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Construction of Recombinant Murine Retroviruses That Express the Human T-Cell Leukemia Virus Type II and Human T-Cell Lymphotropic Virus Type III *trans* Activator Genes

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Recombinant retroviruses containing the *trans* activator genes of human T-cell leukemia virus (HTLV) type II and human T-cell lymphotropic virus type III were constructed. The *trans* activator genes *tat* II and *tat* III were inserted into the murine retroviral vector pZIPNEOSV(X)1. Recombinant plasmids were transfected into the psi 2 and psi AM packaging cell lines that produce murine leukemia virions containing no retroviral RNA. Functional *tat* II and *tat* III gene products were expressed as demonstrated by *trans* activation of HTLV type I and II and human T-cell lymphotropic virus type III long terminal repeat-directed gene expression in the respective infected cells. Use of these recombinant vectors permits high-efficiency gene transfer into a wide variety of cells, thereby providing the opportunity to study the biochemical effects associated with *tat* II and *tat* III gene expression.

Human T-cell leukemia virus (HTLV) type I (HTLV-I) and T-cell lymphotropic virus type III (HTLV-III/LAV) are the etiologic agents of adult T-cell leukemia-lymphoma (10, 20, 31) and acquired immune deficiency syndrome (2, 4, 11, 19, 21, 23), respectively. HTLV type II (HTLV-II), although similar in structure to HTLV-I, was isolated from a patient with a benign T-cell variant of hairy cell leukemia (6, 12, 15). These viruses encode genes designated *tat* I, *tat* II, and *tat* III, the products of which activate in *trans* gene expression directed by the respective long terminal repeats (LTRs) (1, 7, 9, 24, 27, 31). In addition to an autostimulatory role that augments viral gene expression and virion production, it has been suggested that the *tat* gene products mediate some of the pathological effects associated with infection by these viruses (1, 26).

To study the physiological consequence of *tat* gene expression, a high-efficiency vector capable of transferring these genes into cells is required. Disadvantages associated with the standard transfection methods include low efficiency of gene transfer and inability to transfect certain cell types efficiently. In this context, retroviral vectors offer several advantages, including efficient infection and predictable integration configuration (29). Here we describe the construction and activity of Moloney murine leukemia virus-based retrovirus vectors, derivatives of the pZIPNEO vectors developed by Cepko and co-workers (5) that contain the HTLV-II and HTLV-III/LAV *tat* genes.

The defective retroviral vector used for these studies, pZIPNEOSV(X)1 (Fig. 1), contains Moloney murine leukemia virus LTRs, polyadenylation signals, sequences required for reverse transcription and for encapsidation of RNA, as well as the 5′ and 3′ splicing signals that normally produce subgenomic *env* gene messenger RNA (5). The vector also contains the bacterial gene for neomycin resistance (*neo*), which confers a dominant selectable resistance to the antibiotic G418 in eucaryotic cells (28).

The *tat* II gene of HTLV-II was obtained from plasmid pCATLORIIgpt (Fig. 1). This plasmid expresses a fusion protein consisting of nine amino-terminal residues (providing the ATG initiation codon) encoded by the bacterial chloramphenicol acetyltransferase (CAT) gene and the product of the entire *tat* II gene, extending from the splice acceptor to the termination codon present in the 3′ LTR (25). A similar plasmid that contained the *tat* II gene in the antisense orientation relative to pZIPNeotatII was constructed (pZIPNeotatII-A).

The HTLV-III/LAV *tat* III gene was obtained from infectious proviral clone HXBC2 (Fig. 1) and encodes the HTLV-III/LAV associated *trans*-acting factor (1, 26). The recombinant viruses were constructed as outlined in the legend to Fig. 1. DNA was introduced into the psi 2 (ecotropic) and psi AM (amphotropic) cell lines by the calcium phosphate coprecipitation method (30). These lines constitutively produce the murine leukemia virus proteins but cannot package the viral transcripts (8, 18). Two days following transfection, cells were selected with the antibiotic G418 (400 μg/ml for fibroblast lines and 700 μg/ml for lymphocytes). G418-resistant clones were evident in 7 to 10 days. Evidently, insertion of the *tat* regions does not interfere with splicing events required for transcription of the *neo* gene. G418-resistant psi 2 and psi AM clones were isolated, and the virus from clones producing greater than 10^3 infectious units per ml was used to infect the test cells (16). Cells resistant to G418 were observed subsequent to infection of all the cell lines tested.

