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Human Immunodeficiency Viruses Containing Heterologous Enhancer/Promoters Are Replication Competent and Exhibit Different Lymphocyte Tropisms

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The human immunodeficiency virus (HIV) type 1 long terminal repeat (LTR) contains binding sites for nuclear factor κB (NF-κB) and the constitutively expressed transcription factor Sp1, both of which are highly conserved in HIV and simian immunodeficiency virus isolates. To delineate the effects of these motifs on the replicative capacity of HIV and to explore the possibility of extending the virus host range, known heterologous enhancer/promoters were inserted into the HIV-1 LTR in place of the NF-κB and Sp1 binding sites. The effects of these substitutions on viral replication in transfected HeLa cells and on HIV infection of human peripheral blood lymphocytes or continuous T-leukemia cell lines were evaluated. HIVs in which the NF-κB/Sp1 enhancer plus the downstream TATA element were replaced with heterologous enhancer/promoters were also constructed. Viruses containing the human cytomegalovirus immediate-early enhancer exhibited infectious kinetics similar to that of wild-type HIV in activated human peripheral blood lymphocytes and AA2 cells but replicated less efficiently in H9 and CEM cells. These studies indicate that heterologous enhancer elements are capable of restoring Tat responsiveness to the HIV LTR in the context of directing reporter gene expression as well as in the production of infectious progeny virions.

Retroviral long terminal repeats (LTRs) contain multiple *cis*-acting DNA motifs, located upstream of the transcriptional start site, which modulate the synthesis of viral mRNAs. In the case of human immunodeficiency virus type 1 (HIV-1), the structures and functions of several of these elements have been intensively investigated in the context of both LTR-directed reporter gene constructs and full-length proviral DNAs. Some of these elements (e.g., Sp1 and TATA) bind cellular proteins which are constitutively expressed in most eukaryotic cells and influence basal levels of HIV expression (19, 30, 31, 40, 43, 44, 48). Others, such as the nuclear factor κB (NF-κB) and NFAT-1 sites, interact with factors that are induced following T-cell activation, and modulate the basal levels of HIV expression (12, 42, 53, 54, 58).

Much of our knowledge about the functional properties of the HIV LTR comes from transient transfection experiments in which the expression of LTR-driven reporter genes has been evaluated in the presence or absence of the virus-encoded transactivator Tat (2, 15, 17, 55). In many instances, these assays have been conducted in epithelial cell lines such as HeLa or COS or, less frequently, in human leukemic cell lines (44, 48). In addition to delineating the role of cellular factors on LTR-directed expression, these studies have also been useful in dissecting the mechanism(s) underlying Tat-induced stimulation of viral mRNA synthesis. Unlike prototypical transcriptional activators which interact with DNA elements situated upstream of the transcription start site, the HIV-encoded Tat transactivator binds to an RNA stem-bulge-loop structure (designated TAR) located at the 5′ terminus of all HIV transcripts (27, 40, 48). This unorthodox transcriptional activation target has led several groups to propose that the Tat transactivator protein stimulates viral RNA production posttranscriptionally, perhaps by facilitating the elongation of nascent mRNAs (16, 33, 36, 38, 51). However, several reports have shown that in addition to requiring TAR, the responsiveness of an HIV LTR to Tat requires the presence of upstream enhancer elements (26, 44, 48). Mutations affecting both the NF-κB and the Sp1 elements eliminate the Tat responsiveness of LTR-chloramphenicol acetyltransferase (CAT) constructs (4, 37, 44) and render a cloned full-length proviral DNA replication incompetent (37). Replacement of individual enhancer elements or combinations of enhancer elements restores biological function (49). Furthermore, several recent studies, employing Tat fusion proteins, have demonstrated that Tat can transactivate the HIV-1 promoter by binding to DNA elements (4, 32, 56). In two instances, the Tat fusion protein interacted with motifs located upstream of the transcriptional start site (32, 56), and in all cases, Tat transactivation occurred in the absence of TAR (which had been deleted from the reporter construct).

In this study, we investigated whether heterologous enhancer/promoter sequences could be substituted for the HIV-1 Sp1 NF-κB and TATA elements and direct expression from the viral LTR. Although this study was begun by using LTR reporter constructs and Tat supplied in *trans*, we were most interested in determining whether the substituted enhancers could function in the context of the full-length provirus and the viral genome. Functional activity could be restored to CAT constructs following the insertion of certain heterologous enhancer motifs into biologically inactive LTRs. In some cases, the principal effect was a marked elevation of basal levels of LTR-directed expression, while in others, the basal CAT activity was unchanged and increased responsiveness to Tat transactivation was observed. HIV stocks, containing the cytomegalovirus (CMV) immediate-early (IE) enhancer element substituted for the NF-κB and Sp1 motifs, exhibited replication kinetics similar to

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those of wild-type HIV in human peripheral blood lymphocytes (PBLs) and in H9 and AA2 cells, grew slowly in CEM cells, and were unable to infect Molt 3, HL60, and U937 cells.

MATERIALS AND METHODS

Cells. HeLa cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The MT4 (25), H9 (44), and 12D7 (isolated by Guido Poli from the cloned CEM cell derivative A3.01 [18]) continuous human T-leukemia cell lines were maintained in RPMI 1640 medium with 10% fetal bovine serum. AA2 cells, derived from the human B-lymphoblastoid line WIL-2 (8), were obtained from the AIDS Research and Reference Reagent Program of the National Institute of Allergy and Infectious Diseases and propagated in RPMI 1640 medium supplemented with 1× nonessential amino acids and 0.1 mM sodium pyruvate. All other cells were obtained from the American Type Culture Collection (Rockville, Md.). Human PBLs from HIV-seronegative persons were stimulated with phytohemagglutinin (0.25 μg/ml) for 3 days prior to infection and then maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 10% interleukin-2 (Pharmacia).

