MOLECULAR AND CELLULAR BIOLOGY, June 1988, p. 2555–2561
0270-7306/88/062555-07$02.00/0

Vol. 8, No. 6

A Discrete Element 3′ of Human Immunodeficiency Virus 1 (HIV-1) and HIV-2 mRNA Initiation Sites Mediates Transcriptional Activation by an HIV *trans* Activator

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Received 16 December 1987/Accepted 18 February 1988

An important point of regulation in the reproductive growth and latency of the human and simian immunodeficiency viruses (HIV and SIV, respectively) is provided by virally encoded *trans*-activators (*tat*), proteins capable of dramatically increasing viral gene expression. The mechanism of this autostimulatory pathway has remained unclear, however, with substantial effects having been reported at the level of either mRNA accumulation, translational efficiency, or both. Our previous findings indicated that *trans*-activation results primarily from induction of RNA levels but could not distinguish between the roles of transcriptional rate, RNA stabilization, and RNA transport in this event. In addition, the boundaries of *tat*-responding elements, which would be valuable in elucidating the mode of *tat* action, are not precisely known. In this study, HIV-1 and HIV-2 long terminal repeat-directed expression was characterized by using an in vitro nuclear transcription assay to clarify this mechanism, and a detailed mutational analysis was undertaken to localize precisely the sequences participating in this process. Two key findings were revealed: an increased transcription rate was the primary event in *tat*-mediated activation of HIV-1 and HIV-2, and *trans*-activation was impaired by mutations in two regions, the TATA box and sequences between +19 to +42, a region lacking enhancer activity. These results implicate a discrete 3′ regulatory element in the transcriptional activation of the HIVs.

Human immunodeficiency virus 1 (HIV-1) gene expression is regulated by an autostimulatory pathway involving interaction of the HIV-1 *trans*-activator (*tat*) gene product with sequences contained within the HIV-1 long terminal repeat (LTR) (2, 23, 29). HIV-1 *tat* is a polypeptide of M_r 15,000 (23) encoded by two exons of 72 and 14 amino acids (2, 29), the latter of which appears to be dispensable for activity (23, 29). Similarly, HIV-1-related viruses recently isolated, including HIV-2 and a simian immunodeficiency virus (STLV-III), have been found to encode a homologous protein at an analogous position in their genomes (4, 12, 14) and appear to be *trans*-activated by factors produced by HIV-infected cells (1, 12), although it remains to be definitively established that the corresponding *tat* polypeptides are responsible for the observed stimulation. Elucidation of the mode of *tat* action is crucial to understanding viral lytic replication and its relationship to viral latency.

Despite the large volume of information obtained by several groups on the effects of HIV-1 *tat* on the expression of viral and heterologous genes linked to the HIV-1 LTR, the mechanism of *trans*-activation remains unclear. Although initial studies suggested that *tat* dramatically stimulates LTR-directed protein expression with little or no effect on steady-state mRNA levels, indicating that enhancement of expression occurs at the translational level (8, 27), subsequent work has shown that *trans*-activation is accompanied by a significant increase in LTR-directed mRNA which can account partially (5, 33) or primarily (23, 25) for the observed stimulation of protein expression. The latter work could not, however, distinguish between different mechanisms by which *tat* might induce LTR-directed mRNA accumulation, i.e., transcriptional activation or posttranscriptional events such as increased mRNA stability or transport. In addition to this ambiguity, the boundaries of the LTR region(s) mediating the response to *tat*, while known to include sequences 3′ of the mRNA initiation site (23, 26), have yet to be defined. The studies presented in this paper were designed to clarify the nature of *trans*-activation, as well as to localize precisely the sequences involved in this process. We show that *trans*-activation of the HIV-2 LTR by HIV-1 *tat*, like that of the HIV-1 LTR, results in a comparable increase in LTR-directed mRNA and protein steady-state levels. Nuclear runoff transcription experiments reveal that an increased transcriptional rate is the primary event in this induction of HIV-1 and HIV-2 LTR-directed mRNA accumulation by HIV-1 *tat*. Detailed mutational analysis of the HIV-1 LTR indicates that the sequences mediating transcriptional activation are confined largely to a discrete element located 3′ of the RNA start site. This element bears a high degree of nucleotide identity to the functionally equivalent HIV-2 LTR region, providing support for the notion that HIV-1 *tat* activates HIV-1 and HIV-2 by a common mechanism.

