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Enhanced Transformation by a Simian Virus 40 Recombinant Virus Containing a Harvey Murine Sarcoma Virus Long Terminal Repeat

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We have constructed a recombinant simian virus 40 (SV40) DNA containing a copy of the Harvey murine sarcoma virus long terminal repeat (LTR). This recombinant viral DNA was converted into an infectious SV40 virus particle and subsequently infected into NIH 3T3 cells (either uninfected or previously infected with Moloney leukemia virus). We found that this hybrid virus, SVLTR1, transforms cells with 10 to 20 times the efficiency of SV40 wild type. Southern blot analysis of these transformed cell genomic DNAs revealed that simple integration of the viral DNA within the retrovirus LTR cannot account for the enhanced transformation of the recombinant virus. A restriction fragment derived from the SVLTR-1 virus which contains an intact LTR was readily identified in a majority of the transformed cell DNAs. These results suggest that the LTR fragment which contains the attachment sites and flanking sequences for the proviral DNA duplex may be insufficient by itself to facilitate correct retrovirus integration and that some other functional element of the LTR is responsible for the increased transformation potential of this virus. We have found that a complete copy of the Harvey murine sarcoma virus LTR linked to well-defined structural genes lacking their own promoters (SV40 early region, thymidine kinase, and G418 resistance) can be effectively used to promote marker gene expression. To determine which element of the LTR served to enhance the biological activity of the recombinant virus described above, we deleted DNA sequences essential for promoter activity within the LTR. SV40 virus stocks reconstructed with this mutated copy of the Harvey murine sarcoma virus LTR still transform mouse cells at an enhanced frequency. We speculate that when the LTR is placed more than 1.5 kilobases from the SV40 early promoter, the *cis*-acting enhancer element within the LTR can increase the ability of the SV40 promoter to effectively operate when integrated in a murine chromosome. These data are discussed in terms of the apparent cell specificity of viral enhancer elements.

Retroviruses appear to integrate into their host chromosome via pathways analogous to cellular transposons (36, 38). As a model system for transposition, retrovirus integration may help dissect the mechanism involved in these recombination processes in eucaryotic cells, as proviral integration occurs at high frequency. Indeed, the high efficiency of retrovirus oncogenic transformation of cells in culture relative to the DNA tumor viruses may be due in part to this highly efficient mode of recombination. After infection of the cell by a retrovirus, several types of low-molecular-weight DNA accumulate. These proviral DNAs are found in a linear form with a long terminal repeat (LTR) at each end and in at least two circular forms. One canonical circular form contains a single LTR, and the other major form contains two LTRs. The circular molecules containing two LTRs are similar to their linear counterparts in that the LTR of the linear molecules abut one another to form a circle (15, 29, 43). However, linear molecules are not known to contain an AA at the end of each LTR. This dinucleotide duplication can appear in the circular form with two LTRs. It is generally assumed that one of these proviral DNA species integrates into the host chromosome.

Although the chromosomal site of integration of the proviral DNA appears to be random, integration of the provirus into the genome of the host cell occurs at a precise location within

the proviral LTRs (8, 22, 36–38). In addition, a duplication of a few base pairs occurs at the proviral-chromosomal DNA joint (8, 14, 16, 22, 31–33). In its strictest sense, the analogy with bacterial transposons predicts that, with respect to *cis*-acting integration functions, the only proviral DNA sequences required should be those which include and immediately flank the small inverted repeats found within the LTR as these sequences contain the viral attachment site and other sequences potentially involved in the resolution of the recombination intermediates (see reference 9, for example).

In our initial studies, our objective was to determine whether the short inverted repeat DNA sequences at the joint and proximal sequences of the LTRs in the circular provirus serve as the substrate for proviral integration. To test this possibility, we performed the following experiments. First, we constructed a Harvey murine sarcoma virus (HaMuSV) proviral DNA/simian virus 40 (SV40) viral DNA hybrid plasmid. This recombinant viral DNA, when excised from its bacterial plasmid component, could be converted into an infectious SV40 recombinant virus particle in the presence of the appropriate SV40 helper viral DNA. Removal of the LTR from its retroviral position prevents its normal synthesis via reverse transcription. We asked whether the enzymatic machinery for the appropriate nonpermissive cell would recognize the DNA duplex and integrate the recombinant DNA into the host chromosome in a manner analogous to that of a genuine retrovirus provirus. This experimental protocol, which utilizes as the vector an infectious virus particle, avoids the potential artifacts associated with "peklasome" formation during calcium phosphate-mediated gene transfer (26). Furthermore, we infected some NIH 3T3 cells with murine leukemia virus before infection with this SV40/LTR recombinant to determine whether a helper virus function is necessary to drive integration at the LTRs. Thus, we directly tested the notion that the duplex circular proviral DNA serves as a substrate for integrative recombination.

Another objective of these experiments is directed towards the question of which biochemical event (gene expression, stabilization of the viral DNA [via integration], or cell competence) is limiting for the frequency of SV40 transformation of murine cells. If integration is, in part, rate limiting, efficient integration of the SV40 viral DNA at the LTR sequences might lead to an enhanced rate of oncogenic transformation of murine cells by the recombinant virus when compared with morphological transformation of murine cells by SV40 wild-type virus. Alternatively, effective gene expression may play a role in limiting the frequency of morphological transformation. The results presented in this report indicate that gene expression does play some role in limiting these processes.

MATERIALS AND METHODS

Virus sources. Hirt supernatant HaMuSV proviral DNA cloned into pBR322 and pHaMuSV LTR was obtained from Esther Chang and Edward Scolnick. Helper virus DNA, pd13 (dl1001 cloned into pBR322), was obtained from Yasha Gluzman.

Cells and viruses. CV-1 cells (a continuous African green monkey kidney cell line), NIH 3T3 cells (originally a gift of George VandeWoude), and Rat-2 cells were grown in Dulbecco modified Eagle essential medium (DMEM) containing 10% fetal calf serum (FCS) in a 5% CO_2-containing atmosphere. Plaque-purified SV40 (strain 777) was propagated at a multiplicity of 10^{-4} in CV-1 cells.

Generation of recombinant virus stocks. SV40 recombinant plasmid DNAs (pSVLTR1 and pSVLTRΔPRO) were converted into infectious virus particles after digestion of the recombinant plasmid with *Bam*HI, releasing the recombinant DNA from its plasmid component. The *Bam*HI-linearized DNA was mixed with *Bam*HI-linearized helper viral DNA dl1001 (pd13) and transfected into CV-1 cells with the DEAE-dextran technique (24). Transfected cells were overlain with soft agar plus DMEM–10% FCS. Fourteen days post-transfection, infected monolayers were stained with neutral red and individual plaques were picked and carried through a second cycle of plaque purification. Plaques were picked, recombinant viral DNAs were characterized by digestion with various restriction endonucleases, and the resultant fragments were subjected to electrophoresis in a 1% agarose gel.

Helper-independent titration of SV40 recombinant virus stocks. Plate stocks of twice plaque-purified SVLTR1 and SVLTRΔPRO virus were grown on CV-1 cells and titrated as described below. SV40 wild-type virus of known titer (5×10^8 PFU/ml) and SVLTR1 or SVLTRΔPRO virus stocks of unknown titer were serially diluted in DMEM, 1:10, 1:100, and 1:1,000. A 0.5-ml portion of each dilution was placed on CV-1 monolayers at 80% confluency. One hour postinfection, 5 ml of DMEM–10% FCS was added, and the infected monolayer was incubated at 37°C overnight. At 24 h postinfection, Hirt extractions were performed, and the low-molecular-weight DNA was subjected to electrophoresis in a 1% agarose gel. After being stained with ethidium bromide, the gel was photographed and the bands on the negative were subjected to microdensitometric analysis to quantitate viral DNA. Virus dilutions of equivalent titer (determined by equivalent densitometric readings) were used in subsequent transformation studies.

