

## Multiple Transcriptional Regulatory Domains in the Human Immunodeficiency Virus Type 1 Long Terminal Repeat Are Involved in Basal and E1A/E1B-Induced Promoter Activity

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Received 30 May 1989/Accepted 3 August 1989

The human immunodeficiency virus (HIV) type 1 long terminal repeat (LTR) is the site of activation of the HIV *tat* protein. However, additional transactivators, such as the adenovirus E1A and herpesvirus ICP0 proteins, have also been shown to be capable of activating the HIV LTR. Analysis of adenovirus mutants indicated that complete transactivation of the HIV LTR was dependent on both the E1A and E1B proteins. To determine which regions of the HIV LTR were important for complete E1A/E1B activation, a variety of oligonucleotide-directed mutations in HIV transcriptional regulatory domains were assayed both *in vivo* and *in vitro*. S1 nuclease analysis of RNA prepared after transfection of these HIV constructs into HeLa cells infected with wild-type adenovirus indicated that the enhancer, SP1, TATA, and a portion of the transactivation-responsive element were each required for complete E1A/E1B-mediated activation of the HIV LTR. These same promoter elements were required for both basal and E1A/E1B-induced levels of transcription in *in vitro* transcription reactions performed with cellular extracts prepared from cells infected with *dI434*, an E1A/E1B deletion mutant, or wild-type adenovirus. No mutations were found that reduced only E1A/E1B-induced expression without proportionally reducing basal levels of transcription, suggesting that E1A/E1B-mediated induction of the HIV LTR requires multiple promoter elements which are also required for basal transcriptional levels. Unlike activation by the *tat* protein, there was not a rigid dependence on maintenance of the transactivation-responsive stem base pairing for E1A/E1B-mediated activation either *in vivo* or *in vitro*, indicating that activation occurs by a mechanism distinct from that of *tat* induction.

Gene expression of the human immunodeficiency virus (HIV) type 1, a causative agent of the acquired immune deficiency syndrome (5, 15, 40, 41), is regulated by both viral proteins such as *tat* (3, 6, 11, 13, 16, 29, 35, 51), *nef* (2), and *rev* (11, 59) and cellular DNA-binding proteins (19, 32, 33, 46, 63, 64). DNase I footprinting and mutagenesis studies have defined several regions of the HIV long terminal repeat (LTR) important for basal and *tat*-induced regulation in HeLa cells (19). These include the negative regulatory, enhancer, SP1, TATA, and transactivation-responsive (TAR) regions. In addition, a variety of regulatory domains present further upstream are required for activation of the LTR in activated T-lymphoid cells (57). A number of proteins that bind to these transcriptional regulatory regions in the HIV LTR have been characterized. These include SP1 (32), the EBP-1 (64) and NF- $\kappa$ B (37, 46) proteins that bind to the enhancer, and UBP-1 (63), UBP-2 (17), LBP (33), and CTF/NF-1 (33), which bind to the TAR region.

The HIV LTR is activated at the transcriptional level by the 86-amino-acid nuclear protein *tat* (3, 6, 11, 13, 16, 29, 35, 51). Within this protein, multiple Cys-X-X-Cys domains are critical for transactivation (14, 16). Other viral transactivating proteins, such as the 289-amino-acid adenovirus E1A protein, contain similar metal-binding domains which appear to be important in transactivation (7). *tat* has been reported by some investigators to increase HIV gene expression by increasing steady-state RNA levels (6, 19, 22, 44, 51). *In vitro* nuclear runoff experiments have shown that *tat* increases HIV nuclear mRNA levels (29, 52), further support-

ing a role for *tat* in transcriptional activation of the HIV LTR. However, *tat* has also been reported to increase the translation of HIV mRNA (6, 11, 44, 54). Thus, *tat* may have multiple mechanisms of action (6).

Complete activation of the HIV LTR by the *tat* protein requires both upstream regulatory regions, such as the enhancer and TATA elements, and the downstream TAR region (19, 31, 33, 44, 51, 55). Studies have defined a region between +19 and +44 in the TAR domain which was required for activation by the *tat* protein (28, 31). Within the TAR region, a stem-loop structure has been described which appears to be important for HIV gene expression (12, 31, 44, 48). Mutations of the loop or mutations which destabilize the upper portion of this stem greatly reduce *tat*-induced gene expression in chloramphenicol acetyltransferase (CAT) assays (12, 31). However, compensatory mutations which reform the stem structure and restore stem energy (60) result in nearly wild-type (WT) levels of CAT conversion (12, 17). In addition, TAR DNA-binding proteins such as UBP-1, UBP-2, LBP, and CTF/NF-1 also appear to be important in *tat* induction (17, 19, 33). Thus, at least two determinants, the stem-loop structure and TAR DNA-binding proteins, appear to play a role in HIV gene expression.

