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Cytomegalovirus Activates Transcription Directed by the Long Terminal Repeat of Human Immunodeficiency Virus Type 1

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Proteins encoded by a variety of DNA viruses activate gene expression from the promoter within the long terminal repeat (LTR) of the human immunodeficiency virus type 1 (HIV-1). The mechanism by which immediate-early (IE) gene products of human cytomegalovirus (CMV) activate expression from the HIV-1 LTR was examined in transient expression assays in cultures of human cells by using plasmids containing the LTR linked to the bacterial chloramphenicol acetyltransferase (CAT) gene and a plasmid expressing the CMV IE gene. Analysis of clustered site mutations within the HIV-1 LTR revealed that sequences from nucleotides −6 to +20 (relative to the start site of transcription) are critical for responsiveness to transactivation by CMV IE gene products. This region partially overlaps the *trans*-acting response element (+19 to +42) required for function of the HIV-1 transactivator. The CMV IE gene was shown to increase the steady-state levels of both prematurely terminated and full-length transcripts initiated within the LTR. These results support a model in which CMV IE gene products act through a specific regulatory element in the HIV-1 LTR to increase viral transcription.

Viruses represent useful systems for investigating molecular mechanisms regulating gene expression in eucaryotic cells. Numerous viruses encode *trans*-acting factors that regulate expression from homologous and heterologous promoters. Gene expression directed by the long terminal repeat (LTR) of human immunodeficiency virus type 1 (HIV-1), the etiologic agent of acquired immunodeficiency syndrome (AIDS), is activated by regulatory genes of several DNA viruses, including herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) (18, 34, 38, 41, 46), adenovirus (29, 30, 37, 38, 46, 48), cytomegalovirus (CMV) (7, 34, 46), hepadnaviruses (53, 57), pseudorabies virus (PRV) (46, 61), Epstein-Barr virus (28), human herpesvirus 6 (11), and papovavirus (46). Molecular mechanisms by which transactivation of the HIV-1 LTR is mediated, either by regulatory gene products of heterologous viruses or its transactivator (TAT), are not resolved. Transactivation of the HIV-1 LTR by TAT is essential for viral replication (8, 14) and requires the *trans*-acting response element (TAR) located in the LTR between positions +19 and +42 (relative to the start site of transcription at +1) (32, 51). TAT may function by a bimodal mechanism since transcriptional and posttranscriptional effects of TAT have been observed (3, 6, 42, 49, 50). TAT plays a role in the elongation of nascent transcripts (27, 51) and requires an RNA secondary structure within TAR to function (1, 13, 16, 20, 23, 51). Transactivation of the HIV-1 LTR by HSV-1 and human herpesvirus 6 involves nuclear factors that bind to the upstream promoter sequences, i.e., the NF-κB- and Sp1-binding sites (11, 19). The immediate-early (IE) gene of PRV acts through a region of the HIV-1 LTR that includes Sp1 sites and the TATA box (61). Transactivation of the LTR by the E1A/E1B gene of adenovirus requires Sp1 sites and the TATA box as well as some TAR sequences (2, 29) and leads to increased rates of initiation and elongation of viral transcription (30). For other heterologous transactivators, specific regulatory sequences or factors have not been identified (7, 28, 34, 35, 46, 53, 57). Studies of HIV-1 LTR transactivation by heterologous viruses are significant since many HIV-1-seropositive individuals are infected by other viruses that may play a role in AIDS disease progression (10, 39, 45, 58). In particular, the observation that CMV and HIV-1 infect the same cells (39) suggests that activation of the HIV-1 LTR by CMV IE gene products may be relevant to HIV-1 replication and pathogenesis.

This report examines the mechanism by which the IE gene of CMV stimulates expression from the HIV-1 LTR. By using a series of clustered site mutations within the HIV-1 LTR, sequences from nucleotide −6 (relative to the cap site) to +20 were found to be essential for transactivation by CMV IE gene products. Analysis of transcripts initiating in the HIV-1 LTR indicated that CMV IE gene products increase the levels of HIV-1 RNA synthesis.

MATERIALS AND METHODS

Cell lines, transfections, and CAT assays. MRC-5 cells are derived from normal human fetal lung tissue and were obtained from the American Type Culture Collection (Rockville, Md.). Cell cultures were grown according to recommended specifications at subconfluency and routinely monitored for mycoplasma contamination. Cells were passaged 24 h prior to transfection and replated at 3×10^5 viable cells per 35-mm well in six-well plates. The protocol for DEAE-dextran transfection was that of Lopata et al. (31) with modifications. On the day of transfection, cells were refed with 2 ml of minimal essential medium supplemented with 10% Nuserum (Collaborative Research, Waltham, Mass.) (52). Two micrograms of pLTR/CAT plus 3 μg of pSP65, pSVIE, or pSVTAT per well were used for transfection. All plasmids were prepared by alkaline lysis (22) and banded to equilibrium twice in cesium chloride gradients. MRC-5 cells were transfected with 500 μg of DEAE-dextran per ml. The