To test for the presence of *tat* gene transcripts, total cellular RNA was extracted from several clonal isolates of infected NIH 3T3 and HeLa cells. Figure 2A demonstrates that *tat* II and *tat* III gene transcripts were present in the respective infected cells and absent from uninfected controls. This experiment demonstrates that the *tat* genes and the *neo* gene can be transcribed from these vectors.

Recent studies demonstrate that the *tat* II and *tat* I gene products in the absence of other viral proteins activate in *trans* HTLV-I and HTLV-II LTR-directed gene expression

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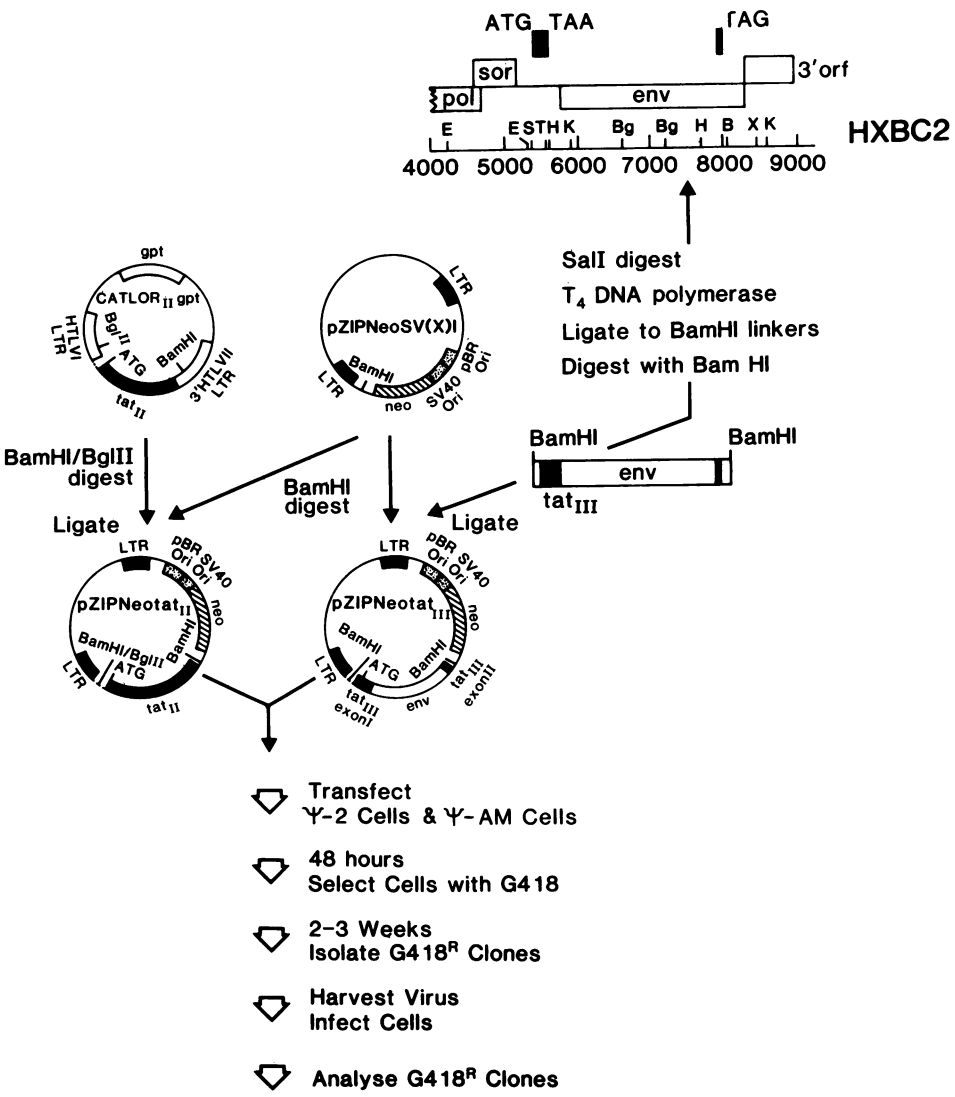

FIG. 1. Construction of pZIPNeotatII and pZIPNeotatIII. The construction of the vector plasmid pZIPNEOSV(X)1 has been previously described (5). The HTLV-II *tat* II gene was obtained from plasmid pCATLORIIgpt. The HTLV-III/LAV *tat* III gene was obtained from the infectious HTLV-III/LAV proviral clone HXBC2 (8a, 26). Plasmids were constructed by standard recombinant DNA methodology (17). Lymphoid cells were infected as described by King et al. (16).