The ability to transactivate the HIV LTR is not limited to the HIV *tat* protein. A number of DNA viruses, such as cytomegalovirus, adenovirus, and herpesvirus, have been shown to be capable of transactivating the HIV LTR (8, 42, 43, 47, 49, 53). Immediate-early proteins from these viruses, such as E1A and ICP0, have been shown to function in this activation (8, 47, 53). One study, in which either E1A- or ICP0-containing plasmids were transfected into *tat*-con-

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taining Jurkat cell lines, suggested that ICP0 required multiple HIV transcriptional regulatory domains for activation while E1A transactivation was dependent only on the TATA element (47). However, mutations of the enhancer region and of the three SP1-binding sites in the HIV LTR also decreased E1A-induced levels of transcription. Transactivation of the HIV LTR was dependent on the 289-amino-acid E1A protein to a much greater degree than on the 243-amino-acid protein (47, 53).

To further study transactivation of the HIV LTR by adenovirus, an analysis of both the adenovirus early regions required for this activation and the HIV LTR regulatory regions involved in this increased gene expression was undertaken. Viral mutants lacking the adenovirus early regions were examined for their ability to transactivate the HIV LTR. Additionally, a variety of mutant HIV LTR constructs were transfected into HeLa cells infected with either WT virus or an E1A/E1B mutant, and their expression was assayed by S1 nuclease analysis. Finally, the effects of mutations in promoter elements of the HIV LTR on transactivation were examined in an *in vitro* transcription system, using extracts prepared from WT and mutant adenovirus-infected cells.

## MATERIALS AND METHODS

**Cells and virus stocks.** WT adenovirus type 5 was grown in HeLa suspension cultures maintained in minimal essential medium plus 5% newborn calf serum, and titers were determined by plaque formation on 293 cells. Adenovirus type 5 mutants *dl312* (34), *dl434* (26), *pm1722* (4), and *pm1520* (4) were grown on monolayers of the complementing 293 cell line (24), and titers were determined by plaque assay on this cell line. HeLa monolayers were maintained on complete Iscoves medium with 5% newborn calf serum containing penicillin and streptomycin.

**Transfections and CAT assays.** HeLa plates were split on the day before transfection so that each 100-mm plate was 50 to 70% confluent at the time of transfection. Cells were infected with WT adenovirus, *dl312* (34), *dl434* (26), *pm1722* (4), or *pm1520* (4) at a multiplicity of infection of 20. At 1 h postinfection, fresh medium with cytosine arabinoside (20 µg/ml) was added (21). At 4 h postinfection, medium was removed and 5 µg of each HIV LTR CAT construct was transfected onto identically prepared plates. Fresh medium containing cytosine arabinoside was added, the medium was changed 12 h later, and the plates were harvested at 36 h posttransfection. CAT assays (23) were then performed, and RNA was prepared for S1 nuclease analysis.

**S1 nuclease analysis.** Fifty micrograms of cytoplasmic RNA prepared from five 100-mm plates of HeLa cells transfected with the indicated HIV LTR construct in the presence of adenovirus infection was hybridized with an end-labeled CAT probe, as described elsewhere (20). The probe was prepared by cutting an adenovirus early region 3 construct containing a portion of the E3 promoter from -85 to +31 fused to the CAT gene with *NcoI*, which cuts 550 nucleotides into the CAT message, labeling with  $\gamma$ -<sup>32</sup>P, cutting with *Bam*HI, and gel isolating the 695-base-pair (bp) fragment. A probe to determine the level of E1A mRNA was prepared from the E1A/E1B-containing plasmid BE5 by cutting this plasmid with *Xba*I, labeling with  $\gamma$ -<sup>32</sup>P, cutting with *Hinf*I, and gel isolating a 550-bp band. After hybridization at either 47°C for the CAT probe or 53°C for the E1A probe, the S1 samples were treated and electrophoresed on an 8 M urea-4% polyacrylamide gel and subjected to auto-

radiography to visualize the 550-bp band specific for the CAT message and 110 bp specific for the E1A message.