Anchorage-independent transformation assays. Low-passage NIH 3T3 cells were plated at a density of 10^5 cells per 60-mm petri plate and infected for 2 h with titrated virus. At 24 h postinfection, the cells were trypsinized, counted, and suspended in soft agar at 10^5 cells per dish as described by Stoker (35). Four weeks later, the plates were photographed and the visible macroscopic colonies were counted.

DNA transformation of NIH 3T3 cells. Morphological transformations of NIH 3T3 cells were assayed by DNA transfection. The experiments were carried out

by using the modification of the method of Graham and van der Eb developed by Wigler et al. (40) for preparing the DNA-calcium phosphate coprecipitate. Carrier DNA extracted from LTK$^-$ cells was diluted with sterile 1 mM Tris (pH 8.1)–0.1 mM EDTA, and plasmid DNA was added followed by 2.5 M $CaCl_2$. After thorough mixing, the DNA in 2× $CaCl_2$ was added dropwise to an equal volume of 2× HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (pH 7.1) while introducing air bubbles at the bottom of the tube with a Pasteur pipette. After 30 min, 0.5 ml of the suspension was added to cultures in 60-mm petri dishes containing 5 ml of growth medium. The cultures were then incubated at 37°C for 16 h, at which time the medium was replaced with fresh DMEM–10% FCS. Cultures were incubated at 37°C for 2 to 3 weeks to allow transformed foci to appear.

Quantitative immunoprecipitation analysis of in vivo labeled SV40 T antigens. Cellular and viral proteins were labeled in vivo by seeding 10^5 cells already in the logarithmic phase of growth on 60-mm petri dishes. The medium was aspirated off and the monolayer was washed with 1× phosphate-buffered saline (PBS). A 5-ml amount of methionine-minus medium was added, and the cells were allowed to incubate for 2.5 h. Postincubation, the medium was removed and replaced with 1 ml of methionine-minus medium containing 100 μCi of [^{35}S]methionine. One hour later, 1 drop of fetal calf serum was added and the cells were allowed to incubate for an additional hour. The medium was then removed and the monolayer was washed two times with 1× PBS. One milliliter of buffer B (120 mM NaCl, 50 mM Tris [pH 7.6], 0.5% Nonidet P-40) was added to the plate, and the cells were removed with a rubber policeman. Lysates were freed of cellular debris by centrifugation. After centrifugation, the supernatant was removed. [^{35}S]methionine incorporation into the cell lysate was measured by spotting 3 μl of lysate onto Whatman 3 MM paper filters. These filters were washed once with ice-cold 10% trichloroacetic acid and once with hot trichloroacetic acid (90°C), rinsed twice with ice-cold acetone, dried, and subjected to scintillation counting in Liquifluor-toluene. In immunoprecipitation experiments designed to quantitate labeled SV40 T antigens in different transformed cell lines, aliquots of cell lysates containing equivalent quantities of incorporated radioactivity were transferred to 1.5-ml conical plastic tubes (Eppendorf), brought to a final volume of 1 ml with buffer B, and immunoprecipitated. To each milliliter of supernatant, quantities of antitumor antibody predetermined to be sufficient to precipitate five times the amount of SV40 T antigen present in control COS-7 cell lysates were added, and the mixture was incubated at 4°C for 1 h. A 30-fold excess of IgGsorb (The Enzyme Center) was then added to the immunoprecipitation mixture and gently agitated for 30 min at 4°C. The immune complexes were isolated by centrifugation at 6,000 × *g* and washed twice in buffer B. Immunoprecipitated protein was eluted by heating at 95°C for 5 min in 30 μl of sodium dodecyl sulfate-elution buffer (20 mM Tris-hydrochloride [pH 9.0], 5% [wt/vol] sodium dodecyl sulfate, 5% β-mercaptoethanol, 20% [vol/vol] glycerol). Electrophoresis was performed by the method of Laemmli, using 4% polyacrylamide stacking gels and 10% polyacrylamide resolving gels, cast in 1-mm-thick slabs (17). Gels were stained with Coomassie brilliant blue, destained, subjected to autofluorography (4), and exposed to Kodak XR-1 film at −70°C.

T-antigen immunofluorescence analysis of DNA-transfected cell monolayers. CV-1 cell monolayers at 30% confluence on 60-mm petri plates were transfected with 2 to 3 μg of various DNAs according to a modification of the methods of Sompayrac and Danna (34) (D. Rio, personal communication). Each cell monolayer was rinsed with 2 ml of sterile PBS and then 2 ml of Tris-buffered saline, after which the Tris-buffered saline was removed by aspiration and replaced with 250 μl of DEAE-dextran at a concentration of 500 mg/ml containing 2 to 3 μg of purified plasmid DNA. The plates were incubated at 31°C and were rocked every 30 min for 6 h. At the end of the incubation period, the monolayer was washed once with Tris-buffered saline and once with PBS, after which 5 ml of DMEM–10% FCS was added and the plates were incubated at 37°C for 24 h. After incubation, the growth medium was removed from the plates and the monolayers were rinsed twice with PBS. Cell monolayers were fixed with 5 ml of ice-cold methanol and allowed to incubate at 37°C for 30 min. Postincubation, the cell monolayer was rinsed twice with PBS, and 10 μl of fluorescein-conjugated goat anti-hamster antiserum (Miles Laboratories) was placed on the area of the fixed monolayer first treated with hamster antitumor antiserum. The covered dishes were incubated again at 37°C for 30 min, after which the stained monolayers were rinsed twice with PBS and allowed to air dry. The stained cell cultures were visualized under oil immersion with a Ziess epiluminescent microscope, and positive nuclei were counted.

RESULTS

A retrovirus LTR enhances transformation of murine cells by an SV40/LTR recombinant virus. Our first experiments used SVLTR1 (Fig. 1), an SV40 recombinant virus that contains one permuted copy of the HaMuSV LTR derived from a cloned circular HaMuSV provirus (see Materials and Methods for details of the construction of the recombinant plasmid DNA).

We generated the recombinant SVLTR1 virus through DNA transfection of CV-1 cell monolayers with a mixture (1:1) of *Bam*HI-cut pSVLTR1 DNA and *Bam*HI-cut plasmid pd13, containing dl1001 viral DNA cloned at the *Bam*HI site of pBR322, using the standard DEAE-dextran transfection method (22, 24). SVLTR1 DNA contains two large deletions in the SV40 late region and thus lacks the DNA sequences encoding the viral capsid proteins and is unable to package in the absence of the appropriate SV40 helper virus. We complemented the late region defect of SVLTR1 with the SV40 deletion mutant dl1001, 4.3 kilobases (kb) in size, which contains an intact late region and a large deletion which spans *Hin*dII + III fragments H and I in its early region (18). This helper