(25). To determine whether the *tat* II gene transcripts present in infected cells direct the synthesis of a functional *tat* product, levels of HTLV-I and HTLV-II LTR-directed gene expression were compared in uninfected and infected cells (Table 1). In a previous study we have shown that the *tat* I and *tat* II gene products are functionally equivalent in *trans*-activation of HTLV-I and HTLV-II LTRs (25). Cells were transfected with plasmids that contained the bacterial CAT gene under control of the HTLV-I LTR (pU3R-I) and the HTLV-II LTR (pU3R-II) (Fig. 3). As controls for specificity of *trans*-activation, the cells were also transfected with plasmids that contained the Rous sarcoma virus LTR (RSVCAT), the HTLV-III/LAV LTR (pU3R-III), or the simian virus 40 early region (pSV2CAT) transcription regulatory sequences upstream to the CAT gene (Fig. 3). The level of CAT activity was measured in cell extracts prepared at 48 h posttransfection. To establish a control for differences in transfection efficiencies that might be associated with the individual clones, the level of CAT activity was normalized to the activity obtained following transfection of the same cells with plasmid pSV2CAT.

The data in Table 1 and Fig. 2B shows that HTLV-I LTR-directed gene expression in several infected Raji clonal lines was approximately 94- to 220-fold greater than that directed by pSV2CAT, a level 165- to 385-fold greater than that observed in uninfected cells. No difference in the level of HTLV-I LTR-directed CAT gene expression was observed between transfected uninfected cells and G418-resistant clones that had been infected with the vector that contains the *tat* II gene in the reverse orientation (ZIPNeotatII-A) (Table 1; data not shown). In addition, HTLV-I LTR-directed gene expression was increased 50- to 120-fold in three ZIPNeotatII-infected G418-resistant Jurkat cell clones. Gene expression directed by the HTLV-II LTR (pU3R-II) was increased 65-fold in ZIPNeotatII-infected Raji cells and 5-fold in infected NIH 3T3 cells compared with the same cells infected with the antisense vector. We noted that no detectable increase in HTLV-II LTR-directed gene