MATERIALS AND METHODS

Cell lines and transfection assays. 293 cells grown in DME/F12 medium containing 10% fetal calf serum were transfected by the calcium phosphate method (32) as described previously (23). From 48 to 60 h later, lysates were prepared from duplicate transfections and assayed for chloramphenicol acetyltransferase (CAT) enzymatic activity in the linear range of chloramphenicol conversion (10) or used for the preparation of RNA. The CAT activities shown in Table 1 represent the averages obtained from duplicate

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TABLE 1. *trans*-Activation of wild-type and mutant HIV-1 and HIV-2 LTRs[a]

cat plasmid	CAT activity (% conversion)		*trans*-Activation (fold)	mRNA induction (fold)
	pUC13	pSVEtat		
Wild type				
HIV-1 (−525/+232)	0.32	22	69	46
HIV-2 (−135/+162)	0.32	27	85	55
HIV-2 deletion mutants				
Control (−135/+162)	1.0	181	174	
Δ1 (−135/+83)	0.86	78	91	
Δ2 (−135/+48)	2.4	89	38	
Δ3 (−135/+33)	1.3	2.4	1.9	
Δ4 (−81/+162)	0.93	69	74	
HIV-1 substitution mutants				
Control (−525/+232)	1.1	188	171	
M1 (sub −28/−26)	0.31	1.7	5.5	
M2 (sub −17/−15)	0.82	158	197	
M3 (sub −11/−9)	1.1	88	80	
M4 (sub −6/−4)	1.6	318	192	
M5 (sub +7/+9)	2.5	158	63	
M6 (sub +10/+12)	4.0	235	58	
M7 (sub +13/+15)	3.1	207	66	
M8 (sub +16/+18)	2.6	174	67	
M9 (sub +19/+21)	1.6	14.3	8.8	
M10 (sub +22/+24)	1.6	15.7	9.8	
M11 (sub +25/+27)	2.9	3.4	1.2	
M12 (sub +28/+30)	2.0	8.0	4.0	
M13 (sub +31/+33)	0.71	2.5	3.5	
M14 (sub +34/+36)	1.5	3.8	2.5	
M15 (sub +37/+39)	1.8	17.2	9.6	
M16 (sub +40/+42)	1.9	6.6	5.5	
M17 (sub +43/+45)	3.7	152	41	
M18 (sub +46/+48)	4.6	295	64	
M19 (sub +52/+54)	3.2	247	77	

[a] 293 cells were transfected with pHIV-1CAT (23), pHIV-2CAT, deletion mutants of pHIV-2CAT, or substitution mutants of pHIV-1CAT in the presence of HIV-1 *tat* (contransfection with pSVEtat [23]) or absence of *tat* (cotransfected with pUC13) and assayed for CAT activity as described in Materials and Methods. The LTR sequences present in pHIV-1CAT, pHIV-2CAT, and pHIV-2CAT deletion mutants are shown in parentheses. pHIV-1CAT substitution (sub) mutants contained LTR sequences between −120 and +80; sequences altered by mutation are shown in parentheses. The mRNA induction values were obtained from the ratio of 1.7-kb *cat* mRNA produced in the presence and absence of *tat*. RNA levels were determined by scintillation counting of the corresponding *cat* mRNA bands from the Northern blots shown in Fig. 1A.

transfections, which generally varied by less than 20%; values are normalized with respect to incubation time.

Plasmid constructions. pHIV-2CAT was derived from pHIV-1CAT (23) by replacing the HIV-1 LTR with a synthetic HIV-2 LTR (−165 to +133 sequences). pSVEtat was derived from pSVETA (23) by deletion of sequences between +8053 to +8593 (22), removing the *art* and pE′ coding regions. HIV-1 substitution mutants were constructed by oligonucleotide-directed mutagenesis (34) of the pHIV-1CAT derivative pHIV-1CAT(−120/+80) (23).

RNA analysis. Total and polyadenylated [poly(A)$^+$] nuclear and cytoplasmic RNA was prepared, electrophoresed on formaldehyde-agarose gels, transferred to nylon membranes (Genescreen; New England Nuclear Corp.), cross-linked by UV irradiation, and hybridized with ^{32}P-labeled probes as described previously (16). The *cat* probe was derived by nick-translation of an *Xho*I-*Bam*HI fragment of the *cat* gene (23). The β-actin probe was prepared by T4 polynucleotide kinase labeling of a 50-mer corresponding to β-actin coding sequences (16). Blots were rehybridized following extensive washing in 50% formamide–0.2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 65°C. Primer extension of RNA samples was carried out as described (6) with a 25- or 35-mer primer corresponding to the antisense strands of *cat* or the β-actin gene.

Nuclear runoff experiments. Nuclei were prepared by hypotonic lysis (31), and nascent transcripts were elongated at 26°C for 10 min as described (19). Transcripts were purified by the method of McKnight and Palmiter (21) except that unincorporated ribonucleotide triphosphates were removed on a Sephadex G-50 column before trichloroacetic acid precipitation. Dot-blot hybridization of runoff transcripts was carried out on nylon membranes at 65°C for 36 to 48 h as described above. Hybridizations (0.3 to 0.5 ml) were performed with duplicate filters containing equal amounts of runoff transcripts prepared from cells cotransfected in the presence or absence of *tat* (3×10^7 to 5×10^7 cpm/ml). Filters were washed in 0.2× SSC–0.1% SDS at 65°C and exposed to Kodak XAR film at −70°C.