Plasmid construction. Plasmid pU3-R-CAT was constructed by ligating the 1.5-kbp *Hin*dIII fragment (mapping between positions 8131 and 9606 [see reference 41 for the proviral DNA numbering system] and designated the U3-R fragment) of the full-length pNL43 HIV cloned proviral DNA (1) and the 1.6-kbp *Hin*dIII-*Bam*HI fragment (containing the CAT gene plus the simian virus 40 polyadenylation signal) of pSV2CAT (21) to the *Hin*dIII-plus-*Bam*HI-digested pT7T318U vector (Pharmacia). pSVtat contains the *tat* gene of HIV_{SF2} (44). Plasmid dl.κB/Sp1-U3-R-CAT, in which the NF-κB and Sp1 sites were deleted, was constructed by M13 mutagenesis (34) using primer 1 (see below), which contains an *Xba*I site plus sequences adjoining the NF-κB-plus-Sp1 deletion shown in Fig. 1.

Heterologous enhancers replacing the HIV NF-κB and Sp1 sites were generated by polymerase chain reaction (PCR) amplification from MT-1 (pBPV [Pharmacia]), CMV-IE(a) (pdlA23cat [28], provided by M. Stinski), CMV-IE(b) (pCMVcat, provided by S. Venkatesan), the murine leukemia virus (MLV) LTR (pMuLV [46], provided by R. Repaske), and the polyomavirus (Py) enhancer A (p1-R [29]) and were individually inserted into the *Xba*I site of dl.κB/Sp1-U3-R-CAT. The oligonucleotide primer pairs (5′ to 3′) used for amplification were primers 2 and 3 (MT-1), primers 4 and 5 [CMV-IE(a)], primers 6 and 5 [CMV-IE(b)], primers 7 and 8 (MLV), and primers 9 and 10 (Py). The amplified fragments were cleaved with *Xba*I or *Nhe*I (for MLV only) and inserted into *Xba*I-digested dl.κB/Sp1-U3-R-CAT.

HIV LTR-directed CAT constructs containing a heterologous enhancer plus heterologous TATA element from either CMV or MLV were also made by PCR amplification. Primers containing CMV sequences (−46 to −1) (the transcription start site is located at position +1) and HIV R-region sequences (+1 to +14 [primer 11]) or MLV sequences (−59 to −1) and HIV R sequences (+1 to +15 [primer 13]) were synthesized. These products were then used individually with primer 12 (containing HIV-1 p17 MA sequences (+402 to +385) to generate by PCR the heterologous enhancer-plus-promoter (E+P) element linked directly to the HIV transcription initiation site plus downstream HIV sequences. The amplified CMV-IE(a)/TATA fragment was cleaved with *Xba*I plus *Hin*dIII and inserted into dl.κB/Sp1-U3-R-CAT containing CMV-IE(a), previously restricted with these two enzymes. The MLV/TATA amplified product was cleaved with *Bss*HII plus *Hin*dIII and inserted into dl.κB/Sp1-U3-R-CAT containing the MLV enhancer, also previously digested with the same two enzymes. All of the heterologous enhancer/promoter inserts generated by PCR were confirmed by DNA sequencing.

Cloned HIV proviruses with heterologous enhancer/promoters were constructed by ligating three fragments from a full-length HIV-1 molecular clone (pNL43 [1]), two fragments isolated from HIV LTR-CAT plasmids containing inserted heterologous enhancer/promoters, and the *Bam*HI-plus-*Pst*I-digested pT7T318U vector. The proviral segments used in the ligation were (i) *env*-U3-R (*Bam*HI-*Sac*I, nucleotides [nt] 8465 to 487 [isolated from a dl.κB/Sp1-U3-R-CAT plasmid containing a replacement enhancer/promoter]), (ii) U5-*gag* [*Sac*I-*Sph*I, nt 487 to 1443 [isolated from pNL43]), (iii) *gag-pol-env* [*Sph*I-*Xho*I, nt 1443 to 8887 [isolated from pNL43]), (iv) *env*-U3-R (*Xho*I-*Hin*dIII, nt 8887 to 531 [isolated from a dl.κB/Sp1-U3-R-CAT plasmid with a replacement enhancer/promoter]), and (v) U5-*gag* (*Hin*dIII-*Pst*I, nt 531 to 1415 [isolated from pNL43]). The structures of the reconstructed HIV proviral DNAs were verified by extensive restriction enzyme mapping, and the LTR regions were checked by nucleotide sequencing.

Transfection and RNA analysis. HeLa cells were transfected by using a modification of the original $Ca_3(PO_4)_2$-DNA coprecipitation procedure (22) when they reached 80 to 90% confluency. Six hours following the addition of DNA (0.5 ml of precipitated DNA in 5.0 ml of medium), the cells were exposed to 20% glycerol in DMEM (without serum) for 5 min, washed twice with fresh medium, and then maintained in DMEM until harvest. In CAT assays, the HeLa cells were transfected with 3 μg of a specific CAT plasmid in the presence or absence of plasmid pSVtat (0.5 μg). For transfections of cloned HIV proviral DNAs, 30 μg of plasmid DNA was added. The $Ca_3(PO_4)_2$ transfections also included 20 μg of sheared salmon sperm DNA as carrier. AA2 cells were transfected by the DEAE-dextran technique as modified by Fisher et al. (17), using 10 μg of CAT plasmid DNA per 10^7 cells. Plasmid pXGH5 (Nichols Institute Diagnostics), which encodes a secreted human growth hormone that can be quantitated by radioimmunoassay, was included (0.5 μg for HeLa cells and 10 μg for AA2 cells) in each transfection assay.

$Poly(A)^+$ RNA, prepared from approximately 3×10^6 cells as previously described (9), was electrophoresed through 1.4% formaldehyde agarose gels, transferred to nitrocellulose membranes (GeneScreen; DuPont), and hybridized to a full-length HIV proviral genomic probe labeled by a random-priming reaction (Promega). The radioactivity on the membranes was quantitated with a phosphoimager (Fuji Bio-imaging analyzer BAS 2000).