**Mutagenesis.** A *Mae*I (-177)-*Hind*III (+83) fragment from the HIV LTR of ARV2 was cloned into *Hinc*II-*Hind*III mp18, and single-stranded DNA template was prepared as previously described (17). Oligonucleotides (19- to 45-mers) were synthesized on a DNA synthesis machine (Applied Biosystems; Molecular Biology Institute and UCLA Jonsson Comprehensive Cancer Center Fermenter/Preparation Core Facility, courtesy of Thomas Sutherland). The oligomers were gel purified, quantitated, and treated with ATP kinase. The kinase-treated oligomers were used in conjunction with a commercial site-directed mutagenesis kit (Amersham Corp.) according to conditions described by the manufacturer. Positive clones were identified by screening and confirmed by sequencing, and *Eco*RI-*Hind*III fragments were isolated for ligation into the CAT vector pJGFCAT18.

**Plasmid constructions.** *Bam*HI-*Hind*III fragments from each of the M13 mutations were ligated into *Bam*HI-*Hind*III pJGFCAT18, which is a Rous sarcoma virus CAT derivative containing a unique *Xho*I restriction site 21 nucleotides downstream from the *Hind*III site of the CAT gene.

**Cell extracts and *in vitro* transcription.** Nuclear extracts were prepared from uninfected, *dl434*-infected, or WT adenovirus-infected HeLa cells at 30 h postinfection, as previously described (9). Transcription reaction mixtures (50 µl) contained 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.9), 50 mM KCl, 6.25 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 10% glycerol, 500 µM each ribonucleoside triphosphate, 850 ng of supercoiled template DNA, and 200 µg of deletion mutant- or WT adenovirus type S-infected HeLa nuclear extract (approximately 20 µl). The reaction mixtures were incubated for 60 min at 30°C, and the reactions were terminated by the addition of 150 µl of stop buffer (200 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate, 250 µg of tRNA per ml). Each reaction mixture was extracted twice with phenol-chloroform and precipitated with 0.3 M sodium acetate-ethanol. The pellets were rinsed with 70% ethanol and dried. Primer extension was performed by a modification of a previously described protocol (33). Each pellet was suspended in 8 µl of 10 mM Tris hydrochloride (pH 7.9)-1 mM EDTA containing approximately 50 fmol of 5'-end-labeled primer (24-nucleotide primer which hybridizes to sequences 4945 to 4968 of Rous sarcoma virus CAT). A 2-µl volume of 10 mM Tris (pH 7.9)-1 mM EDTA-1.25 M KCl was added, and the primer was annealed to the RNA for 60 min at 63°C. A 25-µl sample of a buffer containing 20 mM Tris (pH 7.9), 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 500 µM deoxynucleoside triphosphates, and 50 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc.) was added, and the primer extension reaction mixture was incubated for 90 min at 37°C. Each reaction mixture was extracted with phenol-chloroform, precipitated with 0.3 M sodium acetate-ethanol, suspended in 10 µl of loading buffer (80% formamide-0.01% xylene cyanol-0.01% bromophenol blue in 1× TBE which contains 89 mM Tris borate, 89 mM boric acid, and 2 mM EDTA), and loaded onto a 1-mm-thick 8 M urea-8% acrylamide gel. Dried gels were subjected to autoradiography overnight at -70°C with an intensifying screen. Densitometry was performed with an Ultrascan XL densitometer (LKB Instruments, Inc.).