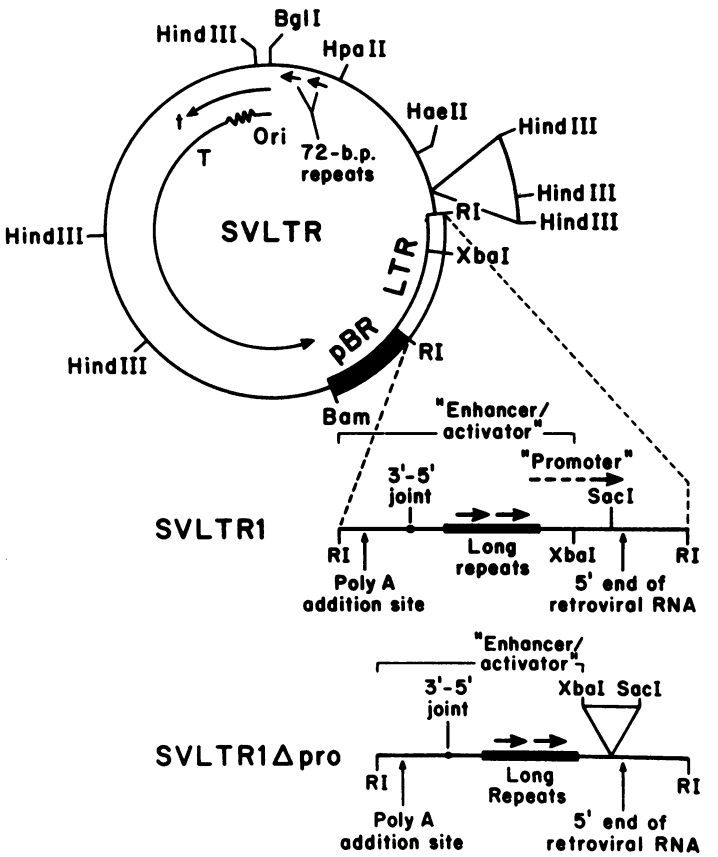

FIG. 1. Physical and functional maps of hybrid SV40/HaMuSV LTR recombinant viruses. Cloned proviral DNA of the HaMuSV containing two copies of the proviral LTRs derived from the Hirt supernatant of infected cells was restricted with *Sma*I, releasing a single permuted copy of the retrovirus LTRs. *Eco*RI (RI) linkers were ligated to this 600-bp fragment, which was then cloned into pBR322 at the *Eco*RI site. This plasmid was digested to completion with *Bam*HI and cut partially with *Eco*RI, and the resultant LTR-containing fragment was mixed with an SV40 molecule which had been digested with *Bam*HI and *Eco*RI. These fragments were mixed with *Bam*HI-cut bacterial alkaline phosphatase-treated pBR322 DNA and ligated with T4 ligase. *E. coli* HB101 cells were transformed with these plasmids, and bacterial colonies were screened for both SV40 early region and LTR DNA sequences. To facilitate efficient packaging of the recombinant DNA as an SV40 virus, the resultant plasmid was partially digested with *Hin*dIII to remove SV40 *Hin*dIII fragments E + F from the SV40 late region. The resultant DNA, when digested with *Bam*HI to release the bacterial plasmid component, is the correct size to package as a recombinant SV40 virus. This plasmid DNA (pSVLTR1) was then restricted with *Bam*HI and converted into an infectious virus particle through DEAE cotransfection of CV-1 cells with SV40 dl1001 (pd13) helper viral DNA, which retains an intact SV40 late region but carries a deletion in the SV40 early region. The SVLTRΔPRO virus was generated in a similar manner. However, pretransfection, pSVLTR1 was digested with both *Xba*I and *Sac*I and the staggered ends of the resultant molecule were treated with T4 DNA polymerase, after which the blunt ends of the recombinant were ligated to generate pSVLTRΔPRO. This recombinant plasmid was then converted into an infectious SV40 recombinant virus particle in a manner identical to that for pSVLTR1. In both cases, numerous plaques were picked and subjected to a second cycle of plaque purification. The viral DNAs derived from 20 of those plaques were analyzed and shown to contain both the helper virus and the desired recombinant viruses with the structures shown above. Poly A, Polyadenylic acid.

viral DNA is itself transformation defective. After 14 days of incubation, we stained the transfected CV-1 monolayers with neutral red and picked 20 plaques. These viral plaques were subjected to a second cycle of purification on CV-1 cells. We picked an individual plaque from each of 20 plates and made small-scale virus stocks from these plaque plugs. Each recombinant virus stock was analyzed by infection of CV-1 cells followed by isolation of Hirt DNA 24 h postinfection and restriction endonuclease analysis. When we digested these Hirt supernatant viral DNAs and analyzed the digestion mixture on a 1% agarose gel, the resultant

pattern was identical to that of the original recombinant pSVLTR1 plasmid with the exception of those fragments that were attached to the *Bam*HI site of the bacterial plasmid component. Further restriction analysis of the viral DNA indicated that the fragment spanning the viral *Bam*HI site was of the size predicted from the original plasmid restriction endonuclease map. No rearrangements had occurred during the packaging process in any of the 20 isolates. We chose two of the isolates for further study. A rapid helper virus-independent titration assay was used with which we were able to measure the titer of the SV40 *A* gene-containing (transformation-competent) SVLTR DNA in our stocks. Total viral DNA was extracted from cultures infected with either titrated SV40 virus stocks or SVLTR stocks, and the DNA mass was analyzed in parallel. With this assay we are able to measure the DNA mass of the 4.9-kb SVLTR1 recombinant viral DNA which is easily resolved from the 4.3-kb helper viral DNA in agarose gels (see Materials and Methods; Fig. 2). This measure of virus titer gave equivalent results when compared with titers estimated from immunofluorescent analysis of infected CV-1 cells.

We infected low-passage NIH 3T3 cells with equivalent amounts of SVLTR1 virus and SV40 wild-type virus and assayed for colony formation in soft agar. We observed an increase in the induction of colony formation by the SVLTR1 virus-infected cultures relative to those cultures infected with SV40 wild-type virus. In separate experiments, the magnitude of the enhancement ranged from 6- to 18-fold (Table 1). When we infected low-passage NIH 3T3 cells with Moloney leukemia virus (MoLV) before infection with SVLTR1 virus, we observed transformation enhancement equivalent to but no greater than NIH 3T3 cells uninfected with MoLV and infected with SVLTR1 virus. That the NIH 3T3 cells were producing MoLV was determined independently by analysis of infected cell culture media for reverse transcriptase activity. These results indicated that the factors responsible for the enhanced transformation phenotype of SVLTR1 virus is independent of any retroviral helper virus (MoLV) function. The two independent virus stocks isolated were tested, and both manifest the enhanced transformation phenotype (experiments 1 to 3 were conducted with one isolate, and experiment 4 was done with the other [Table 1]). Furthermore, the appearance of transformed colonies in both wild-type virus- and SVLTR1 virus-infected cultures followed a strict dose-response relationship (data not shown). To follow the time course of colony formation, we examined SVLTR1 virus-infected 3T3 cell cultures under the microscope and found that abortively transformed

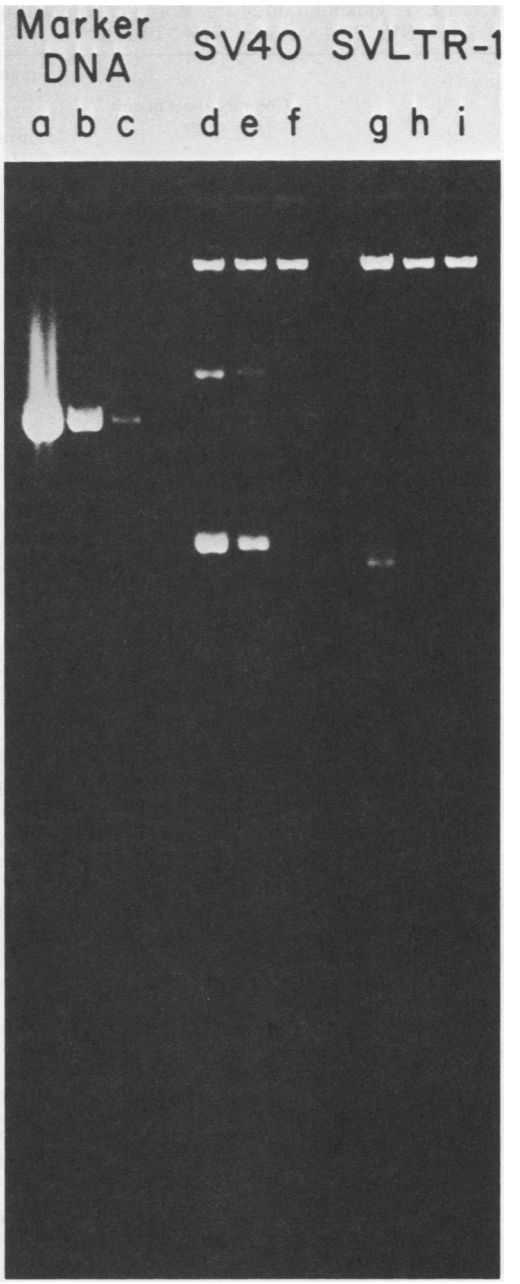

FIG. 2. Helper-independent titration of recombinant virus stocks. Plate stocks of twice plaque-purified virus stocks of SVLTR1 were grown on CV-1 cells and titrated as described in the text. Hirt extracts of defective virus stock- and wild-type virus stock-infected CV-1 cells were prepared. One half of each Hirt extract was suspended in 20 μl of Tris-EDTA buffer and loaded into a slot on a 1% agarose gel. The figure is a photograph of the stained gel. (Lanes a, b, and c) Plasmid pd13 marker DNA dilutions (1, 0.1, and 0.01 μg); (lanes d, e, and f) SV40 wild-type virus dilutions of stock titrated by plaque assays at 5×10^8 PFU/ml; (lanes g, h, and i) SVLTR1 virus dilutions.