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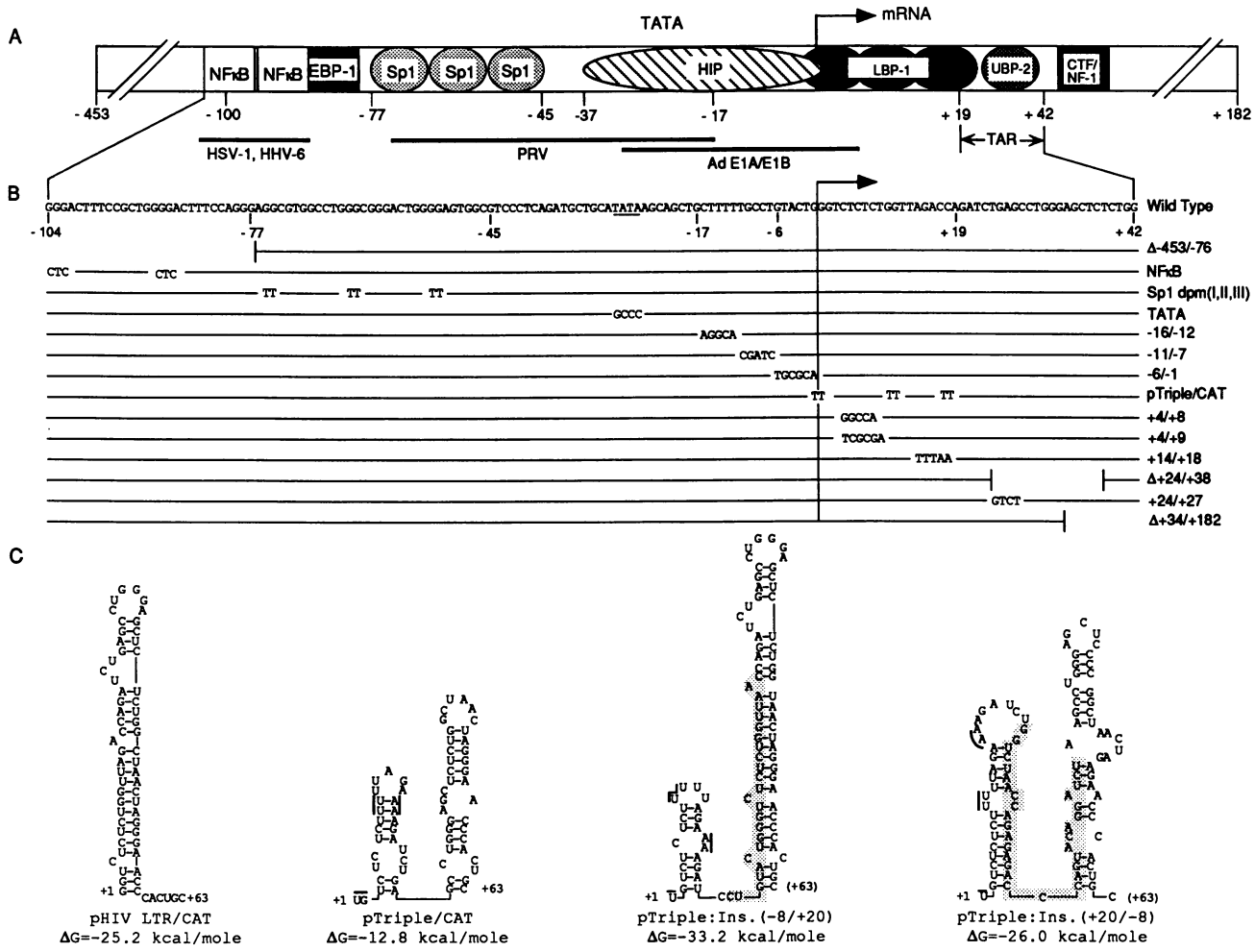

FIG. 1. Regulatory elements and mutations within the HIV-1 LTR. (A) Wild-type HIV-1 LTR (pHIV LTR/CAT) with regulatory elements and cellular proteins demonstrated to bind to the LTR (not to scale). These include NF-κB (37), Sp1 (25), EBP-1 (60), UBP-2 (16), LBP-1, and CTF/NF-1 (26). The protein referred to as HIP (HIV-1 initiator protein) protects the region from −37 to +2 (24). Shown below the LTR (solid lines) are regions involved in transactivation of the HIV-1 LTR by HSV-1 (19), human herpesvirus 6 (HHV-6) (11), PRV (61), and the adenovirus E1A/E1B gene products (Ad E1A/E1B) (2, 29). (B) Sequence of the wild-type LTR from −104 to +42, with the TATA box (underlined) and the start site −76 of transcription (arrow). Also shown are the positions and sequences of the mutations, as well as the endpoints of deletions [Δ(−453/76), Δ(+24/+38), and Δ(+34/+182)], utilized in this study. The mutations are described in the text. (C) Potential RNA structures and free energies of transcripts (9, 15) for pHIV LTR/CAT, pTriple/CAT, pTriple:Ins(−8/+20), and pTriple: Ins(+20/−8). The positions of the mutations that abolish binding of LBP-1 (26) within pTriple/CAT, pTriple:Ins(−8/+20), and pTriple: Ins(+20/−8) are overlined. The wild-type −8 to +20 sequences within pTriple:Ins(−8/+20) and pTriple:Ins(+20/−8) are indicated by shading.