## RESULTS

**E1A/E1B proteins activate the HIV LTR.** Previous results with adenovirus recombinants containing the HIV LTR

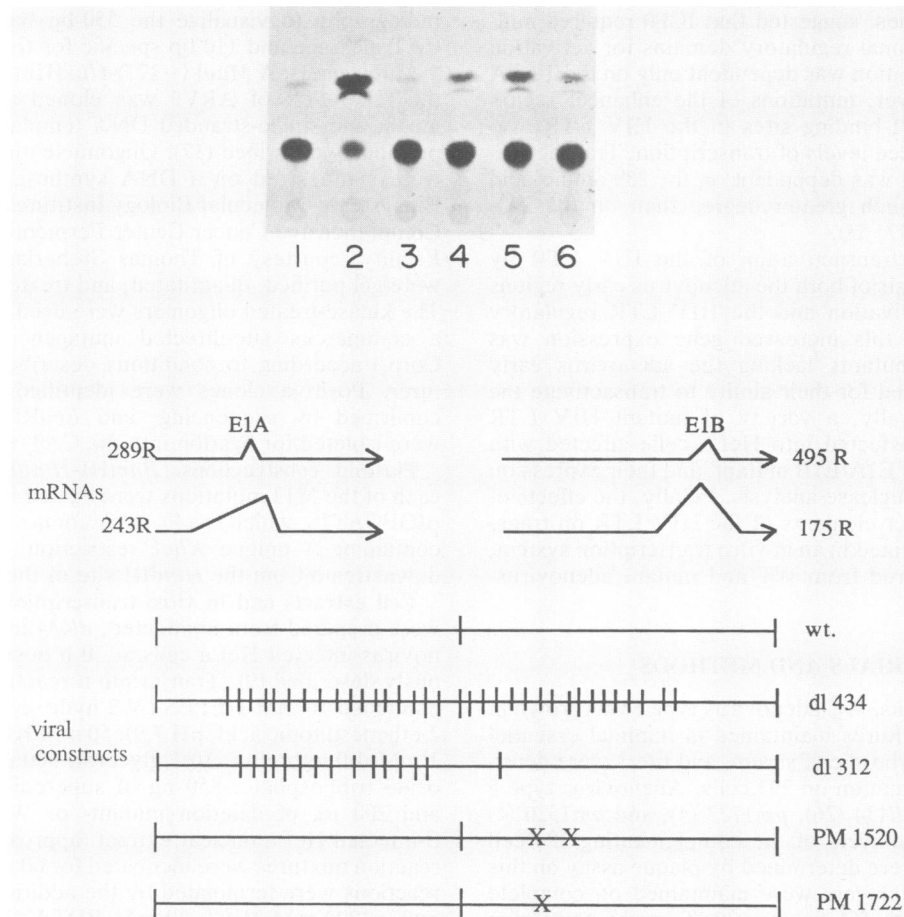


FIG. 1. E1A/E1B proteins activate the HIV LTR. The HIV LTR extending from  $-177$  to  $+83$  was fused to the CAT gene and transfected into HeLa cells either mock infected (lane 1) or infected with WT adenovirus (lane 2), *dl434* (lane 3), *dl312* (lane 4), *pm1520* (lane 5), or *pm1722* (lane 6). Cells were harvested at 36 h posttransfection, and CAT activity was determined. A schematic diagram of the E1A and E1B mRNAs and the mutant viruses used is also shown. The positions of deleted sequences (| | | |) and point mutations (X) are indicated.

fused to the CAT gene indicated that transactivation required the 289-amino-acid E1A protein but not the 243-amino acid protein (47, 53). To determine whether other adenovirus early regions were important in activation of the HIV LTR, viral mutants in the E1B, E2, E3, and E4 regions were tested for activation. An HIV LTR CAT construct extending from  $-177$  to  $+83$  in the LTR fused to the CAT gene was transfected into HeLa cells either mock infected or infected with WT adenovirus; *dl312*, an E1A deletion mutant (34); *dl434*, an E1A/E1B deletion mutant (26); *pm1520*, a double E1B point mutant that eliminates the 55-kilodalton (kDa) E1B protein (4); or *pm1722*, an E1B point mutant that eliminates the E1B 19-kDa protein (4). WT adenovirus infection resulted in a significant stimulation of HIV LTR CAT activity compared with that of mock-infected cells (Fig. 1, lanes 1 and 2). Infection with *dl434* resulted in a decreased stimulation of CAT activity compared with infection with *dl312*, suggesting a role for the E1B proteins in activation of the HIV LTR (Fig. 1, lanes 3 and 4). To ascertain which of the E1B proteins was responsible for this activation, two E1B mutant adenoviruses, *pm1520*, which interrupts the 55-kDa E1B protein, and *pm1722*, which interrupts the 19-kDa E1B protein, were both tested for their role in activation of the HIV LTR. Infection with either *pm1520* or *pm1722* resulted in a marked decrease in activation com-

pared with infection with WT adenovirus (Fig. 1, lanes 5 and 6). Thus, both E1B proteins appear to be involved in activation of the HIV LTR. Mutations in the E2, E3, and E4 regions of adenovirus had minimal effects on activation of the HIV LTR (data not shown). Table 1 shows the results of CAT conversion from three separate experiments.

S1 nuclease analysis with RNA prepared from these transfections was also performed. S1 analysis with an end-labeled CAT probe indicated that WT adenovirus induced

TABLE 1. CAT activity of HIV LTR CAT in the presence of adenovirus mutants

Virus	% CAT conversion <sup>a</sup>	Relative CAT activity
Mock	1.15 $\pm$ 0.35	0.02
WT	48.75 $\pm$ 4.65	1.00
<i>dl434</i>	1.09 $\pm$ 0.25	0.02
<i>dl312</i>	4.76 $\pm$ 0.68	0.10
<i>pm1520</i>	14.10 $\pm$ 1.54	0.29
<i>pm1722</i>	4.35 $\pm$ 0.51	0.09

<sup>a</sup> Both unacetylated and acetylated chloramphenicol (<sup>14</sup>C) were determined by scintillation counting of CAT assays to determine the percent CAT conversion in three separate experiments. The percent conversion for the WT construct was assigned a CAT activity of 1.00.

