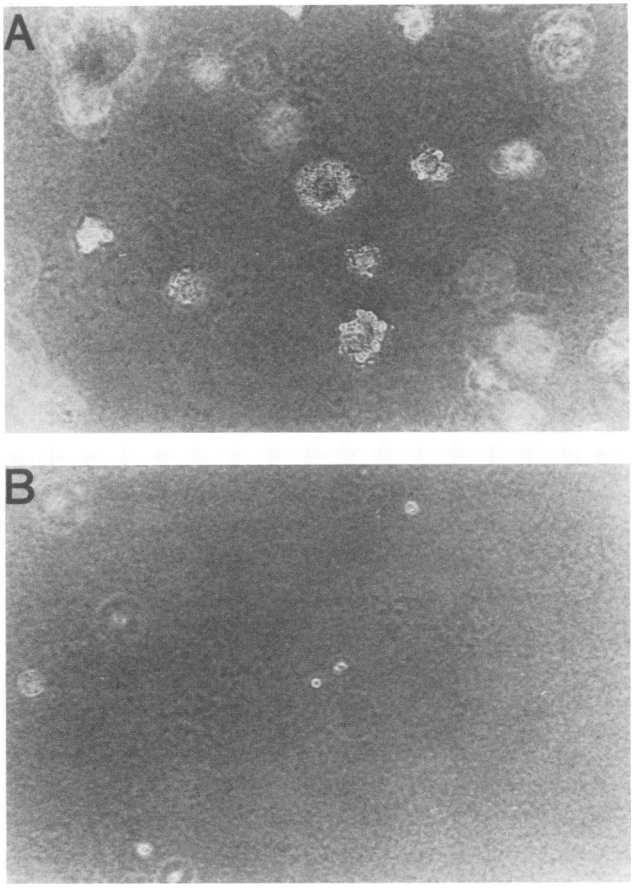

FIG. 4. Microscopic appearance of soft-agar colonies. Typical NTEX6 cells growing in soft agar (A) and control NTEXA2 cells from a parallel culture (B) are shown at 25× magnification. This agar growth experiment was performed as described in Table 1, footnote *a*.

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