DNA–DEAE-dextran mixture in minimal essential medium-Nuserum was maintained on the cells for 4 h at 37°C, at which time the cells were shocked with 10% dimethyl sulfoxide in phosphate-buffered saline for 2 min. After two washes with phosphate-buffered saline, the cells were refed with regular growth media and maintained for 40 to 48 h until harvested. Under these conditions, the transfection efficiencies were 10 to 20% for MRC-5 cells (data not shown).

Whole-cell extracts were utilized for chloramphenicol acetyltransferase (CAT) enzymatic activity according to published protocols (40), except that lysates were prepared by three freeze-thaw cycles in dry ice and 37°C water. Reactions measuring the conversion of [^{3}H]acetyl coenzyme A ([^{3}H]acetyl-CoA) to [^{3}H]acetyl chloramphenicol were carried out for 18 to 20 h and were shown to be within the linear range of the assay (data not shown).

Plasmid constructions. The wild-type HIV-1 LTR/CAT plasmid (pHIV LTR/CAT) was described previously (43). The HIV-1 TAT gene (43), under the transcriptional control of the simian virus 40 early promoter (pSVTAT), was cloned into pUC12. Mutations +4/+9 and +14/+18 (55); TATA, −16/−12, −11/−7, −6/−1, pTriple/CAT, +4/+8, +24/+27, and +30/+33 (26, 51); Sp1-dpm (I,II,III) (25); NF-κB (37); and Δ(−453/−76) (36) have been described previously. The numbers refer to the starting and ending nucleotides for clustered site mutations, or to the ends of a deletion Δ(−453/−76). The plasmid Δ(+24/+38) contains a deletion between the *Bgl*II site (+24) and the *Sac*I site (+38). The plasmid Δ(+34/+182) contains a deletion between the *Sac*I site (+34) and the 3′ end of the LTR (+182). The location and sequence of each mutation relative to regulatory sites within the LTR are shown in Fig. 1A and B.

The plasmid expressing the major protein products of the human CMV IE genes (pSVIE) contains a *Sac*II-*Sal*I restric-

tion fragment from the *Xba*I E fragment of the Towne strain of CMV under the transcriptional control of the simian virus 40 early promoter (generously provided by Richard Spaete, Chiron Corporation, Emeryville, Calif.). It is derived from plasmid pON303 (54). pSVIE contains the transcription units for CMV IE regions IE1 and IE2 (21).

The plasmids pTriple:Ins(−8/+20) and pTriple:Ins(+20/−8) were constructed by ligating a synthetic oligonucleotide corresponding to the wild-type HIV-1/SF2 sequence from nucleotides −8 to +20 into the *Bgl*II site (+20) of pTriple/CAT. The oligonucleotide is as follows:

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GAT CCT GTA CTG GGT CTC TCT GGT TAG ACC A
     GA CAT GAC CCA GAG AGA CCA ATC TGG TCT AG
      |          |                        |
     -8         +1                      +20
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The orientation of the insert was confirmed by DNA sequencing. RNA sequences and potential RNA secondary structures (9, 15) for pHIV LTR/CAT, pTriple/CAT, pTriple:Ins(−8/+20), and pTriple:Ins(+20/−8) are presented in Fig. 1C.

For RNase protection analysis of HIV-1 transcripts, the plasmid pGEM-2/WT was utilized. pGEM-2/WT is similar to pGEM-1/WT (51), except that the *Sca*I-*Hin*dIII fragment from the HIV-1 LTR (−138 to +82) was cloned into pGEM-2 (provided by Mark Selby, University of California, San Francisco). Antisense RNA probes were generated by using T7 polymerase (Promega Biotec, Madison, Wis.) with *Sal*I-digested pGEM-2/WT. Synthesis and purification of RNA probes were performed by the methods of Melton et al. (33).

RNase protection. Total cellular RNA was isolated by the method of Chomczynski and Sacchi (5) and treated with RNase-free DNase (Promega) in the presence of RNasin (Promega) (26). Total RNA (100 μg) was used for RNase protection with HIV-1 LTR antisense RNA probes. The protocol for RNase protection has been described previously (51), except that the total volume for hybridization was 11 μl.

Transactivation assay for CMV IE gene products and HIV-1 TAT. A plasmid containing the CAT gene under the transcriptional control of the HIV-1/SF2 LTR (pHIV LTR/CAT) (43) was cotransfected into mammalian cell cultures with either a plasmid expressing HIV-1 TAT (pSVTAT) (43) or a plasmid containing the CMV IE region downstream of the simian virus 40 early promoter (pSVIE). MRC-5 cells, which are permissive for CMV, were chosen for these studies. A fixed amount of pHIV LTR/CAT (2 μg) was cotransfected with increasing amounts of either pSVTAT or pSVIE. In addition, fixed amounts of pHIV LTR/CAT (2 μg) and pSVTAT (3 μg) were cotransfected with an increasing amount of pSVIE. For each transfection, CAT enzymatic activity was assayed with whole-cell extracts by the method of Nordeen et al. (40), which measures conversion of the substrate [^{3}H]acetyl-CoA to [^{3}H]acetyl chloramphenicol.