RESULTS

***trans*-Activation of HIV-2, like HIV-1, by HIV-1 *tat* leads to a large increase in steady-state nuclear and cytoplasmic RNA levels.** Regulation of HIV LTR-directed CAT expression by the HIV-1 *tat* protein was investigated in a transient expression system found to support a level of in vitro transcription sufficient for nuclear runoff analysis. Human embryonic kidney 293 cells were transfected with expression vectors in which promoter sequences derived from the LTRs of HIV-1 (−525 to +232) and HIV-2 (−133 to +162) were placed immediately 5′ of the *cat* gene. *trans* activator function was provided by cotransfection of plasmid pSVEtat, in which expression of the HIV-1 *tat* protein is under the constitutive control of the simian virus 40 (SV40) early promoter (23). At 48 h posttransfection, parallel cultures of cells were analyzed for levels of CAT enzyme activity and steady-state RNA produced in the presence or absence of *tat*. CAT protein expression directed by the HIV-2 LTR was stimulated by the HIV-1 *tat* protein (70- to 200-fold) to a similar extent as that directed by the HIV-1 LTR, as suggested by Guyader et al. (12) (Table 1).

To determine whether this induction of the HIV-2 LTR by HIV-1 *tat* was due to increased accumulation of LTR-directed RNA, *cat* mRNA was measured by blot hybridization of cytoplasmic poly(A)$^+$ RNA (Fig. 1A). Rehybridization of these blots with a β-actin probe provided an internal reference for the amount of RNA in each lane (Fig. 1A). Cells transfected with pHIV-1CAT, demonstrating a 69-fold increase of CAT enzymatic activity in response to HIV-1 *tat*, displayed a 46-fold increase in the level of 1.7-kilobase (kb) *cat* mRNA (Table 1). Similarly, transfection of cells with pHIV-2CAT led to a similar relative induction in the levels of CAT protein (85-fold) and mRNA (55-fold) by HIV-1 *tat* (Table 1). To determine whether the induced RNA was correctly initiated, primer extension was used to characterize the size and amount of *cat* mRNA, using a primer complementary to the *cat* gene (Fig. 1B). *cat* mRNA was initiated at sites within the HIV-1 and HIV-2 LTRs characteristic of the corresponding viral RNAs produced during HIV infection (12, 23) (Fig. 1B). Identical RNA initiation sites were used in the presence and absence of *tat* for both the HIV-1 and HIV-2 LTRs; in addition, the level of *cat* mRNA directed by each LTR was at least 50-fold greater in the presence of *tat*, confirming the results of Northern (RNA) blot analysis (Fig. 1). These results demonstrate that