CAT and reverse transcriptase (RT) assays. CAT assays were performed as previously described (37). HeLa and AA2 cells were harvested 60 h after transfection, washed three times in phosphate-buffered saline, and subjected to three cycles of freeze-thawing. The protein concentration in cell lysates was determined by using a protein assay kit from Bio-Rad. To obtain results within the linear kinetic range of CAT activity, the amount of cell lysate used in each reaction was adjusted to give a detectable signal within 1 h and less than 50% consumption of the input substrate [^{14}C]chloramphenicol (0.5 μCi; 55 mCi/mmol; ICN). The enzyme concentration was determined by a serial dilution for lysates with high levels of CAT activity.

TABLE 1. Oligonucleotide primers used for amplification or sequencing

Primer	Sequence[a]
1	GCTGCTTATATGTAGCATCTAGAGACTCCGGATGCAG
2	CCTGCAGATCTAGAAGGGGCGGTCCCGCTGTGC
3	CCAGGCCTCTTTATAGTCGTCTAGACGAGTCCGGGCGC
4	TCTACGTATTAGTCATCTAGATTACCATGGTG
5	GGAGCTCTGCTTATATAGACCTTCTAGAGTACACGCCTACCGCCCATTTGC
6	CATTAGTTCATAGCCTCTAGATGGAGTTCCGCG
7	CATAACTGAGAAGCTAGCAGTTCAGATCAAGG
8	CTTTTATTGAGCTCGGCTAGCAGAAGCGC
9	CTGCAGATCTAGATCAGCTTCAGAAGATGGCGG
10	GGATGTCTAGAGGCCAGCTGCGGTCAG
11	GTGTACTCTAGAAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGGGTCTCTCTGGTTA
12	CCCATTTATCTAATTCTCC
13	CTGTTCGCGCGCTTCTGCTCCCCGAGCTCAATAAAAGAGCCCACAACCCCTCACTCGGGGGTCTCTCTGGTTAG
14	CAAGCTTTATTGAGGCTTAAGCAGTG
15	GCCTGCATGGAATGGATGAC

[a] The *Xba*I site is underlined, and the *Nhe*I site is boxed.

RT assays were performed as previously described (49), with the following modifications. The supernatants from transfected cells were spun in a microcentrifuge at 3,000 rpm for 5 min before being added to the reaction mixture. Supernatants from virus infections were removed from cultures after the cells had settled. Each reaction mixture contained 10 μl of supernatant and 50 μl of RT cocktail [60 mM Tris-HCl (pH 7.8), 75 mM KCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM EDTA, 5 μg of poly(rA) per ml, 0.16 μg of oligo(dT) per ml] and was incubated at 37°C for 1 h. The radioactive products generated in the CAT and RT assays were quantitated by using a Fuji phosphoimager. The results obtained were comparable to those derived by scintillation counting.

HIV infections. The wild-type and mutant HIV constructs used in infectivity studies were obtained from the supernatants of transfected HeLa cells and were frozen in 1-ml aliquots. The infections were initiated by incubating 2×10^6 to 5×10^6 cells and similar amounts (as determined by RT activity) of different virus preparations (multiplicity of infection of approximately 0.002) in 2 ml of medium at 37°C for 3 h with occasional shaking. The cells were then washed, resuspended in fresh medium, and split at a 1:3 ratio every 3 to 4 days. In the infections utilizing human PBLs, the cultures were supplemented with fresh cells every 10 days. Virus replication was monitored by RT assay of the supernatant medium.

RT-PCR and DNA sequencing of the reconstructed HIV LTRs. Cell-free particles, present in the supernatants of cells infected with HIV containing reconstructed LTRs, were harvested (100 μl), centrifuged at top speed for 5 min in a microcentrifuge at room temperature, and filtered through a 0.45-μm-pore-size Eppendorf spin filter. The HIV present in the filtrate was dissociated by vortexing in the presence of an equal volume of 8 M LiCl, placed on dry ice for 20 min, transferred to a −20°C freezer for at least 2 h, and centrifuged at top speed in a microcentrifuge at 4°C for 20 min. The RNA pellet was then rinsed with 70% ethanol, dried briefly under vacuum, resuspended in water, and reverse transcribed by using the 3′ LTR primer (primer 14, nt 9611 to 9585) and the RiboClone cDNA synthesis system (Promega) for the synthesis of the first DNA strand. A control reaction excluding the RT was performed in parallel. The cDNA was amplified by PCR, using polymerase and reagents obtained from Perkin-Elmer Cetus; 5′ and 3′ LTR primers (primers 15 and 14, respectively; 0.1 mM each) were added to a reaction mixture containing the cDNA (1/20 of the RT product) and amplified for 30 cycles under the following conditions: 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min. The product obtained was then subjected to asymmetric PCR amplification (i.e., two primers at a 10:1 molar ratio) to generate single-stranded DNA for sequencing as described by Meltzer et al. (39). Excess primers were removed with a Centricon 100 filtration device (Amicon) after each amplification step. Nucleotide sequencing was performed by using Sequenase and protocols supplied by U.S. Biochemical Corp. Sequences of primers used for amplication or sequencing are shown in Table 1.

RESULTS

Insertion of heterologous enhancer elements into the HIV-1 LTR. Previously published studies have shown that mutagenesis of the two NF-κB sites plus the three Sp1 sites renders HIV-1 LTR-directed CAT expression unresponsive to Tat (4, 37, 44) and renders the full-length proviral DNA replication incompetent (37). A 91-bp segment, which included the two NF-κB and three Sp1 sites, was deleted from the U3 region of an HIV-1 LTR-CAT construct, using M13 mutagenesis, and replaced with an *Xba*I cleavage site to permit the subsequent introduction of heterologous enhancer elements approximately 90 nt upstream of the transcriptional start site (Fig. 1). The 5′ border of the deletion in this mutated HIV LTR (designated dl.κB/Sp1) is actually located 24 bp upstream of the 5′-most NF-κB element, extending into a region of the LTR which shares nucleotide sequence homology with a regulatory motif associated with the T-cell receptor alpha gene (23).