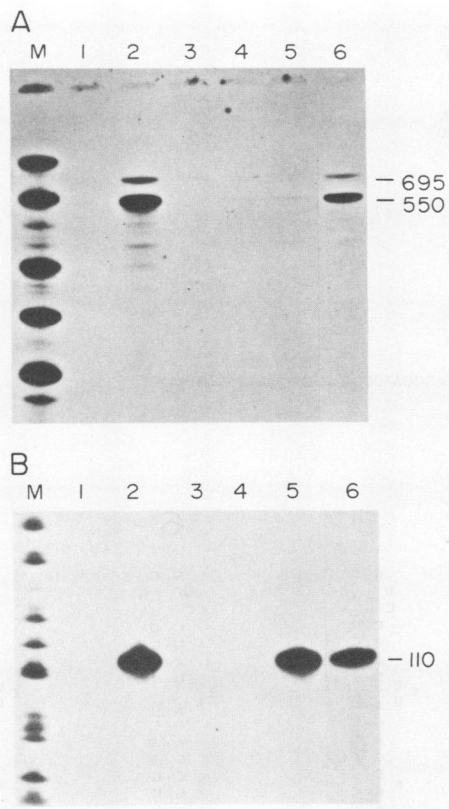


FIG. 2. S1 nuclease analysis of E1A/E1B mutant adenovirus activation of the HIV LTR. (A) HeLa cells were either mock infected (lane 1) or infected with WT adenovirus (lane 2), *dl434* (lane 3), *dl312* (lane 4), *pm1722* (lane 5), or *pm1520* (lane 6). RNA was prepared and used in S1 analysis. Both the 695-bp probe-specific band and the 550-bp CAT-specific band are indicated. (B) RNA from each of the transfections described above was also used for S1 analysis, using a 550-bp E1A probe. The 110-bp E1A-specific protected band is indicated.

HIV LTR CAT activity at least 20-fold compared with induction in mock-infected, *dl312*-infected, or *dl434*-infected cells (Fig. 2A). Prolonged exposures of this gel resulted in the detection of a specific protected band in *dl312*-infected but not *dl434*-infected cells. Infection with *pm1520* resulted in a large induction of CAT-specific message while *pm1722* failed to induce any CAT-specific message (Fig. 2A, lanes 5 and 6). S1 analysis was also performed with an E1A-specific probe. E1A message was present in high levels in cells infected with WT adenovirus, *pm1722*, and *pm1520* (Fig. 2B). These data indicate that the defects in HIV LTR CAT induction by *pm1722* and *pm1520* are not due to marked decreases in E1A mRNA. Thus, it appears that both the 19- and 55-kDa E1B proteins as well as the E1A proteins are involved in transactivation of the HIV LTR, though other early or late proteins may also be involved in this activation.

**Oligonucleotide-directed mutations of the HIV LTR.** Previous studies have defined several regions of the HIV LTR as being important for both basal and *tat*-induced levels of transcription (2, 10, 19, 31, 33, 36, 44, 46, 57). Oligonucleotide-directed mutagenesis was performed to change important nucleotides within the enhancer, SP1, TATA, and TAR regions of the HIV LTR. Mutants in both B motifs in the enhancer region ( $\Delta$ ENH), all three SP1-binding sites ( $\Delta$ SP1), and the TATA sequence ( $\Delta$ TATA) were constructed (Fig.

3A). Mutants were constructed in the TAR region which disrupt either or both CTCTCTGG direct repeats present between +5 and +12 [ $\Delta$ TAR(+11/+14)] and +37 and +44 [ $\Delta$ TAR(+40/+43)]. These repeats serve as binding sites for the cellular protein UBP-1 (64). Mutations that interrupt [ $\Delta$ TAR(+19/+22)] or restore [ $\Delta$ TAR(+19/+22)/(+40/+43)] the stem structure and that mutate the loop sequence [ $\Delta$ TAR(+31/+34)] were also constructed. A schematic diagram of these TAR mutants in terms of changes in RNA secondary structure is shown (Fig. 3B). Each of these constructs was then fused upstream of the CAT gene.