TABLE 1. Quantitation of transforming potential of SVLTR-1 virus on NIH 3T3 cells[a]

Virus	Colonies (no.) in soft agar	Increase relative to SV40 control (fold)
Expt 1		
SV40 wild type	19	1
SVLTR-1	121	6.37
Mock	1	0.05
Expt 2		
SV40 wild type	9	1
SVLTR-1	94	10.44
Mock	0	
Expt 3[b]		
SV40 wild type	9 (10, 7, 4, 13, 11)	1
SVLTR-1	163 (145, 184, 160)	18.1
SVLTRΔPRO	154.25 (154, 140, 175, 148)	17.13
Mock	3.6 (9, 1, 3, 2, 3)	0.43
Expt 4[b]		
SV40 wild type	21.55 (24, 30, 11)	1
SVLTR-1	207.6 (201, 207, 193, 224, 213)	9.58
SVLTRΔPRO	242.75 (242, 238, 251, 240)	11.20
Mock	4.5 (3, 5, 3, 7)	0.20

[a] NIH 3T3 cells were plated at a density of 10^5 cells per 60-mm petri plate and infected with SV40 wild-type virus, SVLTR1 virus, or SVLTRΔPRO virus at equivalent multiplicities. At 24 h postinfection, the cells were trypsinized, counted, and suspended in soft agar at 10^5 cells per dish. Four weeks later, the plates were photographed and the colonies were counted. The numbers of colonies counted on separate plates is shown for each experiment as is the average colony count of all the plates.

[b] For experiments 3 and 4, the value given is the average colony count of plates shown in parentheses.

microcolonies appear earlier and appear to grow faster throughout the course of the experiment than in wild-type-infected control cultures.

Viral DNAs in SVLTR1-transformed cells have nonspecific attachment sites. SVLTR1-transformed foci were picked with cloning cylinders and expanded for analysis. We performed genomic blot analysis on these cellular DNAs isolated from SVLTR1 virus-transformed foci (with and without MoLV). If the recombinant viral genome had integrated within the HaMuSV LTR in a manner analogous to that of a genuine provirus and if no viral genomic rearrangement had occured post-integration, and *Eco*RI digestion of the transformed-cell genomic DNA and subsequent Southern blot analysis and hybridization with ^{32}P-nick-translated SV40 DNA should reveal a unique SV40 DNA-containing fragment of a predicted size, approximately 4.1 kb (Fig. 1). This is because two *Eco*RI sites flank the putative viral attachment site labeled "3'-5' joint" in Fig. 1. We tested 10 SVLTR1 virus-transformed cell line genomic DNAs (Fig. 3), five infected with MoLV before SVLTR1 virus transformation (lanes h to l) and five lines uninfected with MoLV (lanes c to g). All SVLTR1 virus-transformed cell lines appear to contain one to two copies of integrated SVLTR viral DNA, copy numbers similar to those of SV40 wild-type virus-transformed cells. Further, the SV40 DNA-containing bands are disperse, indicating that the SVLTR viral DNA has nonspecific attachment sites. An arrow to the left in Fig. 3

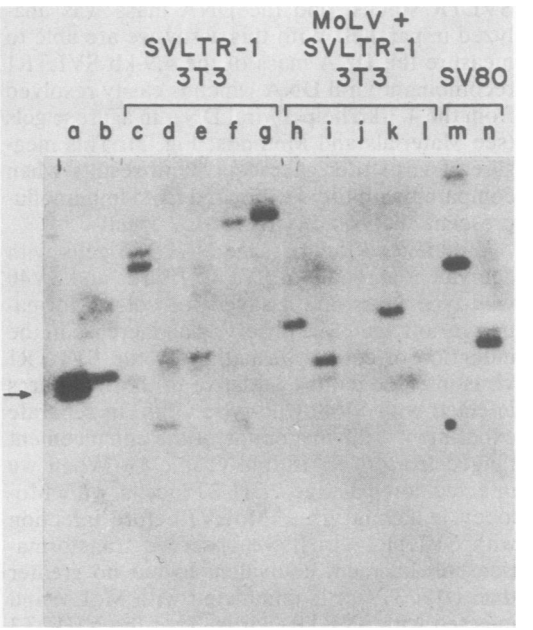

FIG. 3. Genomic blot analysis of SVLTR1-transformed NIH 3T3 cell lines. Genomic DNAs were extracted from NIH 3T3 cell lines either uninfected or infected with MoLV before transformation with SVLTR1 recombinant virus. After cutting with *Eco*RI, the DNAs were analyzed by the Southern technique and probed with ^{32}P-nick-translated pSVLTR1 DNA. This is an autoradiogram of detected DNA bands. The lanes show the detected SV40 fragments, and each lane contained the following: (a and b) 5 pg of pSVLTR1 DNA cut with either *Eco*RI (a) or *Hin*dIII (b); (lanes c to g) 5 μg of DNA from NIH 3T3 cells producing MoLV, transformed by SVLTR1 virus; (lanes m and n) 5 μg of SV80 genomic DNA cut with either *Eco*RI (m) or *Bam*HI (n). Approximately 20 to 30% of the cells in lanes d, j, and i were T-antigen positive. The arrow indicates that the predicted size position of a DNA fragment of *Eco*RI-cut integrated SVLTR1 viral DNA had the viral DNA integrated into the murine genome within the LTR fragment of the SVLTR1 genome and thereafter remained intact.

indicates the length of the *Eco*RI fragment predicted for a correct proviral insertion. When we tested SVLTR1-transformed cell lines that had been infected with MoLV before SVLTR1 transformation, we found the same disparate pattern (Fig. 3). Analysis of the genomic DNA with LTR-specific probes through *Hin*dIII digestion reveals the presence of a band that hybridizes to SV40 DNA and migrates with a *Hin*dIII fragment derived from the original SVLTR-1 virus stock. This fragment contains an intact LTR as well as the 3′ end of the SV40 early region (see Fig. 1). Furthermore, when the genomic DNAs from SVLTR-1-transformed cell lines uninfected with MoLV were hydrolyzed with *Eco*RI and *Bam*HI, the fragment spanning the entire retroviral sequences was detected. These analyses tend to support the notion that the LTR DNA was not involved in the primary recombination event as odd-sized fragments which contain the LTR would be expected to be found even if secondary recombination events obscure the initial events. Finally, we could rescue intact replicating circular SVLTR DNA from four of these cell lines (SVLTR 1 to 4) after cell fusions with permissive CV-1 cells (data not shown). This result implies that at least one complete copy of the monomer genome is found in an integrated array; if indeed one of the attachment sites included a cell-LTR joint, the unit-length 4.1-kb fragment probed for above (see Fig. 1 and 3) would have been detected. These results, coupled with other analyses, suggest that the enhanced morphological transformation phenotype of the SVLTR1 virus is not due to increased integration of the recombinant viral DNA within the HaMuSV LTR.