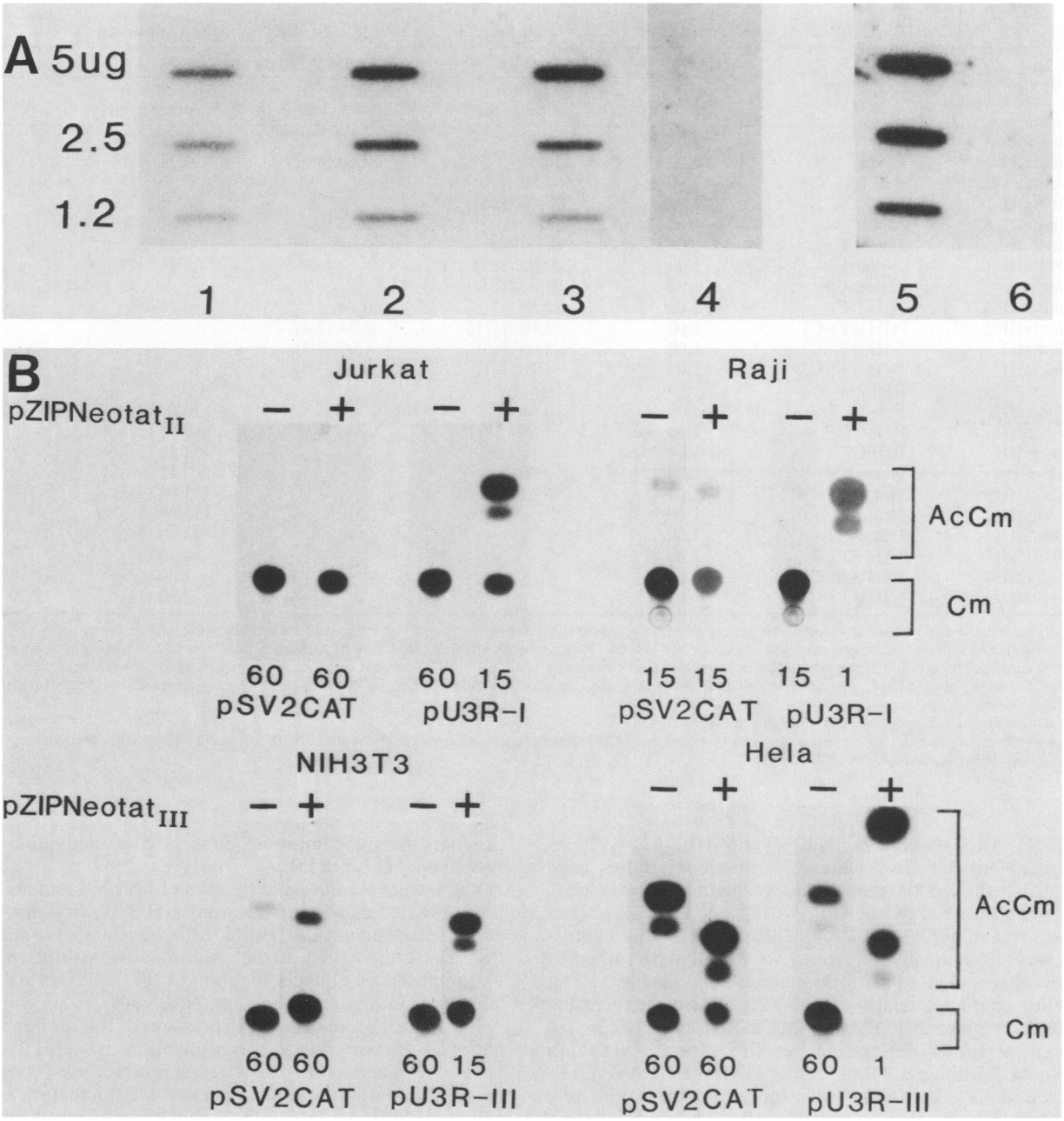

FIG. 2. Analysis of ZIPNeotatII- and ZIPNeotatIII-infected cells. (A) Total RNA was prepared (17) from uninfected NIH 3T3 (lane 4) and HeLa (lane 6) cells. RNA was also prepared from three individual G418-resistant ZIPNeotatII-infected NIH 3T3 clones (lanes 1, 2, and 3) and a pool of G418-resistant ZIPNeotatIII-infected HeLa cells (lane 5). The RNA was spotted onto nitrocellulose and hybridized to either *tat* II (lanes 1 through 4) or *tat* III (lanes 5 and 6) gene ^{32}P-labeled probes (3). (B) The presence of functional *tat* proteins was determined by measuring the level of HTLV-I (pU3R-I) and HTLV-III/LAV (pU3R-III) LTR-directed CAT gene expression following transfection of the respective infected cells (24, 27). The levels of CAT activity obtained following transfection of uninfected (−) and infected (+) cells with the control plasmid pSV2CAT are also shown. The autoradiograms show the level of enzyme activity present in a typical reaction for the time (numbers beneath CAT assays) indicated. Unreacted chloramphenicol (Cm) and acetylated reaction products (AcCm) are shown.

expression was evident in the Jurkat cell line. We have previously observed that in certain cell lines there is no activation of the type II LTR in the presence of *trans*-acting factors (unpublished observations). We attribute this to the lack of additional transcriptional regulatory factors, either cellular or virus encoded, that are required for efficient function of the HTLV-II LTR transcriptional regulatory signals in these cells. The levels of Rous sarcoma virus LTR- and HTLV-III/LAV LTR-directed CAT gene expression were the same in infected and uninfected cells, thereby demonstrating that *trans*-activation is specific for the appropriate HTLV LTR.