Because a goal of these studies was to insert heterologous promoter/enhancer sequences into similarly deleted LTRs associated with full-length HIV proviruses, care was taken to select functional heterologous enhancer elements that would minimally affect LTR size or structure or would be unlikely to perturb the subsequent packaging of the altered viral genome into progeny particles. The heterologous enhancer elements shown in Fig. 1, ranging in size from 111 to 326 bp, were synthesized by PCR as described in Materials and Methods. Each element terminated 10 bp upstream of its associated TATA box except for the Py enhancer (7), which is not situated adjacent to a TATA element. The two CMV-IE elements, designated CMV-IE(a) and CMV-IE(b), overlap one another at their 3′ termini (Fig. 1); each has been

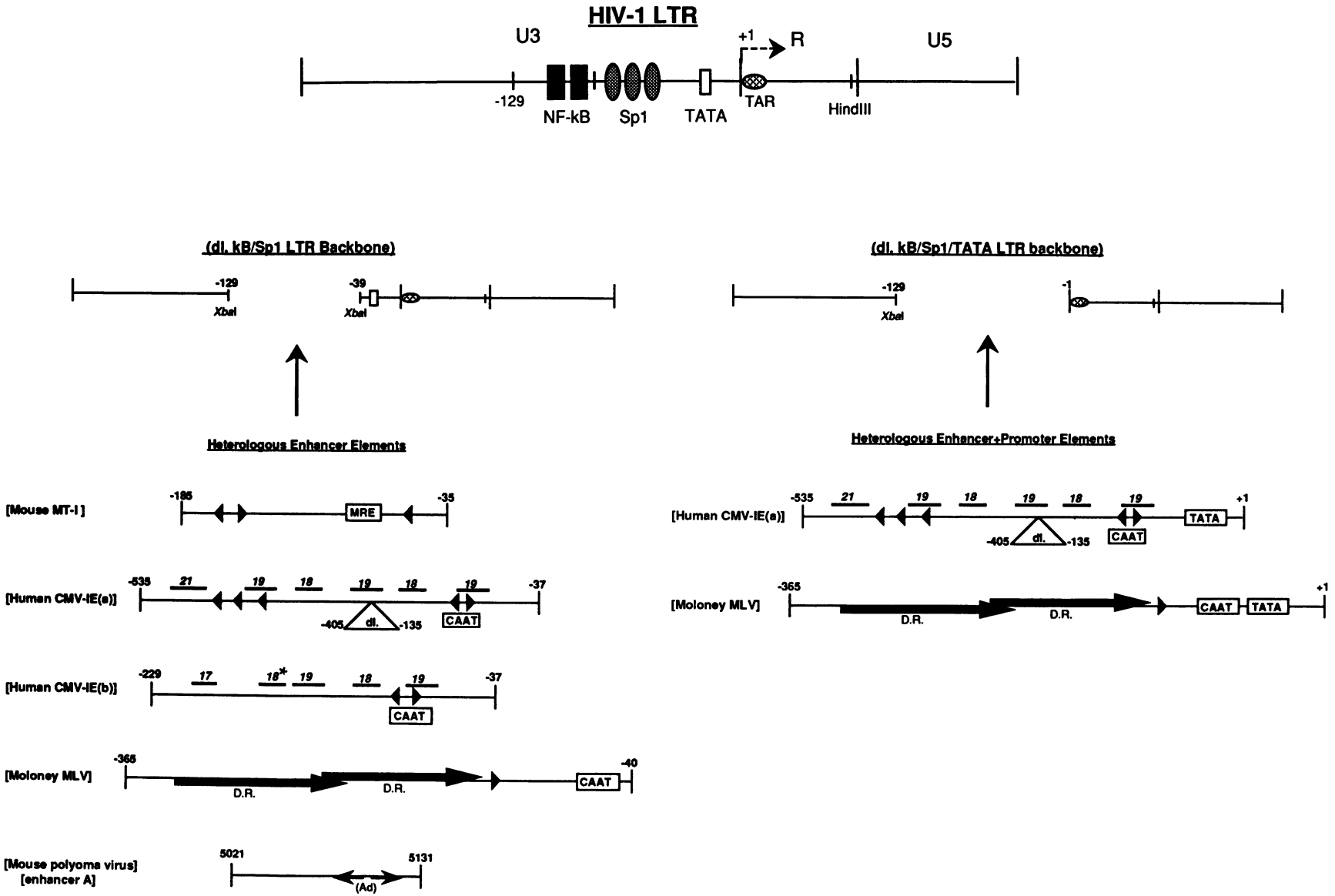

FIG. 1. Schematic representation of the heterologous enhancer/promoters inserted into a deleted HIV-1 LTR. The map positions of the deleted HIV-1 NF-κB, Sp1, and TATA elements as well as the heterologous enhancer/promoter inserts are indicated; +1 designates the transcriptional start site. The presumptive binding sites for the transcription factors are boxed or indicated by arrows, and the bidirectional arrowheads designate putative GC boxes. MRE, metal-responsive element; D.R., direct repeat; Ad, adenovirus enhancer elements (an inverted pair); 18, 19, and 21, repeat sequences of CMV enhancer elements (6, 28, 35); dl., deletion. The sources of the enhancer/promoter elements and the details of their construction are described in Materials and Methods and in Results.