Promotion of the SV40 early region of SVLTR1 from the HaMuSV promoter cannot account for the enhanced transformation rate of SVLTR1 virus. A variety of studies suggest that the retrovirus LTR contains promoter activity (2, 3, 10, 41). We postulated that the retrovirus LTR "promoter" in SVLTR1 viral DNA might function to transcribe the SVLTR1 virus *A* gene mRNA and protein products and that overproduction of the early gene products responsible for morphological transformation might serve to enhance the transformation phenotype of SVLTR1 virus. To test whether the LTR could provide sequences which could serve to promote expression of the SV40 early region, we made the following constructions. *Bam*HI DNA linkers were attached to the SV40 *Bgl*I/*Bam*HI-cut early region DNA fragment. This fragment lacks a functional SV40 promoter but contains an intact structural *A* gene (p121, a gift of C. Thummel). The putative LTR promoter fragments were derived from two sources: cloned circular HaMuSV proviral DNAs first isolated by Hager et al. (12) which contain either one or three copies of the Moloney-specific LTR sequences. In both of these clones the LTR(s) is flanked by an *Eco*RI site (5′) and a *Bam*HI site (3′). These *Eco*RI-*Bam*HI fragments contain sequences derived from both rat and MoLV genomic DNA 5′ to the LTR and approximately 350 nucleotides 3′ to the LTR. This fragment does not transform rodent cells (our unpublished data), as all of the viral sequences encoding for the virus-specific DNA have been deleted (39). We isolated recombinant SV40/HaMuSV proviral DNA plasmids in which the SV40 early region abutted the putative promoter of the LTR in the "correct" (pRETRO-T III + I) and "reverse" (pRETRO-T II) orientations (Fig. 4). These recombinant DNAs were transfected, using the calcium phosphate-mediated gene transfer technique, into NIH 3T3 and Rat-2 cells and assayed for focus-forming activity. We observed transformed foci in both Rat-2 and NIH 3T3 cells only when the *A* gene was inserted in the correct orientation (pRETRO-T III + I). We have made similar observations with the *Bgl*II fragment of Tn*5* DNA which confers resistance to the drug G418 (6) and with the *Bgl*II/*Bam*HI fragment of herpes simplex virus-TK DNA when these fragments are inserted at the HaMuSV *Bam*HI site (Kriegler, Perez, and Botchan, unpublished data). That is, positive selection of the promoter-minus markers is orientation dependent with respect to the LTR. Twenty-five morphologically transformed foci were picked for further analysis and expanded into cell lines. All 25 lines showed classical T-antigen nuclear immunofluorescence, and when several of the cultures were grown in [^{35}S]methionine media, both of the SV40-specific T antigens could be immunoprecipitated from total protein extracts (Fig. 5A; data not shown). The size of these proteins appeared identical with bona fide SV40 proteins which were fractionated in parallel by polyacrylamide gel electrophoresis. These experiments show that the LTR does indeed contain sequence elements capable of promoting *A* gene expression and that functional, SV40 T antigens are the resultant products. We next used pRETRO-T plasmid DNA to crudely delineate the bounds of the LTR promoter sequences from pRETRO-T and then to delete the same sequences from pSVLTR1 DNA in an attempt to possibly separate the "enhancer" activity of the LTR we had identified in the SVLTR1 virus experiments from the promoter activity we had demonstrated above.

Sequence analysis of MoLV-derived LTRs has shown that the 5′ end of the retroviral DNA maps to the rightward 25% of the LTR. Furthermore, in vitro transcription analysis of this DNA implicates the importance of these sequences in

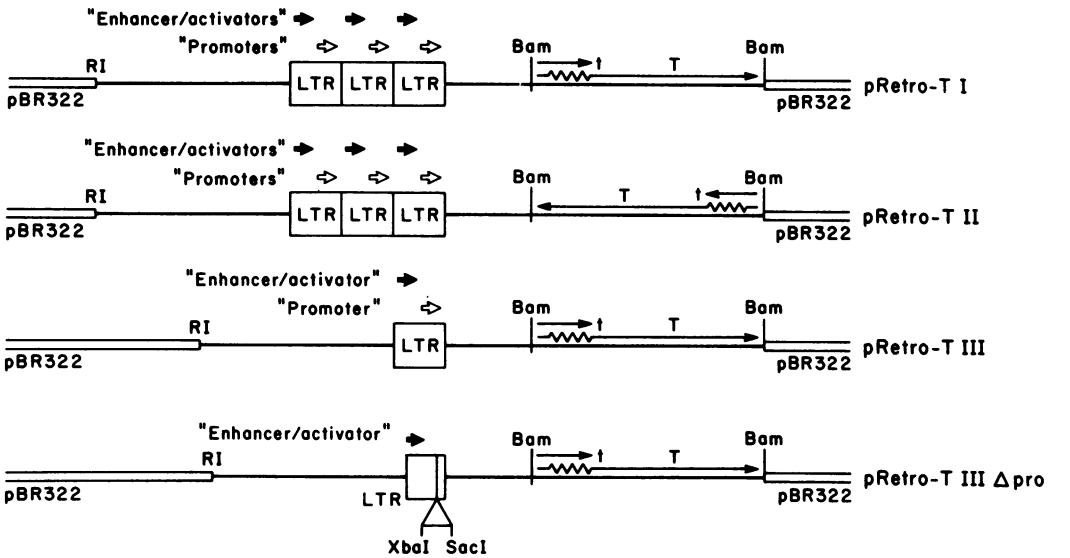

FIG. 4. Structures of RETRO-T plasmids. The following recombinant plasmids were constructed to test the notion that the retrovirus LTRs contained sequences capable of acting as a promoter. An SV40 *BglI*/*Bam*HI fragment (lacking a functional SV40 promoter), to which *Bam*HI linkers had been attached, was inserted into the *Bam*HI site of two distinct *Bam*HI/*Eco*RI (RI) subclones of the HaMuSV provirus (which contained either one or three LTRs and their flanking sequences). Recombinant plasmids containing the SV40 early region in the correct (pRETRO-T I and III) and incorrect (pRETRO-T II) orientations were isolated and assayed for their ability to promote T antigen expression.

vivo (10). In the cloned proviral DNA, 5′ to the 5′ end of the retroviral genomic RNA, at −24, lies an adenine-thymine-rich sequence (TATA box) which is postulated to be the region around which the promoter is situated (28, 37). 5′ to this sequence, at −180, lie two 72-base pair (bp) repeats. 3′ to the retroviral 72-bp repeats and 5′ to the 5′ end of the retroviral RNA genome lie *Xba*I (at −145) and *Sac*I (at −30) restriction sites. We digested pRETRO-T III partially with *Sac*I (which cuts pRETRO-T III twice) and completely with *Xba*I to create a deletion defined by the *Xba*I and *Sac*I sites. The *Xba*I site was filled in, whereas the *Sac*I site 3′ extension was destroyed by the dual action of the T4 DNA polymerase. The ends of this blunt-ended construction were ligated with T4 DNA ligase and recloned in *Escherichia coli*. We transfected Rat-2 cells with this new DNA construction called pRETRO-T IIIΔPRO (see Fig. 4), using the calcium phosphate-mediated gene transfer technique. These experiments showed that this construction was not capable of inducing transformation above background and was therefore at least 100-fold less effective than the parental DNAs. We then made the same *Xba*I-*Sac*I deletion in pSVLTR1 DNA and generated a recombinant SV40 virus stock (SVLTRΔPRO) containing the LTR fragment with these essential promoter sequences deleted (Fig. 1). The procedures used to generate this virus stock were identical to those used to generate SVLTR1 virus. The SVLTRΔPRO virus stocks were carried through two cycles of plaque purification, and the resultant viral DNAs were analyzed. As with SVLTR1 virus, our experiments indicated that no rearrangements had occurred during the packaging process. We measured the ability of SVLTRΔPRO virus to transform low-passage NIH 3T3 cells relative to wild-type SV40 virus and SVLTR1 virus. SVLTRΔPRO and SVLTR1 virus stocks of equivalent titers transformed equal numbers of NIH 3T3 cells (Table 1). Therefore, damage to the LTR promoter had no detectable effect on the ability of the LTR enhancer element to increase the transformation ability of the recombinant virus. Thus, we were able to map the enhancer element to the 300-bp sequence that lies to the left of the *Xba*I site in the LTR of HMSV. These experiments directly demonstrated that the element within the LTR responsible for the enhanced transformation phenotype of SVLTR1 and SVLTRΔPRO is not coincident with a functional retroviral promoter.