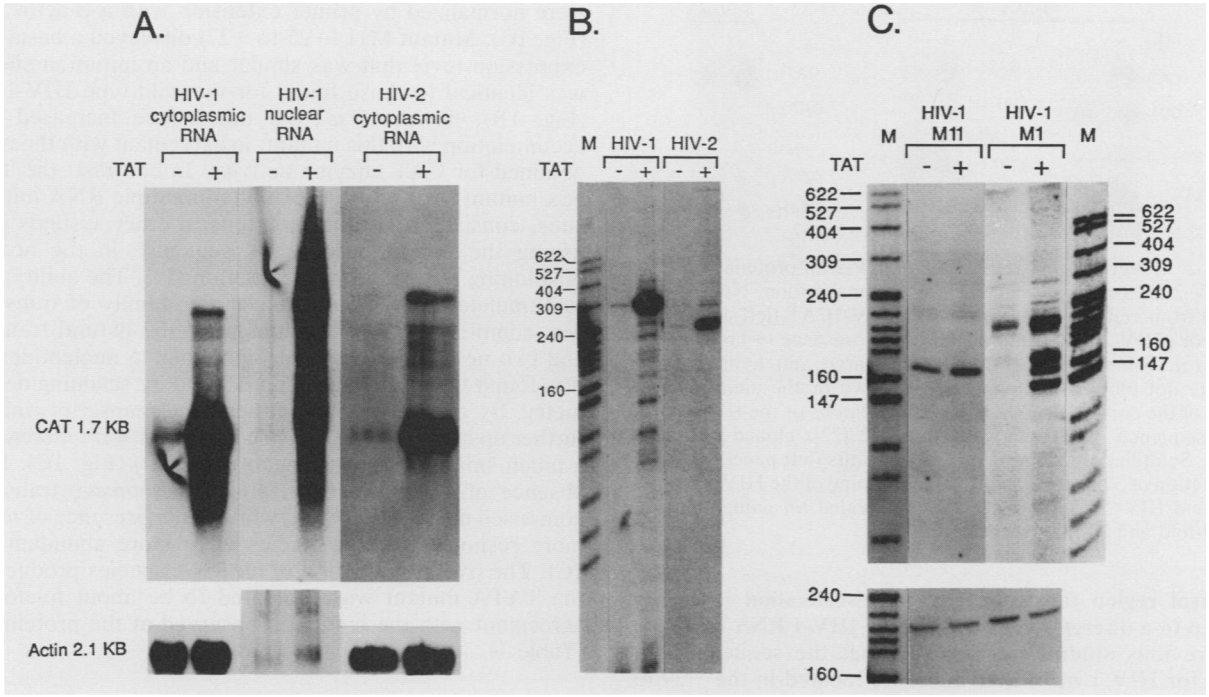

FIG. 1. Effect of *trans*-activation by HIV-1 *tat* protein on HIV-1 and HIV-2 LTR-directed mRNA expression. (A) Northern blot analysis of steady-state levels of HIV-1 and HIV-2 LTR-directed cytoplasmic and nuclear RNA. Cytoplasmic poly(A)$^+$ (5 μg) or total nuclear (25 μg) RNA isolated from cells cotransfected with pHIV-1CAT or pHIV-2CAT in the presence (+ lanes) or absence (− lanes) of HIV-1 *tat* were analyzed on Northern blots and hybridized with ^{32}P-labeled *cat* (upper panel) or β-actin (lower panel) probes. The sizes of the *cat* and β-actin RNA species, determined from the mobility of rRNA markers, are indicated at the left (in kilobases). Overexposure of the nuclear RNA blot was necessary to reveal the level of *cat* RNA in the absence of *tat*. (B) Primer extension analysis of HIV-1 and HIV-2 LTR-directed cytoplasmic RNA. A 1-μg amount of the poly(A)$^+$ RNA samples analyzed in panel A was extended by using the *cat* primer (see Materials and Methods). HIV-1 LTR (−525/+232) or HIV-2 LTR (−135/+162)-directed RNAs are expected to produce a 310- or 240-nucleotide *cat* extension product, respectively. (C) Primer extension analysis of mutant HIV-1 LTR-directed cytoplasmic RNA. Total cytoplasmic RNA (20 μg) prepared from cells transfected with mutant M11 (sub +25/+27) or mutant M1 (sub −26/−28) (see Fig. 3) in the presence (+ lanes) or absence (− lanes) of HIV-1 *tat* were analyzed by using either the *cat* primer (top panel) or β-actin primer (lower panel). Each of the mutant plasmids analyzed contained a 200-nucleotide fragment of the HIV-1 LTR (−120 to +80) (see Table 1), resulting in a 160-nucleotide *cat* extension product. The β-actin primer results in a 210-nucleotide extension product.

trans-activation of the HIV-2 LTR results primarily from increased levels of steady-state LTR-directed mRNA, as previously shown for the HIV-1 LTR (23, 25).

The induction of mRNA accumulation mediated by *tat* could be due to increased transcription or to a posttranscriptional event such as transport or stability. To investigate whether the increase in cytoplasmic *cat* mRNA resulted from more efficient RNA transport, total nuclear RNA transcripts were analyzed as described above. HIV-1 LTR-directed nuclear RNA species, which consisted of the 1.7-kb *cat* RNA and higher-molecular-weight precursors, were coordinately induced by *tat* to at least the same extent as that found for cytoplasmic RNA (Fig. 1A). These results indicate that *trans*-activation does not result from increased mRNA transport but rather from either increased transcription or decreased turnover of nuclear RNA prior to its export to the cytoplasm.

***tat* activates transcription mediated by the HIV-1 and HIV-2 LTRs.** To distinguish between these two possibilities, we measured the relative rate of LTR-directed *cat* transcription in the presence and absence of *tat* with an in vitro nuclear transcription assay. Nuclei were isolated from transfected cells and incubated with ^{32}P-labeled ribonucleoside triphosphates under conditions which permit only the limited elongation of previously initiated RNA chains. To normalize for the efficiency of transfection and rate of transcription in different nuclei preparations, cells were cotransfected with a reference plasmid which expresses the CD4 antigen constitutively (28) under the control of an SV40 promoter which is not affected by *tat* (23, 26). Nuclear runoff transcripts, exhibiting an average size of 100 nucleotides on denaturing acrylamide gels, were quantitated by dot blot hybridization to antisense sequences from the *cat* and CD4 coding regions cloned into single-stranded M13 vectors. This analysis revealed that transcription directed by the HIV-1 and HIV-2 LTRs was increased by at least 30- and 20-fold, respectively, in the presence of the HIV-1 *tat* protein (Fig. 2). In contrast, no significant increase was observed in the level of CD4 transcripts, indicating that transcription directed by the SV40 promoter was similar in the presence or absence of *tat* (Fig. 2). The nuclear transcripts detected in this fashion were specific for the *cat* and CD4 genes and largely initiated from the mRNA sense strand, as revealed by control DNAs present on the dot blots corresponding to the parent M13 vector and M13 clones containing the *cat* and CD4 sense orientations (Fig. 2). A similar increase in HIV-1-driven *cat* transcription by *tat* was detected by hybridization to a dot blot containing the 232-nucleotide HIV-1 leader sequence present in the pHIV-1CAT construction (Fig. 2). These results thus demonstrate that the regulation of HIV-1- and HIV-2 LTR-directed expression by the *tat* protein occurs at the level of transcription.