RESULTS

Transactivation of the HIV-1 LTR by TAT and CMV IE gene products. In MRC-5 cell cultures, cotransfection of pHIV LTR/CAT with either pSVTAT or pSVIE produced large increases in the level of CAT activity (Fig. 2). These results were consistent with published data for similar experiments in other cell types (7, 34). Transactivation of the HIV-1 LTR increased linearly with the amount of cotransfected transactivator plasmid and approached a plateau with 3.0 μg of either pSVTAT or pSVIE (Fig. 2). It is significant

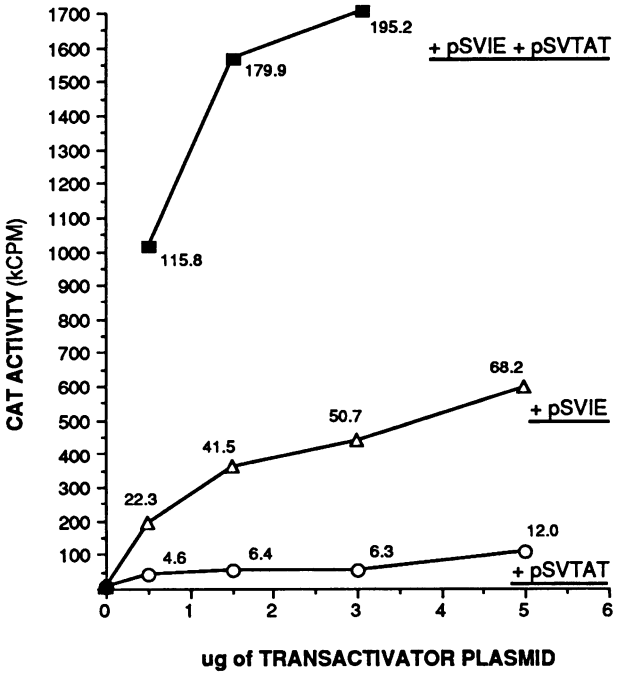

FIG. 2. Transactivation of the HIV-1 LTR by plasmids expressing the IE genes of CMV or HIV-1 TAT. pHIV LTR/CAT (2 μg) was cotransfected into cultures of MRC-5 cells with increasing amounts of pSVIE, expressing CMV IE (△), or with pSVTAT, expressing HIV-1 TAT (○). CAT activity was assayed with whole-cell extracts, as described in the text, and is expressed in kilocounts per minute (kCPM). In addition, 2 μg of pHIV LTR/CAT plus 3 μg of pSVTAT was cotransfected with increasing amounts of pSVIE (■). The numbers next to each datum point represent the relative increase in CAT activity above basal levels for each concentration of transactivator plasmid. Each value represents the average of duplicate transfections; variability between replicate cultures was less than 10%.

that the levels of transactivation of the HIV-1 LTR were five- to ninefold higher with pSVIE than with pSVTAT in MRC-5 cells. The difference between pSVIE and pSVTAT transactivation of the HIV-1 LTR varies by up to 1,000-fold in different cell types (unpublished data). A plasmid derivative of pSVIE, expressing only IE1 of the CMV IE gene (pON308) (4), did not transactivate the HIV-1 LTR (unpublished results).

Cotransfection of pHIV LTR/CAT with both pSVIE and pSVTAT resulted in CAT activities (i.e., transactivation) that were greater than the sum of CAT activities with each transactivator alone; the level of transactivation approached that of the product of both transactivators (Fig. 2). Cotransfection with pSVIE and pSVTAT reached a plateau nearly 200-fold above basal CAT activities (Fig. 2). These data imply that the mechanisms by which pSVIE and pSVTAT transactivate the HIV-1 LTR are different and indicate that the plateau observed with either transactivator alone was not simply due to a saturation of DNA uptake in the transfection procedure.

Sequence at the cap site of the HIV-1 LTR is critical for transactivation by CMV IE gene products. To determine the region of the HIV-1 LTR required for transactivation by pSVIE, phenotypes of clustered site mutations or deletions within the LTR were examined. The wild-type HIV-1 LTR

TABLE 1. Transactivation of plasmids with mutations in the HIV-1 LTR by pSVIE and pSVTAT

Plasmid	CAT activity (cpm)[a]															% Transactivation relative to wild-type LTR[b]	
	Expt 1			Expt 2			Expt 3			Expt 4			Expt 5				
	LTR/CAT	+pSVIE	+pSVTAT	LTR/CAT	+pSVIE	+pSVTAT	LTR/CAT	+pSVIE	+pSVTAT	LTR/CAT	+pSVIE	+pSVTAT	LTR/CAT	+pSVIE	+pSVTAT	pSVIE	pSVTAT
pHIV LTR/CAT	8,496	584,610	48,867	4,534	187,558	40,368	3,376	250,960	39,502	7,766	132,460	31,167	938	185,565	57,758	100	100
Δ−453/−76										5,166	793,280	18,143				950	83
NF-κB				3,540	247,408	37,350										171	121
Sp1-dpm (I,II,III)							3,460	28,048	3,772							10	<1
TATA							3,360	19,061	3,458							6	<1
−16/−12				4,460	221,199	25,096										120	59
−11/−7	9,310	618,850	70,518													97	137
−6/−1	9,384	113,340	61,092													16	115
pTriple/CAT	10,128	78,186	10,828													10	1
+4/+8	7,790	187,575	35,285													34	74
+4/+9	10,324	334,240	47,970													46	76
+14/+18	7,692	284,900	9,747													53	6
Δ+24/+38	10,774	636,180	15,358													86	9
+24/+27				2,314	345,568	3,432										367	6
Δ+34/+182													1,160	170,890	4,598	74	6

[a] Each plasmid containing mutated and wild-type HIV-1 LTR/CAT sequences was cotransfected with either pSVIE or pSVTAT into cultures of MRC-5 cells. CAT activities, in counts per minute (cpm), for the basal levels of LTR/CAT plasmid alone, cotransfection with pSVIE (+pSVIE), and cotransfection with pSVTAT (+pSVTAT) from representative experiments are shown. The mutations and transfection protocols are described in the text. Each value represents the average of two parallel transfections. Variability was less than 10%.