TABLE 2. Relative CAT activities of HIV U3-R-CAT constructs in HeLa and AA2 cells

LTR-CAT construct	Enhancer/promoter insert	CAT activity[a]				Transactivation (fold)	
		−Tat		+Tat			
		HeLa	AA2	HeLa	AA2	HeLa	AA2
U3-R-CAT (wild type)		1.0	0.6	485.0	25.0	485	42
dl.κB/Sp1	None	—[b]	—	—	0.4	—	—
	CMV-IE(a)	182.0	2.0	995.5	19.5	6	10
	CVM-IE(a) (inverted)	17.0	ND[c]	233.0	ND	14	—
	CMV-IE(b)	13.5	2.0	281.0	27.5	21	14
	MT-I	0.9	—	2.0	0.5	2	—
	MLV	0.6	—	38.0	0.7	63	—
	Py	0.7	—	62.5	3.0	89	—
dl.κB/Sp1/TATA	CMV-IE(a)/TATA	88.0	2.0	372.0	15.5	4	8
	MLV/TATA	0.4	0.3	67.5	1.0	168	3

[a] Relative to the CAT activity of uninduced U3-R-CAT in HeLa cells, arbitrarily assigned a value of 1.0. The CAT activities represent reproducible mean values from at least three independent experiments and were normalized to the expression of a growth hormone plasmid construct as described in Materials and Methods.
[b] —, undetectable.
[c] ND, not determined.

shown to be as active as the full-length CMV-IE enhancer (~500 bp) in human epithelial cell lines and activated T cells (6, 28). In all cases, the five heterologous enhancer sequences were inserted into dl.κB/Sp1 LTR 10 bp upstream of the HIV TATA element.

Another mutated LTR (dl.κB/Sp1/TATA; Fig. 1) was constructed to evaluate the activity of a heterologous enhancer plus its associated TATA motif, functioning in the context of the HIV LTR. This recipient dl.κB/Sp1/TATA LTR contained a 129-bp deletion encompassing the NF-κB and Sp1 elements, the HIV-1 TATA box, and downstream sequences extending to the transcriptional start site. As shown in Fig. 1, the 266-bp CMV-IE(a)/TATA and 367-bp MLV enhancer/TATA segments were inserted into dl.κB/Sp1/TATA so that transcription would be initiated near the authentic HIV-1 start site. The structures of all seven reconstructed HIV LTRs were confirmed by nucleotide sequencing.

Tat responsiveness of reconstructed HIV LTRs containing heterologous enhancer elements. As a first step in assessing whether the heterologous enhancers or heterologous E+P elements would restore function, CAT expression, directed by wild-type and reconstructed LTRs, was monitored 60 h following transfection of HeLa or AA2 cells (a $CD4^+$ human B-lymphocyte cell line [8]). Tat transactivated the wild-type HIV LTR approximately 500- and 40-fold in HeLa and AA2 cells, respectively (Table 2); no Tat transactivation of the enhancerless dl.κB/Sp1 LTR vector was detected in either cell type. As shown in Table 2, both of the CMV enhancer elements substantially increased the basal levels of CAT expression directed by the dl.κB/Sp1 LTR in HeLa cells. Although only a sixfold Tat response was observed with the construct containing the CMV-IE(a) enhancer, the final level of CAT activity was nearly twice (995 versus 485) that directed by the wild-type LTR in the presence of Tat. Interestingly, approximately 50- and 100-fold Tat transactivation was observed in HeLa cells when the MLV or the Py enhancer, respectively, was present in dl.κB/Sp1. In AA2 cells, Tat transactivation of the HIV LTR containing heterologous enhancers was marginal at best and was observed only with the two CMV enhancer replacements. The HIV LTR with a replacement metallothionein enhancer failed to respond to Tat in both cell types.

Heterologous E+P elements were also introduced into the HIV LTR to ascertain whether the TATA box, in addition to the Sp1 and NF-κB elements, could be replaced without abolishing responsiveness to Tat. The insertion of the CMV-IE(a) E+P module significantly increased the basal level of CAT; although its response to Tat was only fourfold, CAT expression in the presence of Tat was nearly 80% that of the wild-type LTR (Table 2). In contrast, the HIV LTR containing a replacement MLV E+P module directed low basal levels of expression which could be transactivated approximately 170-fold by Tat in HeLa cells. The latter construct, however, had little if any activity in the human B-cell line AA2.

Because of the relatively long half-life of CAT protein, the measurement of CAT activity may not accurately reflect HIV LTR-directed RNA production (47), particularly in experiments such as these in which heterologous enhancer/promoter elements are being evaluated. Consequently, Northern (RNA) blot hybridization (with subsequent quantitation by using a phosphoimager) was used to monitor the synthesis of CAT RNA in HeLa cells directed by the wild-type HIV LTR or the seven reconstructed LTRs containing replacement enhancer or E+P modules. As shown in Table 3, several of the constructs expressed substantial levels of RNA in the presence of Tat. In the case of the dl.κB/Sp1 LTR containing the CMV-IE(a) enhancer, the basal level of CAT RNA production was 12-fold higher than the wild-type levels. In contrast, insertion of the CMV-IE(b) enhancer led to only a marginal elevation of basal RNA synthesis and an additional 17-fold increase in the presence of Tat. The transcriptional start sites of RNAs expressed from the LTR chimeric constructs containing the CMV enhancer were shown to be located at the authentic initiation site by an RNase mapping experiment (data not shown). The LTR containing the CMV-IE(a) E+P module initiated transcription at position −1 (also a G residue); the LTR containing the MLV E+P module initiated transcription at position −2 (also a G residue; data not shown).

Replicative properties of HIV proviral DNAs with reconstructed LTRs containing heterologous enhancers or E+P elements. A more physiologically relevant assessment of the reconstructed HIV LTRs would be an examination of their functional properties as regulatory components of cloned proviral DNAs. This was evaluated by first transferring the equivalent of the dl.κB/Sp1 or the dl.κB/Sp1/TATA LTR

TABLE 3. Relative RNA expression of HIV U3-R-CAT constructs in HeLa cells

LTR-CAT construct	Enhancer/promoter insert	RNA level[a]		Trans-activation (fold)
		−Tat	+Tat	
U3-R-CAT (wild type)		1	34	34
dl.κB/Sp1	None	—[b]	—	ND[c]
	CMV-IE(a)	12	61	5
	CMV-IE(a) (inverted)	ND	ND	ND
	CMV-IE(b)	3	50	17
	MT-I	1	1	1
	MLV	2	8	4
	Py	3	21	7
dl.κB/Sp1/TATA	CMV-IE(a)/TATA	3	26	9
	MLV/TATA	1	3	3