SVLTR1-transformed 3T3 cells contain amounts of T antigen similar to wild-type SV40 transformants. The enhancer element might serve to increase the amount of *A* gene product generated by the SV40 early region in SVLTR1 virus by some unknown mechanism. To test this

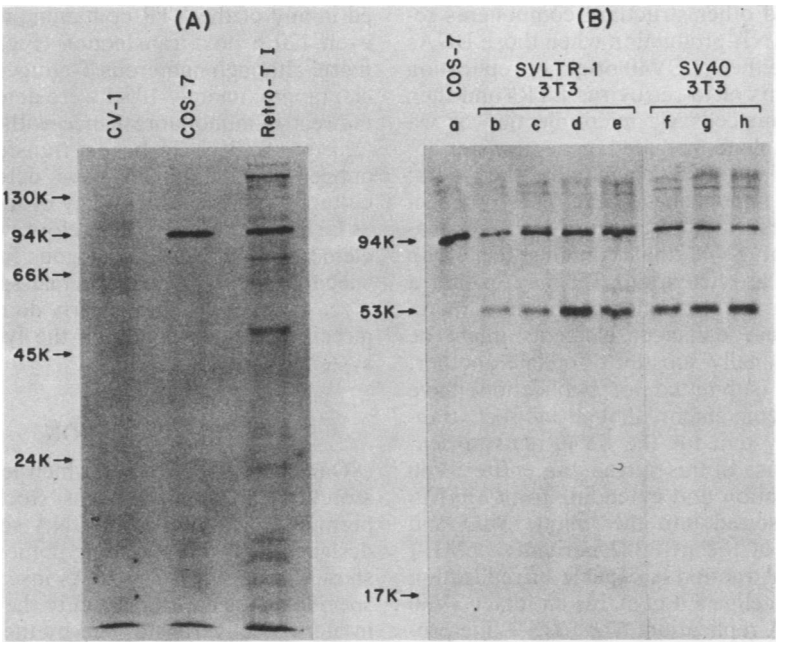

FIG. 5. Immunoprecipitation analysis of pRETRO-T- and SVLTR1-transformed cell lines. (A) Immunoprecipitation analysis of pRETRO-T-transformed NIH 3T3 cells. CV-1-, COS-7-, and pRETRO-T I-transformed NIH 3T3 cells were plated at 8.0×10^5 cells per 60-mm plate, incubated for 1 h in methionine-free medium, and labeled for 3 h with 50 μCi of [^{35}S]methionine (400 Ci/mmol). The labeled cells were harvested and lysed in immunoprecipitation buffer. Incorporated counts were measured, and equivalent amounts of trichloroacetic acid-precipitable counts were treated with anti-D2 antiserum and *Staphylococcus* A bacteria. Immunoprecipitated proteins were eluted and subjected to electrophoresis in a 10% polyacrylamide gel. An autoradiogram of that gel is shown above. Large T antigen is detected as a band at 94K in the pRETRO-T I sample as well as in the COS-7 sample. Small t antigen is not immunoprecipitated by anti-D2 antiserum and is therefore not detected in this experiment. (B) Immunoprecipitation analysis of SVLTR1 virus-transformed NIH 3T3 cells. SV40- and SVLTR1-transformed NIH 3T3 cells were analyzed as described above; however, antitumor serum had been used to immunoprecipitate large and small T antigens. Large T antigen is detected as a band at 94K, and small t antigen can be readily detected at 17K in longer exposures of this autofluorogram in both the SV40 and the SVLTR1 samples.

hypothesis, we performed quantitative immunoprecipitation assays on SV40-transformed NIH 3T3 cells and on SVLTR1 virus-transformed cells picked as individual colonies in soft agar. Each line was tested for T-antigen immunofluorescence and all were found to be 100% T-antigen positive. These transformed cell lines were generated in the same experiment and had been carried through the same number of passages. To ensure quantitative immunoprecipitation of the SV40 T antigens from the isolated transformants, we used a fivefold excess of hamster antitumor serum to precipitate the [^{35}S]methionine-labeled tumor antigens. The transformed cells were labeled for 2 h with [^{35}S]methionine and lysed in 1 ml of lysing buffer, and we removed an aliquot for scintillation counting as described in Materials and Methods. Equivalent numbers of incorporated counts were subjected to immunoprecipitation with antitumor serum, and the proteins were displayed by standard polyacrylamide gel electrophoresis and autoradiographic protocols. The intensity of the autoradiographic bands in each lane directly reflects the percentage of [^{35}S]methionine counts incorporated into T-immunoreactive polypeptides relative to total [^{35}S]methionine incorporation in each cell culture. The results are quite consistent from cell line to cell line when those cell lines have been transformed with the same virus. The results of that assay are shown in Fig. 5B. We observed no increase (less than twofold) in the amount of immunoreactive large T (94K) polypeptide in the SVLTR1 virus transformants when compared with the SV40 transformants analyzed under the same conditions.

The retroviral enhancer element does not directly substitute for the SV40 enhancer element. Several groups have recently reported observing enhanced transformation of cells with various biochemical marker DNAs which contain intact

promoters and other structural components required for mRNA production when those DNAs are attached either to SV40 origin of replication DNA fragments or to retrovirus LTRs and then transfected into cells via microinjection or via calcium phosphate-mediated gene transfer (5, 19; Robbins and Botchan, unpublished data; Luciw et al., unpublished data). These parallel findings suggest that the origin of DNA replication region of SV40 that contains the 72-bp repeats and the retrovirus LTR may exhibit a common type of "enhancing principle." Accordingly, these enhancer elements might be able to functionally substitute for one another. Lusky et al. (submitted for publication) have developed a convenient, though indirect, transient assay system for the SV40 activator/enhancer function. In this system, an entire SV40 early transcription unit extending from *Sph*I to *Bam*HI is inserted into the unique *Sph*I and *Bam*HI sites of the pBR322 derivative pML-I (21). This construction is capable of replication in the COS-7 cells as it contains an intact SV40 origin of DNA replication. The COS-7 line provides the required SV40 gene product for replication as it contains an integrated proviral copy of SV40 (11). However, as the construction deletes the SV40 activator sequence distal to *Sph*I, this DNA is not capable of producing the *A* protein by itself in CV-1 cells and therefore does not replicate in normally permissive simian cells. This plasmid, which is called pJYMΔSph, can be restored to replication competence (and T-antigen expression) by reinsertion of activator fragments from either SV40 or bovine papilloma virus into the vector, in an apparently position- and orientation-independent manner with respect to the SV40 early transcription unit (Lusky et al., submitted; Fig. 7). To test for functional equivalence between the SV40 enhancer and the retroviral enhancer elements, we inserted the LTR enhancer fragments into pJYMΔSph both 5′ and 3′ to the SV40 early region in both orientations. These constructions are diagrammed and described in detail in Fig. 6 and its legend.

We tested the ability of these recombinant plasmids to replicate in CV-1 cells through DEAE–dextran-facilitated DNA transfection (34). Hirt supernatant DNA was prepared at 0, 36, 96, and 120 h post-transfection and assayed for DNA replication by Southern blot analysis. We detected copious amounts of replication of the pSV3′72-containing samples 36 h post-transfection. This recombinant DNA has the SV40 activator-containing fragment over 2.5 kb distal from the early transcription start sites. The kinetics and extent of replication of this construction are independent of the orientation of the enhancer fragment. No replication is detected in any of the LTR-containing clone samples even 120 h post-transfection (Fig. 7). Furthermore, although numerous T-antigen-positive nuclei (approximately 10%) were detected through indirect immunofluorescence with either pJY-1 (21) or pSV3′72 24 h post-transfection, no T-antigen-positive nuclei were detected in cell cultures transfected with any of the pJYMΔSph + LTR constructions. Therefore, whereas these elements might serve analogous functions for a specific virus in a given animal species or in a given tissue type, they clearly do not substitute directly for one another in the lytic SV40 cell system.