**Multiple HIV LTR regulatory regions required for complete E1A/E1B activation in vivo.** Each of these HIV LTR CAT constructs was transfected into HeLa cells infected with either WT adenovirus or *dl434*. Analysis of RNA prepared after transfection of the WT HIV LTR construct into *dl434*-infected cells resulted in a faint S1-protected band (Fig. 4A, lane 1). Transfection of the mutant HIV LTR constructs into *dl434*-infected cells did not result in detectable S1-protected bands (data not shown). Expression of the WT HIV LTR construct was stimulated 30-fold upon transfection into HeLa cells infected with WT adenovirus compared with transfection into *dl434*-infected cells (Fig. 4A, lane 2).

Mutation of both direct repeats in the enhancer region resulted in an approximately threefold decrease in steady-state RNA levels (Fig. 4A, lane 3). A dramatic decrease (>30-fold) in steady-state RNA levels was found for  $\Delta$ SP1 and  $\Delta$ TATA constructs compared with the WT HIV LTR construct when either was transfected into HeLa cells infected with WT adenovirus (Fig. 4A, lanes 4 and 5). Surprisingly, all mutations of the TAR region also gave lower levels of steady-state mRNA in the presence of WT adenovirus relative to the WT HIV LTR construct. Mutations in the loop sequence [ $\Delta$ TAR(+31/+34)], which serves as a DNA-binding site for UBP-2, and both CTCTCTGG direct repeats, which serve as DNA-binding sites for UBP-1 [ $\Delta$ TAR(+11/+14)/(+40/+43)], resulted in 12- and 8-fold decreases, respectively, in steady-state mRNA levels (Fig. 4A, lanes 8 and 10). Mutants which disrupt the stem structure [ $\Delta$ TAR(+40/+43) and  $\Delta$ TAR(+19/+22)] but which, when coupled, reform the stem structure [ $\Delta$ TAR(+19/+22)/(+40/+43)] were also tested. Each of these three mutants resulted in a two- to threefold reduction in the E1A/E1B-induced level of transcription (Fig. 4A, lanes 7, 9, and 11). Quantitation of the S1 analysis by densitometry is shown in Table 2. We note that we are unable to rule out an effect of these TAR mutations on mRNA stability in vivo. We have shown that these mutant RNAs have the same stability as the WT construct when synthesized in vitro (see below). In vitro nuclear runoff assays will be required to conclusively determine whether these mutations alter mRNA stability.

S1 analysis of E1A mRNA in each set of transfections was also performed (Fig. 4B). The same quantities of RNA from each of the transfections which was added with the HIV LTR CAT probe was also used with the E1A probes. This RNA was hybridized at 47°C (rather than 53°C, as for the CAT probe), which is the optimal temperature for hybridization for the E1A probe. The level of E1A mRNA was about equal (within twofold) in each transfection (Fig. 4B).

Taken together, these results indicate that the enhancer, SP1, TATA, and TAR regions are all necessary for WT E1A/E1B-induced levels of transcription. The decrease in transcription seen with mutants  $\Delta$ TAR(+11/+14)/(+40/+43), which interrupts UBP-1 binding sites (17), and  $\Delta$ TAR(+31/+34), which interrupts the UBP-2-binding site (17), indicates that these factor-binding sites are required for