[a] Measured from radioactive signals on RNA blots by using a phosphoimager and normalized by quantitating actin RNA; values are relative to the activity of uninduced U3-R-CAT, which is arbitrarily assigned a value of 1.
[b] —, undetectable.
[c] ND, not determined.

deletion into the full-length HIV-1 infectious molecular clone pNL43 as described in Materials and Methods and then introducing the heterologous enhancer or E+P elements into the 5′- and 3′-deleted LTRs of a full-length proviral DNA clone. The expression of such HIV proviruses was then monitored by the production of poly(A)$^+$ viral RNA and RT activity in transfected HeLa cells. This assay measures only one aspect of HIV infection cycle: the synthesis of viral RNA and progeny virions. As shown in Fig. 2 and Table 4, the two CMV-IE enhancer elements restored replication competence to the HIV provirus in which the NF-κB and

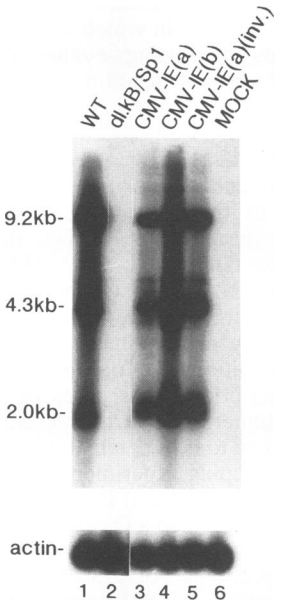

FIG. 2. HIV RNA synthesis in transfected HeLa cells. Proviral DNA and the human growth hormone plasmid (pXGH5) were transfected into HeLa cells as described in Materials and Methods. Cytoplasmic poly(A)$^+$ RNAs were purified from the equivalent of one half of a T25 flask and hybridized to a full-length HIV probe. The amounts of endogenous actin RNA present in each lane were used for sample normalization. WT, wild type; inv., inverted.

TABLE 4. Relative levels of poly(A)$^+$ viral RNA and RT activity of HIV-1 proviral mutants expressed in HeLa cells

HIV construct	Poly(A)$^+$ RNA[a] (%)	RT[b] (%)
Wild type	100	100
dl.κB/Sp1	2	0.003
CMV-IE(a)	50	12
CMV-IE(a) (inverted)	60	13
CMV-IE(b)	110	35
MLV	2	0.003
Py	2	0.003
CMV-IE(a)/TATA	4	0.6
MLV/TATA	2	0.3

[a] Measured from radioactivity on RNA blots by using a phosphoimager. Values are relative to the wild-type RNA level, which is arbitrarily given a value of 100%.
[b] Determined at the time when samples for poly(A)$^+$ RNA analysis were harvested; assayed by using 10 μl of transfected HeLa cell supernatant.

Sp1 elements had been deleted. In these experiments, the quantitation of viral RNA synthesis (Table 4) has been normalized both for transfection (by human growth hormone cotransfection) and for the number of cells harvested (by probing for endogenous actin RNA [Fig. 2]). Note that the provirus containing MLV and Py enhancer sequences failed to generate detectable HIV RNA following transfection, whereas cloned proviral DNA carrying the CMV-IE(a) enhancer in the inverted orientation directed the synthesis of substantial amounts of viral RNA. The proviral DNA constructs harboring the CMV-IE(a) and MLV E+P modules (in place of the HIV enhancer plus the HIV TATA motifs) both failed to express significant levels of viral RNA despite the robust levels of CAT expression directed by an identically reconstructed LTR (Table 2). As expected, the sizes of the three major species of viral RNA directed by LTRs containing the CMV-IE enhancers were each larger than those present in cells transfected by the wild-type pNL43 cloned provirus (Fig. 2), reflecting the presence of an enlarged U3 region (and confirmed by sequencing; see below).

The HIV proviral DNAs containing the three different CMV enhancer elements (but with the HIV TATA box still present) all released RT activity into the supernatant medium following their transfection into HeLa cells (Table 4). In all three cases, however, the production of progeny virions was lower than with the wild-type proviral DNA clone.

Infectivity of HIV stocks containing heterologous enhancers in human T cells. Although the CMV-IE(a) (in both orientations) and CMV-IE(b) enhancer elements restored HIV replicative capacity in HeLa cells, this type of assay (transient transfection) monitors only a single cycle of virus growth and not a spreading infection of CD4$^+$ cells. To determine whether HIV containing heterologous enhancers and E+P elements could productively infect CD4$^+$ T cells, activated human PBLs were incubated with the cell-free virus present in the supernatants of transfected HeLa cells. In each instance, equivalent amounts of HIV, as determined from RT activity, were used as inocula. As shown in Fig. 3A, virus containing the substituted CMV-IE(a) and CMV-IE(b) enhancer elements readily established a spreading infection in cultures of human PBLs. Infectivity was also observed, albeit with delayed kinetics, with virus in which the CMV-IE(a) enhancer was present in an inverted orientation.

Since HIV mutants containing mutated LTRs have previously been reported to exhibit variable replicative capacities