To determine whether the *tat* III gene is functional, ZIPNeotatIII-infected cells were transfected with plasmid

TABLE 1. Effect of ZIPNeotatII and ZIPNeotatIII infection on HTLV LTR-directed gene expression

Virus	Cell line[b]	Relative CAT activity with plasmid[a]:				
		pSV2CAT	pU3R-I (stimulation)[c]	pU3R-II (stimulation)[c]	pU3R-III (stimulation)[c]	RSVCAT
ZIPNeotatII-A	Raji	1.00	0.57	0.15	0.24	0.75
ZIPNeotatII	Raji-P	1.00	220.00 (385)	10.00 (65)	0.23	0.48
ZIPNeotatII	Raji-C1	1.00	102.00 (179)			
ZIPNeotatII	Raji-C2	1.00	94.00 (165)			
ZIPNeotatII-A	Jurkat	1.00	1.80	<0.01	0.61	
ZIPNeotatII	Jurkat-C1	1.00	83.00 (46)	<0.01	0.48	
ZIPNeotatII	Jurkat-C2	1.00	220.00 (122)			
ZIPNeotatII	Jurkat-C3	1.00	91.00 (50)			
ZIPNeotatII-A	NIH 3T3	1.00	0.58	0.14	0.20	4.50
ZIPNeotatII	NIH 3T3-C1	1.00	3.90 (6.7)	0.34 (2.4)		6.10
ZIPNeotatII	NIH 3T3-C2	1.00	5.00 (8.6)	1.05 (7.5)		5.10
ZIPNeotatII	NIH 3T3-C3	1.00	4.10 (7.1)	0.42 (3.0)		4.20
None	Raji	1.00	0.62		0.24	0.81
ZIPNeotatIII	Raji-P	1.00	0.68		530.00 (2,208)	1.40
ZIPNeotatIII	Raji-C1	1.00			650.00 (2,708)	
ZIPNeotatIII	Raji-C2	1.00			427.00 (1,779)	
None	HeLa	1.00	0.50		0.12	6.00
ZIPNeotatIII	HeLa-P	1.00	0.61		115.00 (958)	3.00
ZIPNeotatIII	HeLa-C1	1.00			135.00 (1,125)	8.50
ZIPNeotatIII	HeLa-C2	1.00			92.00 (766)	5.70
ZIPNeotatIII	NIH 3T3-P	1.00			4.70 (23)	5.20
ZIPNeotatIII	NIH 3T3-C1	1.00			1.30 (6.5)	4.20
ZIPNeotatIII	NIH 3T3-C2	1.00			2.20 (11)	6.00

[a] Uninfected cells and retrovirus-infected clonal cells or pools were transfected with 2 μg of the indicated plasmid DNA (described in the text). CAT assays were performed 48 h posttransfection as previously described (14, 24). Reaction products were separated by ascending thin-layer chromatography and quantitated by liquid scintillation counting of the spots cut from the thin-layer chromatographic plates. The percent conversion of chloramphenicol to its acetylated products per minute is normalized to the CAT activity obtained following transfection with plasmid pSV2CAT in the same cells. The results represent the average of at least two individual transfections.

[b] C, Clonal isolates; P, pooled cells from more than 10 clones.

[c] The numbers in parentheses represent the fold stimulation of CAT gene expression in infected compared with uninfected cells, all normalized to the CAT activity obtained with plasmid pSV2CAT.

pU3R-III. This plasmid contains HTLV-III/LAV LTR sequences 5′ to the CAT gene (27). Previous studies have shown that the *tat* III gene product activates in *trans* gene expression directed by the HTLV-III/LAV LTR (26, 27).

The level of HTLV-III/LAV LTR-directed gene expression was dramatically increased in ZIPNeotatIII-infected HeLa cells compared with uninfected cells (Table 1; Fig. 2B). For example, relative to pSV2CAT, gene expression directed by plasmid pU3R-III was increased between 760 and 1,100 times depending on the clone tested. In ZIPNeotatIII-infected Raji cells, HTLV-III/LAV LTR-directed gene expression was increased approximately 2,000-fold relative to control Raji cells. The level of CAT activity was increased by 6.5- to 23-fold in infected NIH 3T3 cells. The levels of Rous sarcoma virus LTR- and HTLV-I LTR-directed CAT gene expression were the same in infected and uninfected cells.

From these experiments we conclude that infection of cells with the ZIPNeotatIII and ZIPNeotatII viruses leads to the synthesis of functional *tat* gene products. Moreover, the level of *trans*-activation in many of the infected cells approximates the level of *trans*-activation obtained with the respective HTLV infection.