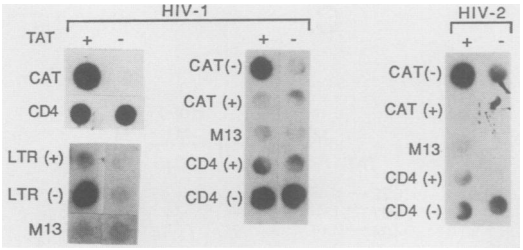

FIG. 2. Effect of *trans*-activation by HIV-1 *tat* protein on HIV-1 and HIV-2 LTR-directed nuclear runoff transcription. Nuclei were isolated from cells cotransfected with pHIV-1CAT (left and center panels) or pHIV-2CAT (right panel) in the presence (+) or absence (−) of *tat*. Runoff transcripts were prepared and hybridized to duplicate dot blots containing the sense (+) or the antisense (−) strands of the *cat* (10) or CD4 (28) coding regions or the HIV-1 LTR leader sequence (nucleotides +1 to +232 [22]) cloned into M13 vectors. Scintillation counting of HIV-1 results (left panel) revealed *tat* induction of 32-fold; densitometric scanning of the HIV-1 (center panel) and HIV-2 (right panel) results revealed *tat* induction of at least 30-fold and 20-fold, respectively.

Control region for *tat*-induced *trans*-activation is largely confined to a discrete segment 3′ of the HIV-1 RNA initiation site. Previous studies have shown that the sequences required for HIV-1 *trans*-activation are located in the −29 to +54 region of the LTR (23), while sequences upstream of this position primarily regulate the basal expression level (23) and activation by mitogens and phorbol esters (24). While a critical role for sequences 3′ of the cap site was suggested, the involvement of sequences between the TATA box and cap site was not investigated (23, 26), nor has the analogous responding region for HIV-1 *tat* in the HIV-2 LTR yet been determined. As shown in Fig. 3, the HIV-2 LTR had 60% identity with HIV-1 between nucleotides −104 and +89. To determine the approximate location of the HIV-2 *tat*-responding element, a series of deletions were introduced into the HIV-2 LTR (Fig. 3). Deletion of HIV-2 core enhancer sequences did not significantly affect the basal expression level or alter the response to *tat*, indicating that, as for HIV-1, these sequences are dispensable for *trans*-activation (Table 1). A set of deletions with the HIV-2 R region revealed a crucial role for sequences between +34 and +48 in *trans*-activation, suggesting that the homologous sequences found 3′ of the HIV-1 and HIV-2 RNA cap sites mediate the *tat* response (Table 1).

To localize the *tat* activation site more precisely, we constructed a set of clustered point mutations within the HIV-1 LTR covering the region from −29 to +54 and altering sequences most similar to those found in HIV-2 (Fig. 3). Substitutions located 5′ of the cap site affected neither the basal expression level nor *trans*-activation, with the exception of a mutation within the TATA box, which led to a dramatic reduction in *tat* stimulation (Table 1). Mutations 3′ of the cap site had little effect on basal expression but differed significantly in their effects on *trans*-activation. Substitution of sequences between +19 and +42 substantially reduced induction by *tat*, with alterations between +25 and +36 virtually abolishing *trans*-activation, while mutations outside this region had little effect (Table 1). This analysis thus establishes the boundaries of one region critical in *trans*-activation to a discrete element completely 3′ of the HIV-1 mRNA initiation site that has 84% nucleotide identity with the HIV-2 LTR and identifies another in the TATA box.

The effects of deleterious mutations from both regions was further studied by primer extension analysis; RNA levels were normalized by primer extension with a β-actin probe (Fig. 1C). Mutant M11 (+25 to +27) displayed a basal RNA expression level that was similar and an initiation site that was identical to those found for the wild-type HIV-1 LTR (Fig. 1B); however, *tat* did not induce increased RNA accumulation with this mutant, in agreement with the results obtained for CAT enzyme activity. In contrast, the TATA box mutant (−28 to −26) exhibited multiple RNA initiation sites, consistent with observations in other systems establishing the importance of this sequence in the accurate positioning of transcription initiation (11). The ability of *tat* to stimulate the accumulation of this family of transcripts was complex (Fig. 1C). Induction of the natural transcript and two new transcripts starting within 15 nucleotides of it was found to be approximately 15-fold by scanning densitometry. By contrast, several other novel transcripts initiated further upstream in the TATA box mutant were increased to a much smaller extent (two- to threefold) (Fig. 1C). In the absence of *tat*, these relatively nonresponsive transcripts composed most of the RNA, while in the presence of *tat*, the more responsive RNA species were more abundant (Fig. 1C). The overall induction of all RNA species produced by the TATA mutant was estimated to be about fivefold, in agreement with the induction observed at the protein level (Table 1).