DISCUSSION

Our initial experiments which led to the construction of an SV40 virus stock carrying a permuted retroviral LTR DNA sequence were designed to test a simple hypothesis: could we show that these SV40 DNAs inserted in a site-specific manner with respect to the viral genome in murine cells transformed by the recombinant virus? This notion derives from the perhaps oversimplified view that the retroviral attachment site is encoded entirely by the short inverted repeats and flanking DNA at the ends of the LTRs and that these *cis*-acting sequences would be sufficient for specific insertion in a manner analogous to bacterial transposition. Insofar as our results were negative (that is, we could not provide evidence for site-specific insertion), the results by themselves do not define the sequence elements required for retroviral proviral insertion. Quite unexpectedly, however, we have found that these SV40 virus stocks transform NIH 3T3 cells in a colony assay with 10- to 20 times the efficiency of SV40 wild-type virus. The major points established in this report can be summarized as follows.

(i) The enhanced morphological transformation of this SV40 virus does not appear to be due to retrovirus-like insertion or to enhanced integration as insertion copy number is low (see below for further discussion of this point).

(ii) The LTR can serve as an effective promoter for a variety of genes including the SV40 *A* gene. Recombinant plasmids that contain an intact SV40 early region (minus the SV40 viral promoter region) linked to the LTR only in the correct orientation yield DNAs capable of morphological transformation of 3T3 cells. These morphologically transformed lines produce what appear to be intact SV40 T antigens (Fig. 5A). DNA constructions with SV40 early region inserted in the reverse orientation with respect to the LTR are at least 100-fold less efficient in transformation. A large deletion of nucleotide sequences within the LTR inactivates this pro-

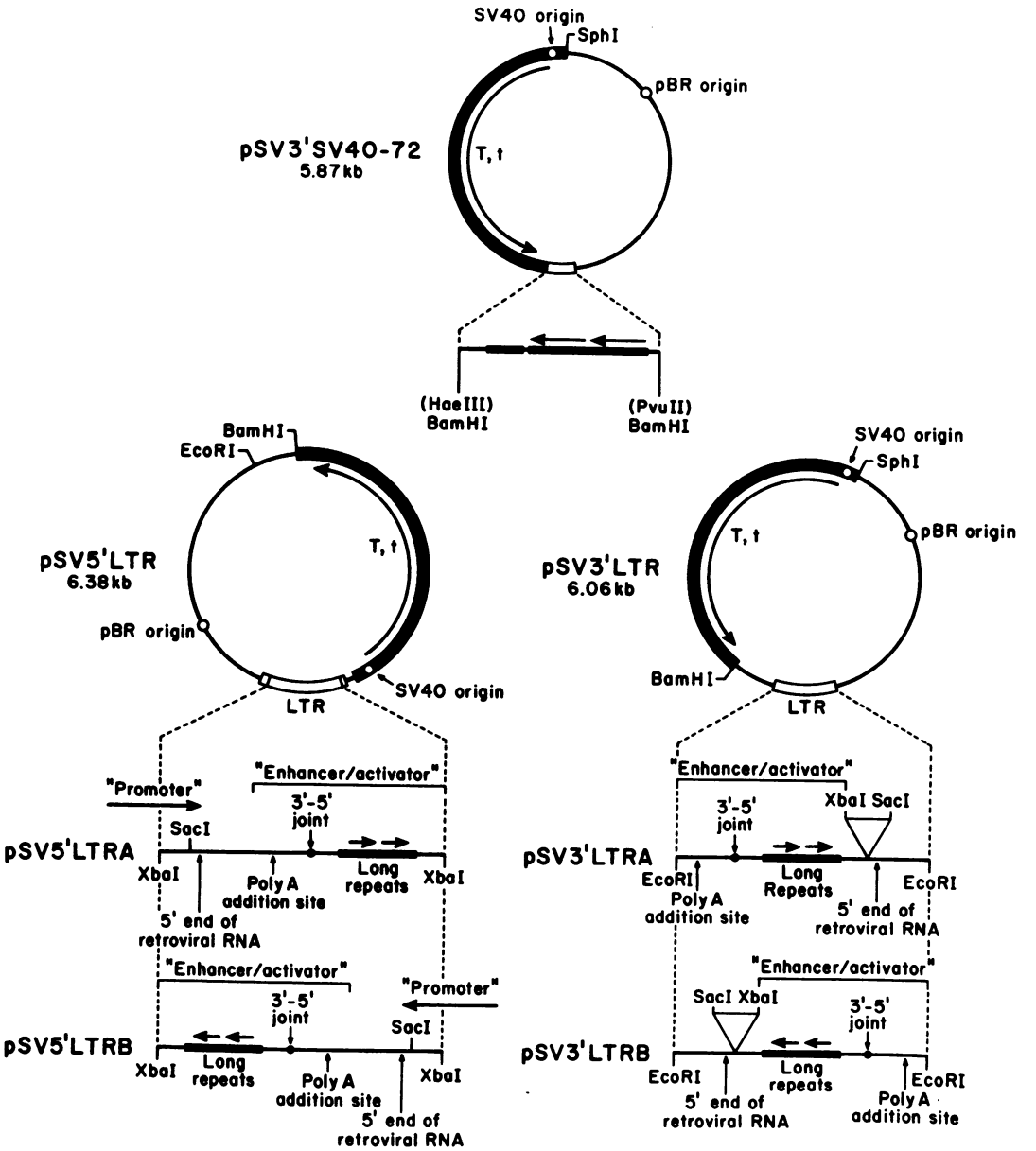

FIG. 6. Physical and functional maps of pSV3'-72 and LTR + pJYMΔSph constructions. Cloned pSV3'72 DNA was used as a positive control in replication assays. This construction is composed of pJYMΔSph DNA which has been cut with *Bam*HI and into which the *Pvu*II/*Hae*III SV40 enhancer fragment (with *Bam*HI linkers attached) has been inserted. Specific HaMuSV retroviral LTR fragments were inserted into cloned, replication-defective pJYMΔSph or pJYMΔSph polylinker DNAs. These DNAs were used in replication studies in CV-1 cells. pJYMΔSph polylinker (where a polylinker fragment derived from plasmid pi-vx has been inserted into the *Sac*I site of pJYMΔSph) was digested with *Xba*I (which cuts once in the polylinker fragment), and the 600-bp *Xba*I-cut permuted unit-length LTR fragment was inserted in both orientations (clones A and B). To construct the pSV3' LTR subclones, pJYMΔSph was digested with *Eco*RI and the 480-bp LTRΔPRO fragment (shown to have enhancer activity in other assays) inserted in both orientations (clones A and B). Poly A, Polyadenylic acid.

moter function but nevertheless leaves the LTR enhancer function intact.

(iii) The levels of SV40 early proteins produced by SVLTR1 morphologically transformed cell lines are equivalent to those levels normally produced in wild-type SV40-transformed lines.

(iv) The LTR enhancer may manifest its effect in concert with the SV40 activator in these

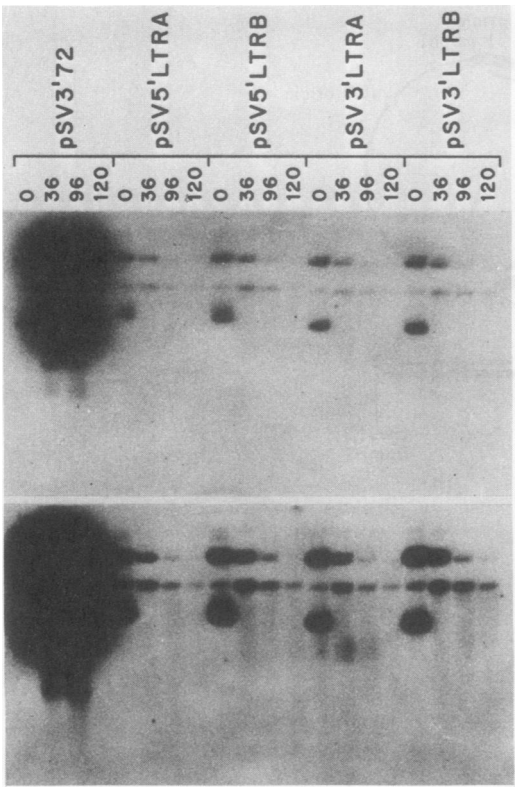

FIG. 7. Analysis of replication competence of LTR + pJYMΔSph constructions in CV-1 cells. CV-1 cell monolayers were transfected with 10 ng of pSV 3'72, pSV5'LTR-A, pSV5'LTR-B, pSV3'LTR-A, or pSV3'LTR-B DNA (see Fig. 6 for details) with the DEAE-dextran facilitated transfection method. Hirt supernatant DNAs were prepared at 0, 36, 96, and 120 h post-transfection and replication was assayed by Southern blot analysis as described previously (21). Two exposures of that autoradiogram of the Southern blot are shown. (Upper) 12-h exposure; (lower) 5-day exposure.

constructions in murine cells, but it cannot substitute for the SV40 enhancer in simian (CV-1) cells.