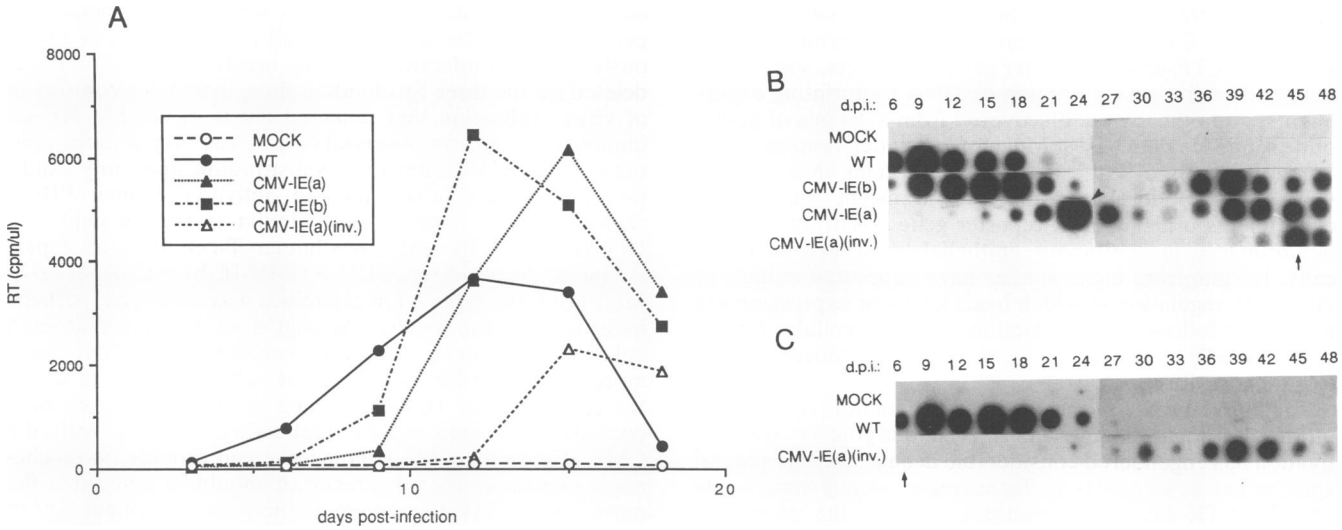

FIG. 3. Infectivity of HIV containing heterologous enhancer/promoter elements in PBLs and H9 cells. (A) The viruses containing the indicated promoter/enhancers were harvested from the supernatants of transfected HeLa cells (following normalization for RT activity [6×10^4 cpm]) and were adsorbed to activated PBLs (3×10^6) at 37°C for 3 h. Supernatant medium was collected every 3 days, and fresh PBLs (3×10^6) were added at 7- to 10-day intervals. (B) H9 cells were infected with HIV containing CMV-IE enhancer/promoters as described for panel A for activated PBLs. The arrowhead indicates the viral progeny that was sequenced. The arrow depicts the time point when the CMV-IE (a) inverted (inv.) virus stock was harvested and used as the inoculum for a second passage in H9 cells, shown in panel C. The wild-type (WT) virus used in panel C was derived from the infection of H9 cells shown in panel B. d.p.i., days postinfection.

depending on the human T-cell line examined (37, 49), the infectivity of HIV harboring heterologous enhancer elements was also evaluated in several different human leukemia cell lines. Figure 3B shows virus production in H9 cells following infection with three HIV LTR mutants containing replacement CMV enhancer motifs. The HIV stock with the CMV-IE(b) enhancer exhibited infection kinetics very similar to that of wild-type virus in H9 cells, while the replication of virus containing the substituted CMV-IE(a) enhancer was delayed by approximately 10 days. HIV containing the replacement CMV-IE(a) enhancer in the inverted orientation was also infectious in H9 cells, although progeny virus production was markedly delayed (bottom row in Fig. 3B).

As a consequence of the error-prone properties of the HIV RT, nucleotide substitutions appear with great frequency during passage of virus both in vitro and in vivo (3, 24). We have, in fact, previously reported that the appearance of virus at very late times (>3 weeks) following infection with HIV mutants frequently heralds the emergence of second-site revertants (49). Therefore, to ascertain whether the reconstructed LTR had changed during some of the prolonged replication cycles, viral RNA was purified from the particles released from H9 cells (day 24; arrowhead in Fig. 3B) that had been previously infected with HIV containing the CMV-IE(a) enhancer and was analyzed by RT-PCR and subsequent nucleotide sequencing. No changes were detected in the sequence of the −204 to −39 region of the reconstructed U3 LTR. The replication of HIV containing the inverted CMV-IE(a) enhancer continued to be markedly retarded (bottom row in Fig. 3C) during a second cycle of infection of H9 cells when the day 45 virus from the initial infection (indicated by the arrow in Fig. 3B) was used as the inoculum. The results presented in Table 5 indicate that of the human lymphocyte cell lines tested, AA2 cells were the most susceptible to infection by HIVs containing reconstructed enhancer or E+P modules. Even virus containing an inverted CMV-IE(a) or an MLV enhancer element was able to replicate in these cells. Most interestingly, HIV containing the entire CMV-IE(a) E+P module in place of HIV sequences located between −129 and −1 (which includes the HIV NF-κB, Sp1, and TATA elements) produced progeny virions in AA2 cells. Molt 3, HL60, and U937 cells were resistant to infection by all of the LTR-mutant HIVs constructed for this study.

DISCUSSION

RNA synthesis in eukaryotic cells is modulated by transcription activator proteins which bind to DNA motifs located upstream of their respective promoters. Like other eukaryotic promoters, the HIV LTR contains its own en-

TABLE 5. Time required for peak RT production in continuous human lymphoid cell lines by HIVs containing heterologous enhancer/promoter elements

Construct	Time (days) needed for peak RT production[a]		
	H9	CEM (12D7)	AA2
Wild-type HIV-1	9, 9	9, 9	6, 6
Enhancer mutants			
dl.κB/Sp1	—	—	—
CMV-IE(a)	17, 17	33, 34	6, 6
CMV-IE(a) (inverted)	39, 42	—	3, 3
CMV-IE(b)	9, 9	17, 17	6, 6
MLV	ND	21, 24	14, 17
Py	ND	ND	—
Enhancer/promoter mutants			
dl.κB/Sp1/TATA	ND	ND	ND
CMV-IE(a)/TATA	—	ND	10, 10
MLV/TATA	ND	—	—

[a] Results from two experiments are given. —, RT activity not detected during 2 months of infection; ND, not determined.

semble of DNA elements to which transcriptional regulatory factors bind. Some of these were identified from mutagenesis studies of LTR-driven reporter gene constructs, some were found following gel retardation or DNA footprinting experiments, and others were discovered from searches of nucleotide sequence data bases. Much of our understanding of the functional roles of viral and cellular proteins that regulate HIV gene activity has come from transient transfection experiments utilizing LTR-reporter gene constructs carried out primarily in continuous epithelial lines such as HeLa cells. Results from these studies have supported models of viral gene regulation in which basal levels of expression are modulated following the induction of certain cellular factors (e.g., NF-κB) or the synthesis of the HIV-encoded proteins (e.g., Tat or Rev).