[b] Levels of transactivation of the mutated plasmids by pSVIE and pSVTAT relative to those of the wild-type LTR (arbitrarily set at 100%) are shown.

with known regulatory and protein-binding sites and the mutated HIV-1 LTR/CAT constructions are depicted in Fig. 1A and B. Mutated LTR/CAT constructions were transfected into MRC-5 cell cultures, and the responsiveness of each to pSVTAT and pSVIE was determined by measuring CAT activity.

An HIV-1 LTR with mutations either in the three Sp1 sites [Sp1-dpm (I,II,III)] (25) or in the TATA sequence (26) was transactivated by pSVIE to only 10 and 6% of wild-type levels, respectively (Table 1). However, mutations in these same sites also abolished transactivation by pSVTAT (less than 1% of wild-type levels) (Table 1). Mutations in these basic promoter elements probably affect all LTR-directed promoter function, and thus the Sp1 and TATA sequences are not specifically involved in transactivation by pSVIE. Mutations around the start site of transcription, between nucleotides −6 and +20 in the HIV-1 LTR, define the boundaries of the region responsive to pSVIE. While the TATA box mutation abolished transactivation by both pSVTAT and pSVIE, the mutations −16/−12 and −11/−7 did not diminish transactivation by pSVIE (120 and 97% of pHIV LTR/CAT levels, respectively) (Table 1). However, the mutation −6/−1 retained only 16% of wild-type levels of transactivation by pSVIE, whereas it remained fully responsive to transactivation by pSVTAT (Table 1). Thus, mutation −6/−1 discriminated between basal promoter elements (such as the Sp1 sites or the TATA sequence) and elements specific for transactivation by pSVIE. pTriple/CAT contains three dinucleotide mutations and was designed to abolish binding of a cellular factor designated leader-binding protein 1 (LBP-1) (26). The mutations in pTriple/CAT also impaired potential RNA secondary structure of the nascent transcript compared with RNA from the wild-type LTR (Fig. 1C). pTriple/CAT exhibited a dramatically impaired response to transactivation by pSVIE and pSVTAT (less than 10 and 1% of wild-type levels, respectively) (Table 1). The observation that the mutations within pTriple/CAT abolished transactivation by pSVTAT appears to be in contrast to the results of Malim et al. (32), who observed no loss of transactivation by TAT with a similar, although not identical, mutation. Clustered mutations extending out to the Δ(+24/+38) deletion all exhibited a lower level of transactivation by pSVIE such that mutations more distal from the start site, e.g., Δ(+24/+38) and Δ(+34/+182), had less of an impact on transactivation by pSVIE (Table 1). The +24/+27 mutant was fully responsive to pSVIE; in fact, the level of transactivation by pSVIE was above that observed with the wild-type LTR (Table 1), a consistent phenotype for transfections in different cell types (unpublished results). Clustered site mutations immediately downstream of the start site also diminished transactivation by pSVTAT, although the more severe mutations (with the exception of pTriple/CAT) began downstream of mutation +4/+9. Mutation +24/+27 retained less than 6% of wild-type levels of transactivation by pSVTAT (Table 1). The phenotype of these mutants with respect to transactivation by pSVTAT was in general agreement with studies of other cell types (23, 51).

Interestingly, a deletion mutation beginning at the 5′ end of U3 (−453) and extending to position −76 [Δ(−453/−76)] cotransfected with pSVIE resulted in more than a nine-fold increase in CAT activity over that of the wild-type LTR, whereas it had little effect on transactivation by pSVTAT. In other experiments, the increase in transactivation by pSVIE with the Δ(−453/−76) mutation has ranged from four- to ninefold (unpublished results). Similarly, mutations within both NF-κB-binding sites led to an increased transactivation

by pSVIE relative to the wild-type LTR (171% of wild-type levels; Table 1). This result has been consistently observed in multiple experiments (unpublished results). The NF-κB mutation led to a small increase in transactivation by pSVTAT in MRC-5 cells (Table 1).