DISCUSSION

The results presented here provide evidence that *trans*-activation of HIV-1 and HIV-2 LTR-directed expression by an HIV *tat* polypeptide is primarily a transcriptional event. Our findings bear resemblance to the recent report by Hauber et al. (13), indicating that an increased rate of transcription from the HIV-1 LTR is mediated by *tat*, but differ in that these investigators also observed a substantial effect of *tat* in enhancing translation of HIV-1 LTR-directed mRNA. This difference is possibly attributable to the use by these investigators of a replicating expression vector in COS-7 monkey cells, since findings similar to those we have reported here for 293 cells, demonstrating the primary effect of *tat* on LTR-directed mRNA accumulation, have been obtained with other human cell lines, including Hela and HUT-78 (23, 25). In addition, we have shown in the present work that the sequences mediating this transcriptional activation are confined largely if not exclusively to a discrete element located 3′ of the RNA initiation site. In most examples studied to date, transcription at RNA polymerase II-dependent promoters is regulated by sequences located 5′ of the RNA cap site (7). A notable exception to this is the existence of downstream enhancers in a number of cellular and viral genes (7). We have previously found that a cassette containing the *tat*-responding element of HIV-1 (−17 to +80 sequences) displays an absolute functional dependence on its orientation and position, indicating that this region does not have the characteristic properties of an enhancer (23, 25). The mechanism by which the *tat* protein, or a factor induced by it, affects transcription at this element, while thus apparently distinct from the actions of enhancers, is presently unclear. The increase in nuclear runoff transcription mediated by *tat* is consistent with an increase in the rate of transcriptional initiation or, alternatively, with the facilitation of transcript elongation. In the latter model, sequences within the HIV leader might contain a block to RNA elongation causing pausing or premature termination unless relieved by *tat*, as recently suggested by Kao et al. (17). This possibility seems less likely, given that deletions spanning