Tat is required for the efficient production of progeny virions. Although the mechanism(s) underlying Tat transactivation has engendered considerable debate, there is general agreement on two points: (i) Tat increases steady-state levels of HIV LTR-directed transcripts, and (ii) the promoter-proximal positioning of Tat (via its interaction with TAR) is required for transcriptional activation. The latter requirement suggests but does not prove that the function of TAR may be only to deliver Tat to the initiation complex during its assembly, where it might interact with transcriptional activators. Several studies have, in fact, demonstrated Tat transactivation in the absence of TAR in systems in which Tat was targeted to the promoter as a chimeric protein containing known binding sites for RNA (52, 57) or DNA (4, 32, 56). It has also been shown that an HIV LTR, lacking upstream enhancer elements, is not responsive to Tat (4, 37) and fails to direct progeny virion production when present as a component of full-length proviral DNA (49). Restoration of homologous (NF-κB or Sp1 [37]) or heterologous (Ap1, Oct 1, ATF, or USF [4, 56]) elements to the LTR resulted in Tat-responsive reporter gene expression; the introduction of an Sp1 or NF-κB binding motif into an enhancerless HIV-1 LTR led to the synthesis of virus particles (49).

In this work, we have shown that replacement enhancer elements from CMV, MLV, or Py can direct CAT expression from an HIV LTR lacking the two NF-κB and three Sp1 elements. In some assays (CMV enhancers), the final augmented response to Tat was equal to or exceeded that of the wild-type LTR, while in others, the level of Tat transactivation was reduced (MLV and Py enhancers) or undetectable (MT-1). This result was not unexpected in view of the previously reported effects of heterologous enhancers on HIV LTR-directed reporter gene expression (44). What was unanticipated, however, was the restoration of replication competence to noninfectious stocks of HIV by heterologous enhancers. As noted above, we had previously reported that a cloned HIV provirus containing no enhancers failed to generate virus progeny and that the inclusion of a single NF-κB or Sp1 element was required for particle production in transient transfection assays (49). A sequence analysis of the heterologous enhancer motifs used in this study indicated that the MLV and Py enhancer replacements contained no sequences with homology to either the NF-κB or Sp1 element. However, the CMV-IE(a) and CMV-IE(b) enhancers each contain copies of an 18-bp repeat (Fig. 1) capable of binding NF-κB in phytohemagglutinin-phorbol myristate acetate-stimulated Jurkat cells (50). Similarly, it has recently been demonstrated that the 19-bp repeat of the CMV-IE enhancer binds Sp1; however, mutagenesis of this binding site failed to affect the promoter function (35).

The biological activity of HIVs containing heterologous enhancer elements varied greatly among the various host cells used for infection. This result is reminiscent of previously reported infectivity studies involving HIV-1 mutants deleted for the three Sp1 binding sites, in which a continuum of virus replication that reflected levels of cellular NF-κB binding activity was observed (49). Virus preparations containing the CMV-IE enhancer substitutions uniformly exhibited the highest infectivities in activated human PBLs, replicating with kinetics quite similar to that of wild-type HIV (Fig. 3A). In continuous human T-cell lines, an apparent hierarchy [wild-type HIV > CMV-IE(b) > CMV-IE(a) > MLV] of virus replication capacities was observed, perhaps indicative of the relative strengths of the reconstructed enhancer/promoters. This difference in virus infectivity was more pronounced in H9 and CEM cells (Table 5), in which the replication of HIV containing the CMV-IE(a) element consistently lagged behind replication of virus with the CMV-IE(b) enhancer. Although we can provide no mechanistic explanation for this result, it should be noted that the more active CMV-IE(b) element includes an element (18* in Fig. 1) with Ap1 and NF-κB binding sites [missing from CMV-IE(a)] and lacks the 21-bp motif present in CMV-IE(a).

The infectivity of HIV containing a substituted MLV enhancer could be demonstrated only in AA2 cells. These cells were originally derived from the $CD4^+$ B-cell line WIL-2 and have been reported to be exquisitely sensitive to HIV-1 infection (8). Although not commonly used for infectivity studies, AA2 cells generate up to 100-fold more virus than do CEM cells and undergo extremely rapid cytopathic effect. As shown in Table 5, AA2 cells were also permissive for HIV containing the inverted CMV-IE(a) enhancer as well as for virus with the CMV enhancer plus the CMV TATA elements.

These results are consistent with previously reported studies that have evaluated the role of upstream regulatory elements on retroviral tropism, infectivity, and cytopathicity. For example, it is now recognized from work involving Friend and Moloney MLVs that *cis*-acting sequences, located in the U3 region of their respective LTRs, are the major determinants of whether these retroviruses induce an erythroleukemia or a lymphocytic leukemia/lymphoma (10). In other experiments, it has been reported that the replacement of the Moloney MLV enhancer with a Py regulatory element results in a nonpathogenic virus (14). Among the human retroviruses, it has been shown that sequences located upstream of the transcriptional start site in human T-cell leukemia virus type II (HTLV-II) limit expression to human T and B cells; no HTLV-II-directed RNA was detected in several human epithelial and fibroblast cell lines (11). On the other hand, functional studies evaluating HIV-1 LTR-directed gene activity thus far indicate little if any cell type or species restriction imposed by elements located in U3. This very likely reflects the fact that experiments evaluating HIV LTR function have all been conducted in tissue culture systems. The physiological role(s) of the multiple regulatory motifs as well as the possibility that HIV LTR-directed expression may be restricted to or modulated in certain subsets of the lymphocyte/monocyte lineage remain to be elucidated.

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