Nucleotides around the HIV-1 cap site confer responsiveness to CMV IE gene products. Many promoters are transactivated by CMV IE gene products (44). We determined whether a promoter that was nonresponsive to pSVIE could be made responsive to the CMV transactivator. A synthetic oligonucleotide corresponding to sequences from nucleotides −8 to +20 of the HIV-1 LTR, the target for pSVIE, was cloned in both orientations into the *Bgl*II site at position +20 of pTriple/CAT (see Materials and Methods). The recombinant plasmids, pTriple:Ins(−8/+20) and pTriple:Ins(+20/−8), contained the wild-type sequence from nucleotides −8 to +20 in the pTriple/CAT background in the sense and antisense transcriptional orientations, respectively (Fig. 1C). Also shown in Fig. 1C are the potential RNA secondary structures for these two recombinants (9, 15). RNA for pTriple:Ins(−8/+20) is capable of forming a stem-loop structure identical to the wild-type mRNA, except for the presence of two additional base pairs at the base of the stem. The free energies (ΔG) for the stem-loop structures in the wild-type LTR and in pTriple:Ins(−8/+20) are −25.2 kcal (ca. −105,/437J)/mol and −33.2 kcal (ca. −138,/909J)/mol, respectively. In addition, the location of the stem-loop in pTriple:Ins(−8/+20) is shifted downstream by 29 bases. RNA for pTriple:Ins(+20/−8) is also potentially capable of forming a stable secondary structure (ΔG = 26.0 kcal [ca. −108,784 J]/mol), although the structure exhibits no apparent similarity to the wild-type stem-loop in TAR. In comparison, RNA for pTriple/CAT has a lower potential free energy (ΔG = −12.8 kcal [ca. −55,555 J]/mol; Fig. 1C). These plasmids were transfected into MRC-5 cells, and responsiveness to pSVTAT and pSVIE was assayed as described above.

When the −8 to +20 region of the wild-type LTR was cloned in the sense orientation into pTriple/CAT, the result was an increase in transactivation by pSVTAT but not by pSVIE. pTriple:Ins(−8/+20) was transactivated by pSVTAT at a level 30% that of the wild type, whereas pTriple/CAT was transactivated by pSVTAT to 7% that of the wild type (Table 2). When pTriple:Ins(+20/−8) was transfected into MRC-5 cells, there was a five- to sixfold increase in transactivation by pSVIE to 58% of wild-type activity. This is compared with 11% of wild-type LTR activity for pTriple/CAT. pSVTAT did not transactivate pTriple:Ins(+20/−8) above the level observed with pTriple/CAT. In these constructions, sequences from positions −8 to +20 of the HIV-1 LTR partially restored the responsiveness to transactivation by pSVIE but only when cloned in the antisense orientation within pTriple/CAT. These results reinforce the conclusion reached from Fig. 2 and Table 1 that the mechanisms of transactivation by pSVTAT and pSVIE in MRC-5 cells are different.

Transactivation by CMV IE gene products leads to increases in levels of steady-state HIV-1 RNA. Data presented here demonstrate that transactivation of the HIV-1 LTR by pSVIE increased the level of protein, as measured by CAT activity in extracts of transfected cells. We also examined effects of transactivation by pSVIE on levels of steady-state RNA directed by the LTR. There were no RNase-protected transcripts utilizing total RNA isolated from mock-transfected MRC-5 cells (Fig. 3; lane 1). Transfection of MRC-5 cells with the wild-type LTR alone (lane 2) gave rise to terminated transcripts 54 to 59 nucleotides in length and few of the full-length protected transcripts 80 nucleotides in length (detectable on long-term exposures only). The low number of full-length transcripts was consistent with the low level of CAT enzymatic activity for transfections with pHIV LTR/CAT only (Fig. 3). In agreement with data published previously (27, 51, 56), cotransfection with pSVTAT caused a shift from short to full-length transcripts. When pHIV LTR/CAT was cotransfected with pSVTAT, there was an increased intensity of full-length transcripts and an almost

TABLE 2. Transactivation of pTriple/CAT, pTriple:Ins(−8/+20), pTriple:Ins(+20/−8), and pHIV LTR/CAT by pSVIE and pSVTAT

Plasmid	CAT activity				
	cpm[a]			Relative transactivation[b]	
	LTR/CAT	+ pSVIE	+ pSVTAT	pSVIE	pSVTAT
pHIV LTR/CAT	10,184	372,360	40,371	100	100
pTriple/CAT	8,692	42,436	10,357	11	7
pTriple:Ins(−8/+20)	6,594	33,330	12,661	12	30
pTriple:Ins(+20/−8)	6,606	144,285	6,385	58	<1

[a] pTriple/CAT, pTriple:Ins(−8/+20), pTriple:Ins(+20/−8), and pHIV LTR/CAT were transfected into cultures of MRC-5 cells, and levels of CAT activity in counts per minute (cpm) were measured for LTR/CAT alone, LTR/CAT plus pSVIE (+ pSVIE), and LTR/CAT plus pSVTAT (+ pSVTAT). Each value represents the average of two parallel transfection experiments. Less than 10% variation was observed in these assays.

[b] Levels of transactivation of pTriple/CAT, pTriple:Ins(−8/+20), and pTriple:Ins(+20/−8) by pSVIE and pSVTAT relative to that of the wild-type LTR (arbitrarily set at 100%) are shown.