Our results suggest that the sequences present in our permuted LTR are insufficient to drive integration of the proviral DNA at the viral attachment site when presented as a supercoiled DNA duplex. It seems highly likely that the retrovirus proviral integration is not strictly analogous to the integration of bacterial transposons. An unidentified intermediate of the reverse transcription process, the intact linear form, the circular form containing one LTR, or a circular form containing two complete LTRs, may serve as the integrative intermediate in proviral integration. Alternatively, the process may require enzymes which are packaged along with the viral RNA and that these proteins only work in *cis*. In other words, the integrative recombination enzymes may not be provided in *trans* by the MoLV helper virus or the cell. One caveat to these arguments is that we cannot dismiss the notion that the retroviral DNA did indeed serve as a target for integrative recombination early in the SVLTR-transforming infection and that subsequent chromosomal rearrangements obscured these events.

The enhanced transformation of NIH 3T3 cells by the SVLTR virus stocks can be the result of effects upon different sorts of in vivo phenomena. In general, the affected processes may involve the initial steps in the stabilization of the viral genome within the cells; these could involve either transport or compartmentalization of the viral DNA or its integration into the chromosome. Alternatively, the enhancement phenomena may be a manifestation of more effective gene expression. A corollary of this enhanced gene expression hypothesis is that integration and stabilization processes are not the only rate-determining steps in limiting viral transformation and that the insertion of cryptic genomes is common. The results presented in this report cannot distinguish between these hypotheses. It has been assumed that during abortive transformation of murine cells by SV40 viruses, where essentially all of the cells transiently express characteristics of the stably transformed lines, the altered phenotypes are not permanently inherited because the viral DNA is not stably integrated. However, the results of cotransformation studies to be reported elsewhere (Kriegler et al., unpublished data) indicate that this may not be a satisfactory explanation. We and others (13, 27) have observed that when the thymidine kinase (TK) gene is used as a selective marker for DNA transformation-competent cells only 5% of the TK^+ transformants are morphologically transformed. In these cases, blot analysis indicates that multiple copies of the SV40 DNA have integrated into the host chromosome but that the early region is not expressed at levels high enough to manifest morphological transformation. In keeping with this notion is the finding that TK^+ transformants need only express the marker at RNA levels at least two orders of magnitude below those levels required for SV40 transformation. In other words, the low phenotypic index for cotransformation can be explained simply by understanding that the selection requires vastly different levels of gene expression. In contrast, however, when the murine retroviral LTR is used to promote the SV40 early region (see Fig. 4) or when the promoter-minus LTR is linked to the intact SV40 early region (carrying an intact promoter/enhancer of

its own), almost all of the TK^+ transformants are morphologically transformed, with no difference in SV40 DNA copy levels. We therefore hypothesize that the LTR enhancer helps the penetrance of SV40 early region genes and that cryptic insertions are indeed common for wild-type SV40-integrated copies. In this context, it is important to note that Khoury and his colleagues (personal communication) have found that the transient transcription of the chloramphenicol acetyltransferase gene promoted by early SV40 DNA sequences is markedly activated in a position- and orientation-independent manner by the Moloney sarcoma virus LTR in murine 3T3 cells and that this transcriptional activation is markedly greater than that affected by the SV40 72-bp repeats in these cells. Transcriptional activation of remote promoters by a viral regulatory DNA was first described by Banerji et al. (1), and it seems highly likely that the enhanced transformation of stable markers manifested by these elements when they are linked is another experimental measure of the same in vivo phenomenon. Whereas this equivalence of enhancer and activator may not always be valid, we conclude that it is so for the HaMuSV LTR. Similar conclusions have been reached for the enhancer/activator associated with bovine papilloma virus (Lusky et al., submitted) and for the avian sarcoma virus LTR (Luciw, Varmus, and Capecchi, personal communication).

The enhancer may affect the expression of linked genes by either providing a site for entry of factors which may migrate to promoters required in a positive sense for transcription (25) or providing a *cis*-acting signal that in concert with a protein factor prevents certain promoters from being actively shut off. In any of these models, markers linked to an appropriate enhancer would be more likely to work in a given chromosomal domain. Our finding that the amounts of SV40 early protein detected in SVLTR versus SV40 wild-type transformants are equivalent is consistent with these arguments as selection coupled with *A* gene autoregulation determines the window of expression. Below a threshold level of early protein expression, the cell remains untransformed, and above a certain level the *A* protein represses its own synthesis. It is important to note here that the RNAs detected by Northern blot analysis of an SVLTR-1-transformed cell line show only the two early 19S SV40 mRNAs at levels equivalent to wild-type SV40 transformants (Singh and Botchan, unpublished data).

The HaMuSV LTR will not provide an activator function which can substitute for the SV40 activator in the lytic simian cell system (Fig. 7). These results are superficially in contradiction to those reported by Levinson et al. (20). These workers have created an SV40 virus stock wherein the SV40 72-bp repeats and other flanking sequences are removed and in their place are substituted sequences from the murine sarcoma virus LTR. (Both the HaMuSV and murine sarcoma virus are derived from the MoLV and have nearly identical LTRs.) This virus, however, grows slowly and will not plaque. We have obtained cloned DNA of this construction (provided by Peter Gruss and George Khoury) and have assayed for replication and T-antigen expression. These constructions (either inserted in pML2 or released from the vector) do not express detectable levels of T antigen nor can we detect replication after 120 h post-transfection. One likely resolution to these apparently contradictory results would be that a very low level of transcription is allowed by the LTR insertion and that, upon high-multiplicity infection, enough *A* protein is made to allow for viral DNA replication. This multiplicity presumably would be difficult to reach via transfection and would be difficult to detect in a single-step biochemical assay such as ours. Alternatively, their viral stock may contain low levels of a complementing defective genome. These considerations aside, it is clear that activators or enhancers or both have cell type specificity. It is unlikely that these specificities are related to species differences as a bovine papilloma virus activator will substitute for the SV40 activator in our single-step SV40 DNA replication assay in simian cells (Lusky et al., submitted) and the murine LTRs will not. Along these lines, Conrad and Botchan (7) have found that a fragment from the human genome will enhance TK transformation in certain human cell lines and not in other murine TK^- lines. However, these results may reflect differences in cell lineages as much as or more than species differences. Reinforcing these notions of cell type specificity for activators is the finding that when polyoma virus adapts to grow in an undifferentiated mouse cell type, a frequent alteration is a change in nucleotide sequence within the enhancer/activator element of this virus (see review by Yaniv [42]). Further delineation in the different elements of the actual sequence domains required for these activities may provide a clue as to when one enhancer/activator may substitute for another.

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ADDENDUM IN PROOF

J. de Villiers and W. Schaffner reported earlier that activators exhibit cell type specificity, in accord with our findings (Abstracts of the 15th Annual Meeting of the Union of Swiss Societies of Experimental Biology, Experientia 37:6, 1981). See also Laimins et al. (L. A. Laimins, G. Khoury, C. Gorman, B. Howard, and P. Gruss, Proc. Natl. Acad. Sci. U.S.A. 79:6453–6456).

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