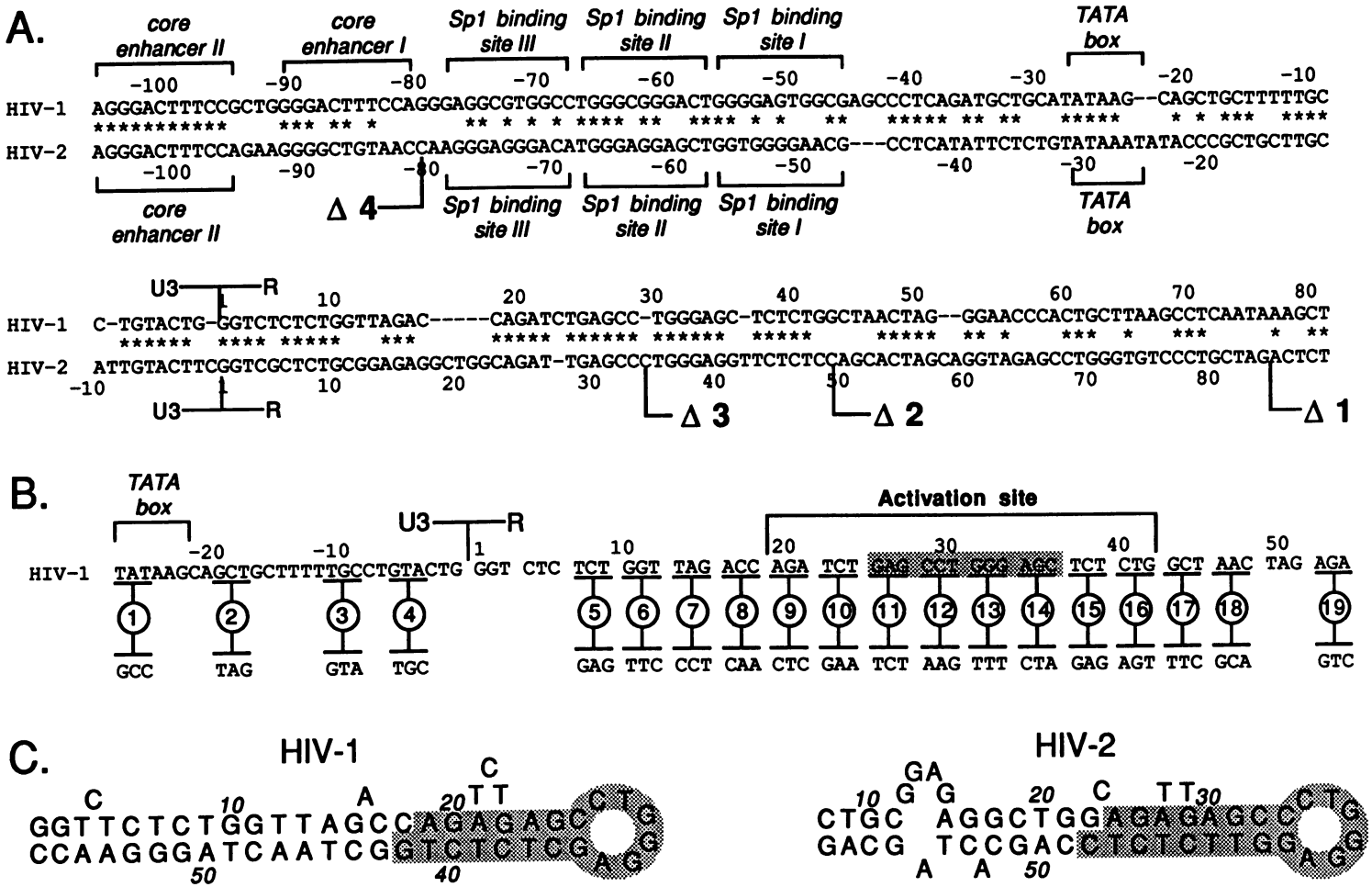

FIG. 3. (A) Sequence comparison of the HIV-1 and HIV-2 LTRs. Nucleotide identities between the HIV-1 LTR (22) and HIV-2 LTR (12) are indicated by asterisks. Gaps were introduced to optimize the alignment. Consensus sequences corresponding to the core enhancers, Sp1-binding sites, and TATA box are bracketed. The RNA initiation sites (U3-R junction) of HIV-1 (22) and HIV-2 (12) are indicated. Arrows show the endpoints of HIV-2 LTR deletion mutants described in Table 1. Sequences deleted in mutants Δ1, Δ2, Δ3, and Δ4 correspond to +83 to +162, +48 to +162, +33 to +162, and −82 to −135, respectively. (B) Mutational analysis of the *tat*-responsive region of the HIV-1 LTR. The upper sequence represents the wild-type HIV-1 LTR. HIV-1 LTR substitution mutants were created by oligonucleotide-directed mutagenesis (34); each of the mutations contains a cluster of three nucleotide changes corresponding to transversions; substituted sequences are indicated below the line. The site of *tat* activation (+19 to +42) is indicated by brackets; the inner core element (+25 to +36) is shadowed. (C) Experimentally determined secondary structure of HIV-1 leader mRNA (23) is compared with predicted HIV-2 leader mRNA structure; *tat*-responsive sequences are shadowed.

the entire HIV leader region do not lead to an increase in the basal level of expression (3, 12), as would be expected if this region contained a *cis*-acting site of negative regulation. Nevertheless, these observations do not exclude the possibility that *tat* may have a more general role in transcription antitermination.

The common mode of transcriptional activation by *tat* observed for HIV-1 and HIV-2 appears to be due to the high degree of conservation of the 3′ activation element found in the respective LTRs (Fig. 3). We previously demonstrated the existence of a stable stem-loop structure in HIV-1 leader RNA, the tip of which encompasses this element (23). A similar stem-loop is expected to form in the HIV-2 RNA leader, with the exposed loop having identical residues (Fig. 3). While it was suggested that a twofold symmetry in the stem of the HIV-1 hairpin might constitute the binding site for *tat*, or a factor induced by *tat* (23), the present results which show that most of the stem is dispensable, suggest that the relevant structure may instead consist of the loop itself and eight or nine proximal base pairs of the stem.

In contrast to the coordinate decrease in LTR-directed RNA and protein observed for mutations in the 3′ element, a mutation in the HIV-1 TATA box decreased *trans*-activation in a transcript-specific fashion, in that RNA initiated more than 20 nucleotides 5′ of the normal cap site was poorly induced by *tat*, while RNA initiated further 3′ was induced in a nearly normal fashion. This finding indicates that the interaction of transcription factors with the TATA box is not obligatory for *trans*-activation and suggests that the distance between the 3′ *tat* activation element and the RNA initiation site might be critical to the interaction between the factor which recognizes this element and the transcriptional complex. Sequences 3′ of the RNA start site affecting transcription have been described for RNA polymerase I-regulated genes such as 5S rRNA and tRNA (3, 9). Downstream sequences have also been implicated in transcriptional regulation of the adenovirus major late promoter (18, 20) and in the regulation of mRNA levels by the *Drosophila* heat shock gene, *hsp22* (15), and the bovine papillomavirus P1 promoter (30); however, the 5′ boundaries of the downstream elements found in these RNA polymerase II promoters have not yet been defined. Further insight into the mechanism of HIV *trans*-activation will require an analysis of the cellular and/or viral factors which recognize this 3′ element and their interaction with the cellular transcriptional apparatus.

ACKNOWLEDGMENTS

We thank Scot Marsters and Charu Agrawal for technical assistance, Ernest Peralta, Arnon Rosenthal, and Justus Cohen for comments on the manuscript, Mark Vasser, Peter Ng, and Parkash Jurhani for oligodeoxynucleotide synthesis, and Carol Morita for preparation of the figures.