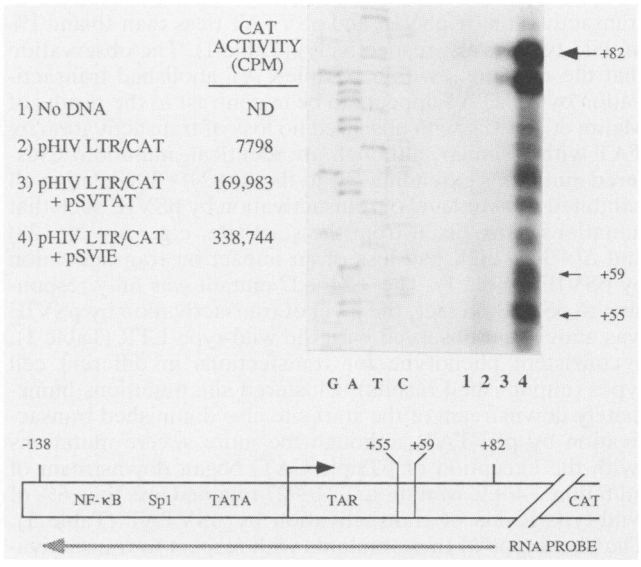

FIG. 3. HIV-1 transcripts in transfected MRC-5 cell cultures. Cultures of MRC-5 cells were transfected with no DNA (lane 1), pHIV LTR/CAT (lane 2), pHIV LTR/CAT plus pSVTAT (lane 3), and pHIV LTR/CAT plus pSVIE (lane 4). Samples of transfected cell extracts were utilized for CAT activity and RNA analysis, as described in the text. CAT enzymatic activity is given in counts per minute (CPM). ND, Not done. RNA analysis by RNase protection is shown at the right, with the 55- and 59- (short arrows) and the 82-nucleotide (long arrow) protected transcripts. DNA sequencing reactions of the HIV-1 LTR were used as size markers (GATC). The antisense RNA probe is shown at the bottom in relation to the HIV-1 LTR.

complete absence of short transcripts (lane 3), compared with transfection with pHIV LTR/CAT alone (lane 2). A second band corresponding to 79 nucleotides also was observed for full-length transcripts (Fig. 3); the sequence composition of the 79-nucleotide transcript remains to be determined. In these transfections, CAT activity was stimulated nearly 20-fold by pSVTAT (Fig. 3). pSVIE increased both short and full-length transcripts. The intensity of full-length transcripts upon cotransfection with pSVIE was increased, compared with that from transfection with pHIV LTR/CAT alone (lanes 2 and 4) or with that from cotransfection with pSVTAT (lane 3) and was consistent with the increase in CAT activity (Fig. 3). In addition, cotransfections with pSVIE led to a large increase in the quantity of short transcripts, in contrast to that observed with pSVTAT (lanes 3 and 4). The predominant short transcript was 55 nucleotides in length, although additional species of short transcripts between 54 and 80 nucleotides in length were also observed. Thus, pSVIE caused an overall increase in the steady-state number of both terminated and elongated transcripts, whereas pSVTAT increased only the amount of elongated species. Similar results were obtained when an RNA probe from −17 to +182 was utilized (unpublished results). These observations further differentiated the effects of TAT and CMV IE gene products on the HIV-1 LTR.

DISCUSSION

IE gene products of human CMV regulate expression from CMV promoters and several heterologous viral and cellular promoters (7, 34, 44, 46). We have demonstrated that CMV IE gene products require sequences of the HIV-1 LTR between positions −6 and +20 to increase gene expression by a mechanism distinct from transactivation by TAT (12, 16, 20, 23, 27, 36, 43, 51). Mutation −6/−1 identified a *cis*-acting regulatory element required for transactivation by CMV IE since this was the only mutation which decreased the level of transactivation by pSVIE, but it did not affect transactivation by pSVTAT (Table 1). Mutations in the Sp1 sites and the TATA sequence severely reduced transactivation by both CMV IE and TAT gene products (Table 1). These *cis*-acting elements are components of the basal promoter and probably affect all LTR-directed promoter function. Mutations within these two promoter domains also diminish transactivation by the adenovirus E1A/E1B gene (29, 38) and the PRV IE gene (61). Mutations which increased transactivation by pSVIE [i.e., NF-κB, Δ(−453/−76), and +24/+27; Table 1] are intriguing but have not been investigated further. Transactivation of these mutations by pSVTAT was unaffected [(NF-κB, Δ(−453/−76)] or severely reduced (+24/+27) relative to the wild-type LTR (Table 1). These observations in MRC-5 cells support the notion that responsiveness of the HIV-1 LTR to CMV IE gene products also may involve negative-acting domains as well as positive elements described herein.

Localization of an element responsive to CMV IE gene products between positions −6 and +20 is consistent with previous reports on transactivation of the HIV-1 LTR by CMV. Davis et al. (7) reported that the region of the LTR between positions +29 and the end of U5 (+182) was not required for transactivation by CMV IE gene products. Mosca et al. (34) reported a lack of specific sequences in the HIV-1 LTR required for mediating transactivation by CMV. All of the deletion mutations in the HIV-1 LTR analyzed in these two reports (7, 34) contained the region described here (nucleotides −6 to +20) and therefore should have been transactivated by CMV IE gene products.

LBP-1 or other factors which remain to be identified may be a critical component in transactivation by CMV IE gene products (26). LBP-1 is implicated since pTriple/CAT is not responsive to CMV IE gene products in MRC-5 cells (Tables 1 and 2) and in HuT 78, HeLa, and murine L929 cells (unpublished results). This issue needs further resolution since both pTriple:Ins(−8/+20) and pTriple:Ins(+20/−8) contain an intact LBP-1-binding site yet only pTriple:Ins(+20/−8) exhibited increased transactivation by CMV IE gene products. Binding of LBP-1 to this portion of the LTR may be a necessary component for gene expression but is not sufficient for transactivation by CMV IE gene products. These results suggest that transactivation by CMV IE gene products may require that the region of the HIV-1 LTR from positions −6 to +20 be in a precise architecture and context with respect to sites that bind other regulatory factors (17, 25, 26, 32, 37, 59, 60).