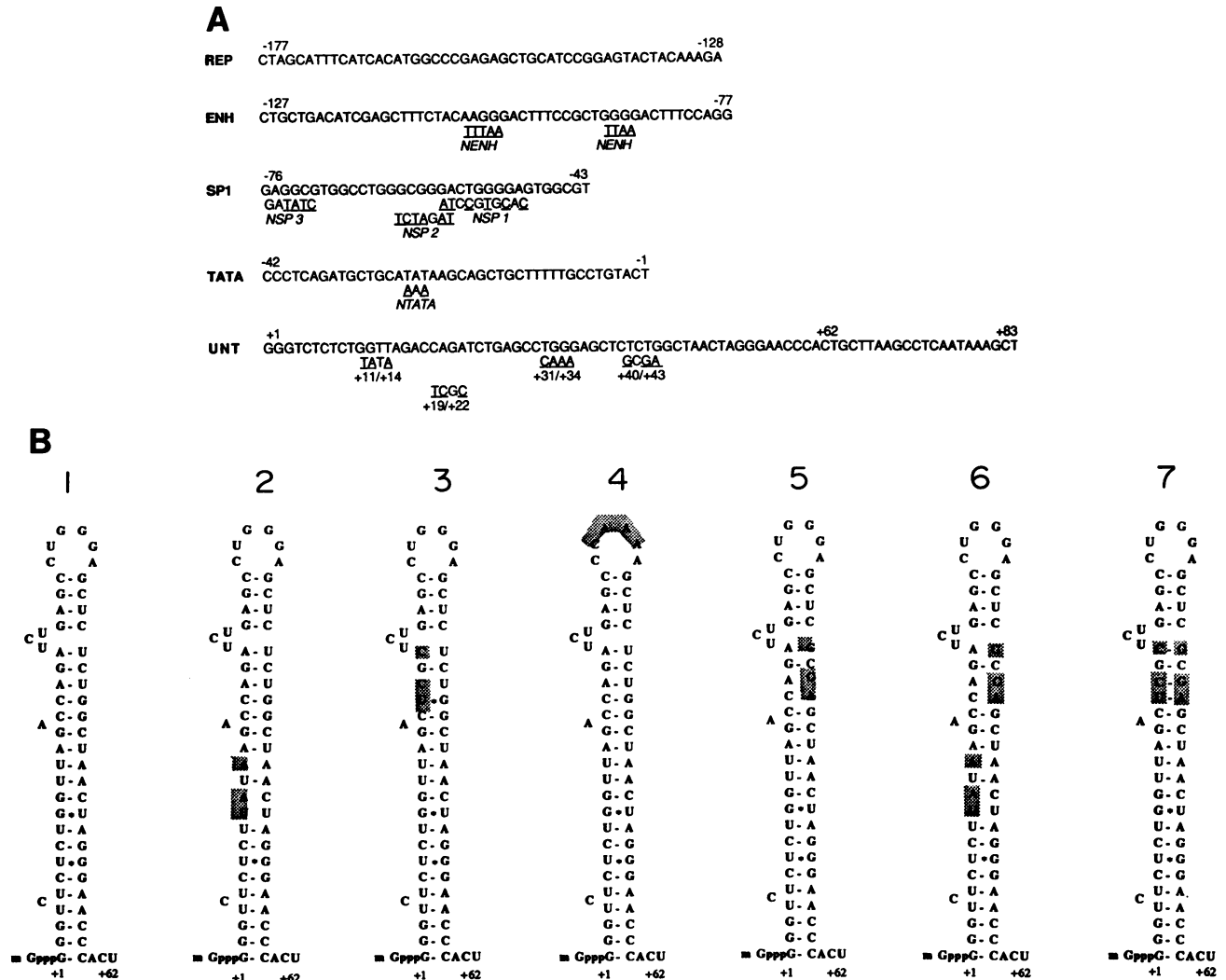


FIG. 3. HIV LTR sequences and oligonucleotide-directed mutations. (A) The HIV LTR extending from  $-177$  to  $+83$  and base substitutions introduced by oligonucleotide-directed mutagenesis in the enhancer (ENH), SP1, TATA, and TAR (UNT [untranslated]) regions are shown. (B) The stem-loop structure of the HIV LTR extending from  $+1$  to  $+62$  is shown for a series of constructs which extend to  $+80$  in the HIV LTR and were then fused to the CAT gene. 1, WT; 2,  $\Delta$ TAR(+11/+14); 3,  $\Delta$ TAR(+19/+22); 4,  $\Delta$ TAR(+31/+34); 5,  $\Delta$ TAR(+40/+43); 6,  $\Delta$ TAR(+11/+14)/(+40/+43); 7,  $\Delta$ TAR(+19/+22)/(+40/+43).

complete E1A/E1B-mediated activation. In contrast to *tat*-mediated induction, a strong dependence on base pairing in the stem structure was not observed for E1A/E1B-mediated activation. We emphasize that our inability to quantitate steady-state mRNA levels for the mutant LTR constructs in *dl434*-infected cells prevents us from determining whether any of the HIV LTR mutations decreases transcription in WT adenovirus-infected cell extracts to a greater degree than in *dl434*-infected cell extracts. Thus, we are unable to determine whether any HIV LTR promoter element(s) contributes disproportionately to transactivation by the E1A/E1B proteins.

**Multiple regulatory domains activate the HIV LTR in vitro.** We next wished to determine whether the *in vivo* activation of the HIV LTR by the E1A/E1B proteins could be reproduced in an *in vitro* transcription reaction using extracts containing the E1A/E1B proteins. As a first step, each of the HIV LTR mutants was tested in an *in vitro* transcription assay using uninfected HeLa cell nuclear extracts. The RNA from each reaction was analyzed by primer extension anal-