LITERATURE CITED

1. **Arya, S., B. Beaver, L. Jagodzinski, B. Ensoli, P. Kanki, J. Alberts, E. Fenyo, G. Biberfeld, J. Zagury, F. Laure, M. Essex, E. Norrby, F. Wong-Staal, and R. C. Gallo.** 1987. New human and simian HIV-related retroviruses possess functional transactivator gene. Nature (London) **328**:548–550.
2. **Arya, S., C. Guo, S. Joseph, and F. Wong-Staal.** 1985. *trans*-Activator gene of human lymphotropic virus type III (HTLV-III). Science **229**:69–73.
3. **Bogenhagen, D., S. Sakonju, and D. Brown.** 1980. A control region in the center of the 5S RNA gene directs specific initiation of transcription. II. The 3′ border of the region. Cell **19**:27–35.
4. **Chakrabarti, L., M. Guyader, M. Alizon, M. Daniel, R. Desrosiers, P. Tiollais, and P. Sonigo.** 1987. Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. Nature (London) **328**:543–547.
5. **Cullen, B.** 1986. *trans*-Activation of human immunodeficiency virus occurs via a bimodal mechanism. Cell **46**:973–982.
6. **Dynan, W., and R. Tjian.** 1983. Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. Cell **32**:669–680.
7. **Dynan, W., and R. Tjian.** 1985. Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. Nature (London) **316**:774–778.
8. **Feinberg, M., R. Jarrett, A. Aldovini, R. Gallo, and F. Wong-Staal.** 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. Cell **46**:807–817.
9. **Galli, G., H. Hofstetter, and M. Birnstiel.** 1981. Two conserved sequence blocks within eukaryotic tRNA genes are major promoter elements. Nature (London) **294**:626–631.
10. **Gorman, C., L. Moffat, and B. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
11. **Grosschedl, R., and M. Birnstiel.** 1980. Identification of regulatory sequences in the prelude sequences of an H2A histone gene by the study of specific deletion mutants in vivo. Proc. Natl. Acad. Sci. USA **77**:1432–1436.
12. **Guyader, M., M. Emerman, P. Sonigo, P. Clavel, L. Montagnier, and M. Alizon.** 1987. Genome organization and *trans*-activation of HIV-2. Nature (London) **326**:662–669.
13. **Hauber, J., A. Perkins, E. Heimer, and B. Cullen.** 1987. *Trans*-activation of human immunodeficiency virus gene expression is mediated by nuclear events. Proc. Natl. Acad. Sci. USA **83**: 6364–6368.
14. **Hirsch, V., N. Riedel, and J. Mullins.** 1987. The genome organization of STLV-3 is similar to that of the AIDS virus except for a truncated transmembrane protein. Cell **49**:307–319.
15. **Hultmark, D., R. Klemenz, and J. Gehring.** 1986. Translational and transcriptional control elements in the untranslated leader of the heat-shock gene hsp22. Cell **44**:429–438.
16. **Jakobovits, A., M. Schwab, J. Bishop, and G. Martin.** 1986. Expression of N-myc in teratocarcinoma stem cells and mouse embryos. Nature (London) **318**:188–190.
17. **Kao, S., A. Calman, P. Luciw, and B. Peterlin.** 1988. Antitermination of transcription within the long terminal repeat of HIV-1 by *tat* gene product. Nature (London) **330**:489–493.
18. **Lewis, D., and J. Manley.** 1985. Control of adenovirus late promoter expression in two human cell lines. Mol. Cell. Biol. **5**:2433–2442.
19. **Linial, M., N. Gunderson, and M. Groudine.** 1985. Enhanced transcription of c-myc in bursal lymphoma cells requires continuous protein synthesis. Science **230**:1126–1132.
20. **Mansour, S., T. Grodzicker, and R. Tjian.** 1986. Downstream sequences affect transcription initiation from the adenovirus major late promoter. Mol. Cell. Biol. **6**:2684–2694.
21. **McKnight, G., and R. Palmiter.** 1979. Transcription regulation of the ovalbumin and conalbumin genes by steroid hormones in chick oviduct. J. Biol. Chem. **254**:9050–9058.
22. **Muesing, M., D. Smith, C. Cabradilla, C. Benton, L. Lasky, and D. Capon.** 1985. Nucleic acid structure and expression of the human AIDS retrovirus. Nature (London) **313**:450–458.
23. **Muesing, M., D. Smith, and D. Capon.** 1987. Regulation of mRNA accumulation by a human immunodeficiency virus *trans*-activator protein. Cell **48**:691–701.
24. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates the expression of human immunodeficiency virus in T lymphocytes. Nature (London) **326**:711–713.
25. **Peterlin, B., P. Luciw, P. Barr, and M. Walker.** 1986. Elevated levels of mRNA can account for the *trans*-activation of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **83**:9734–9738.
26. **Rosen, C., J. Sodroski, and W. Haseltine.** 1985. The location of cis-acting regulatory sequences in the human T-cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. Cell

41:813–823.

27. **Rosen, C., J. Sodroski, W. Goh, A. Dayton, J. Lippe, and W. Haseltine.** 1986. Post-transcriptional regulation accounts for the *trans*-activation of the human T-lymphotropic virus type III. Nature (London) **319**:555–559.
28. **Smith, D., R. Byrn, S. Marsters, T. Gregory, J. Groopman, and D. Capon.** 1987. Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. Science **238**:1704–1705.
29. **Sodroski, J., R. Patarca, C. Rosen, F. Wong-Staal, and W. Haseltine.** 1985. Location of the *trans*-activating region on the genomic of human T-cell lymphotropic virus type III. Science **229**:74–77.
30. **Stenlund, A., G. Bream, and M. Botchan.** 1987. A promoter with an internal regulatory domain is part of the origin of replication in BPV-1. Science **236**:1666–1671.
31. **Weber, J., W. Jelinek, and J. Darnell.** 1977. The definition of large viral transcription unit late in Ad2 infection of Hela cells: mapping of nascent RNA molecules labeled in isolated nuclei. Cell **10**:611–616.
32. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76**:1373–1376.
33. **Wright, C., B. Felber, H. Paskalis, and G. Pavlakis.** 1986. Expression and characterization of the *trans*-activator of HTLV-III/LAV virus. Science **234**:988–992.
34. **Zoller, M., and M. Smith.** 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. Nucleic Acids Res. **10**:6487–6500.