The mechanism by which CMV IE gene products transactivate the HIV-1 LTR is fundamentally different from transactivation by TAT. The target site for transactivation by CMV IE (nucleotides −6 to +20) gene products was different from the TAR element, essential for transactivation by TAT (positions +18 or +19 to +42 or +44) (12, 16, 20, 23, 27, 36, 43, 51). In addition, the multiplicative effects of cotransfection with pSVIE and pSVTAT (Fig. 1), compared with transfection with either transactivator plasmid alone, demonstrated that the mechanisms by which CMV IE gene products and TAT increase gene expression must be independent. An important feature of this experiment was that the multiplicative effects were observed at conditions where transfection with pSVIE or pSVTAT alone gave near-saturating CAT activities. These results differ from those of Davis et al. (7), who reported that the effects of TAT and CMV IE gene products were additive. Finally, analysis of RNA transcripts directed by the HIV-1 LTR also distinguished transactivation by CMV IE gene products from that by TAT, both qualitatively and quantitatively. In MRC-5 cells, transactivation by pSVIE increased the steady-state quantity of short as well as full-length transcripts (Fig. 3). In contrast, transactivation by TAT reduced the steady-state level of terminated transcripts and increased the quantity of elongated transcripts (27, 51, 56). The observation that TAT augmented elongation of transcripts in MRC-5 cells extends the cell types in which TAT acts at the elongation level to include nontumorigenic human cell strains derived from primary cells. The net phenotype of both transactivators was an increase in CAT activity. These data are consistent with a model in which CMV IE gene products function at or prior to initiation of transcription from the HIV LTR, distinct from the function of TAT. Nuclear run-on experiments are required to measure directly the frequency of transcription initiation regulated by CMV IE gene products. The observation that CMV IE gene products increased the levels of both full-length and short transcripts is similar to the phenotype of transactivation of the HIV-1 LTR by the adenovirus E1A/E1B gene product (30). As with transactivation by CMV IE gene products, the region of the LTR responsive to adenovirus E1A/E1B extends downstream from the cap site (29), leading to the possibility that CMV IE and adenovirus E1A/E1B gene products transactivate the LTR by similar mechanisms.

Implicit in the postulate that CMV IE gene products function at or prior to initiation of transcription in the LTR is the issue of how CMV IE gene products abrogate the block to elongation to generate full-length transcripts. The elongation block in the LTR may not be absolute, in that a certain

percentage of transcription complexes will elongate, even in the absence of TAT, to give a basal level of expression. Any increase in the loading of RNA polymerase II transcription complexes due to CMV IE gene products would result in an increase in the number of full-length transcripts. This could be due to a limitation of factors involved in premature termination, through increased RNA polymerase loading by CMV IE gene products, with the result that the elongation block is less efficient. Alternatively, CMV IE gene products may alter the transcription complex in some fashion to render the complex less sensitive to premature termination. Whereas TAT is very efficient at facilitating elongation in MRC-5 cells (i.e., all transcripts are full-length; Fig. 3, lane 3), the potentiation of expression from the HIV-1 promoter by CMV IE gene products is ultimately dependent on the efficiency of elongation.

Expression of IE genes after infection by CMV involves multiple transcripts and proteins as a result of differential splicing (21). Transactivation of heterologous promoters by CMV IE gene products appears to be a function primarily of the IE2 gene product (7, 21, 44). While the IE1 gene product demonstrates little or no transactivation capabilities by itself with heterologous promoters (7, 21, 44), IE1 appears to function with IE2 to increase transactivation above that observed with IE2 alone (21). The IE1 gene has been demonstrated to stimulate expression from its own promoter through a regulatory element within the enhancer (4). We have not addressed directly the role that the IE2 gene product played in transactivation of the HIV-1 LTR, although expression of the IE1 gene product alone did not transactivate the HIV-1 LTR (unpublished results).

Infection of a nonimmunocompromised individual by CMV, a member of the herpesvirus family, is usually innocuous and asymptomatic (10). However, in AIDS patients, almost all of whom harbor CMV, a large percentage of individuals suffer debilitating or life-threatening complications due to CMV (10, 47). A direct effect of DNA viruses on the pathogenesis of AIDS is implied by the demonstration that the genes coding for the transactivators of many, but not all (7, 11, 18, 28, 34, 35, 37, 38, 41, 46, 48, 53, 57, 61), DNA viruses are capable of stimulating expression from the HIV-1 LTR. Thus, superinfection of cells harboring a latent HIV provirus by a heterologous virus may activate HIV-1 replication. In vivo evidence for coinfection has been demonstrated for human CMV and HIV-1 in cells from the white matter of brains of AIDS patients (39). The results in this report are significant since they describe a molecular mechanism that may occur in cells coinfected with HIV-1 and CMV. Potentially, in vivo the immediate-early gene products of CMV act through a regulatory element at the cap site in the LTR to increase initiation of HIV-1 transcripts.

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