The results reported here demonstrate that use of the recombinant retroviruses that express the *tat* II and *tat* III genes permits efficient transfer of these genes into a wide variety of cell types, including human lymphoid lines. The level of *tat* gene activity in recombinant virus-infected cells approximates that of cells infected with HTLV-I and HTLV-III/LAV. The results also demonstrate that the *tat* gene products are sufficient to account for *trans*-activation of the respective HTLV LTR.

The low level of *trans*-activation of HTLV-I and HTLV-II LTR-directed gene expression in NIH 3T3 fibroblasts compared with human cells (HeLa, Raji, and Jurkat) is noteworthy. These data suggest that additional transcriptional factors required for efficient function of HTLV LTRs are either lacking or in low abundance in these cells.

It has been proposed that expression of the *tat* I and *tat* II genes is required for immortalization by HTLV-I and HTLV-II, whereas the *tat* III gene product may contribute to the cytopathic effects associated with infection of $T4^+$ cells by HTLV-III/LAV. The availability of retrovirus vectors that express the *tat* genes permits high-efficiency transfer of these genes into a wide variety of cells and should provide an opportunity to study the biochemical events associated with *tat* gene expression in vivo. In addition to high-efficiency transfer of genes, use of these defective recombinant viruses eliminates the possibility of virus spread in the absence of helper virus. By substitution of the Moloney LTRs with transcriptional regulatory regions that display a unique tissue preference, the consequence of *tat* gene expression in cells of different hematopoeitic lineage can be assessed.

Cell lines that express high levels of these *tat* gene products are essential for the development of high-level gene expression systems. For example, the desired genes under control of the respective HTLV LTR can be introduced either transiently or stably into the *tat* cell lines and, in turn, should express a large quantity of the desired gene product.

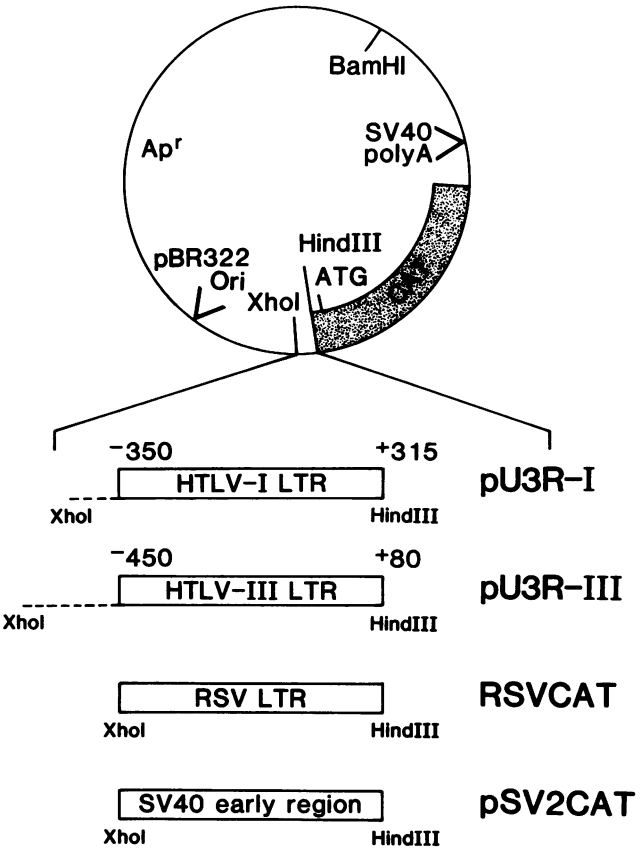

FIG. 3. Plasmids used to assess *trans*-activation. Plasmids pU3R-I (24) and pU3R-III (27) contain the indicated portions of HTLV-I and HTLV-III/LAV LTR sequences, respectively upstream to the CAT gene. Plasmids pSV2CAT (14) and RSVCAT (13) contain simian virus 40 (SV40) and Rous sarcoma virus (RSV) transcriptional regulatory sequences, respectively, upstream to the CAT gene. Plasmid pU3R-II (not shown) contains HTLV-II LTR sequences upstream to the CAT gene (24).

Such a system may be useful for the overproduction of HTLV *env* proteins, which in turn might be useful clinically for diagnostic or prophylactic purposes.

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