ysis, which generated a 163-bp transcript. A truncated WT HIV LTR template which generates a 142-bp transcript was included as an internal control (Fig. 5). Both of these products were sensitive to treatment with  $1 \mu\text{g}$  of alpha-amanitin per ml (Fig. 6C). Mutation of the enhancer sequences ( $\Delta$ ENH) resulted in a slight decrease in *in vitro* transcription from the HIV LTR (Fig. 5, lane 2). Mutations of the three SP1-binding sites ( $\Delta$ SP1) or the TATA element ( $\Delta$ TATA) resulted in no detectable primer-extended product (Fig. 5, lanes 3 and 4). Mutation of a portion of the first direct repeat in the TAR region, which serves as the binding site for UBP-1 (64) [ $\Delta$ TAR(+11/+14) or  $\Delta$ TAR(+11/+14)/(+40/+43)], also resulted in a significant decrease (approximately fivefold) in *in vitro* transcription of the HIV LTR (Fig. 5, lanes 5 and 10). Other mutations of the TAR region had little or no effect on transcription *in vitro*. These results suggest that multiple regions of the HIV LTR are required for full basal transcriptional activity *in vitro* (32, 33).

**Adenovirus E1A/E1B proteins activate the HIV LTR in vitro.** To study E1A/E1B-induced activation *in vitro*, tran-

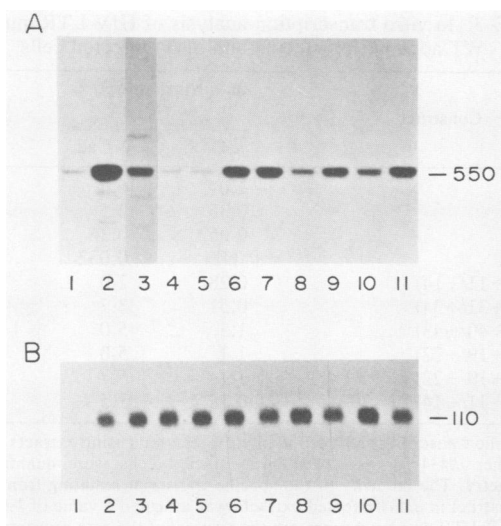


FIG. 4. S1 nuclease analysis of HIV LTR CAT constructs. (A) HeLa cells infected with *dl434* (lane 1) or WT adenovirus (lanes 2 to 11) were transfected with HIV LTR CAT constructs WT (lanes 1 and 2),  $\Delta$ ENH (lane 3),  $\Delta$ SP1 (lane 4),  $\Delta$ TATA (lane 5),  $\Delta$ TAR(+11/+14) (lane 6),  $\Delta$ TAR(+40/+43) (lane 7),  $\Delta$ TAR(+31/+34) (lane 8),  $\Delta$ TAR(+19/+22) (lane 9),  $\Delta$ TAR(+11/+14)/(+40/+43) (lane 10), and  $\Delta$ TAR(+19/+22)/(+40/+43) (lane 11). RNA was prepared and used in S1 analysis. The 550-bp CAT-specific S1-protected band is indicated. (B) RNA from each of the transfections in panel A was also used for S1 analysis using a 550-bp E1A probe. The 110-bp E1A-specific S1-protected band is indicated.

scription reactions were performed with each of the mutant HIV LTR constructs, using nuclear extracts prepared at 30 h postinfection from HeLa cells infected with either WT adenovirus or *dl434*. Extracts containing the E1A/E1B proteins were approximately sixfold more active for transcription from the WT HIV LTR template than extracts prepared from *dl434*-infected cells (Fig. 6A and B). Similar levels of stimulation were observed with nuclear extracts prepared in four independent experiments. Stimulation was observed in vitro over a wide range of template and extract concentrations (data not shown). We note that we are unable to determine whether the E1A/E1B proteins stimulate transcription from the HIV LTR directly or whether the E1A/E1B products result in the accumulation of other viral proteins which are, in turn, responsible for this stimulation. However, our in vivo analysis indicates that both the E1A and E1B proteins are required for this stimulation.

TABLE 2. S1 nuclease analysis of HIV LTR mutants in WT adenovirus-infected HeLa cells

Construct	S1 activity (% WT) <sup>a</sup>
WT	100.0
$\Delta$ ENH	34.5
$\Delta$ SP1	2.8
$\Delta$ TATA	2.3
$\Delta$ TAR(+11/+14)	46.0
$\Delta$ TAR(+19/+22)	30.2
$\Delta$ TAR(+40/+43)	44.4
$\Delta$ TAR(+19/+22)/(+40/+43)	44.0
$\Delta$ TAR(+11/+14)/(+40/+43)	13.5
$\Delta$ TAR(+31/+34)	9.1

<sup>a</sup> Densitometry of S1 nuclease mapping of HIV LTR constructs transfected into WT adenovirus-infected HeLa cells. The intensity of the S1-protected band for the WT HIV LTR construct was assigned a value of 100%.

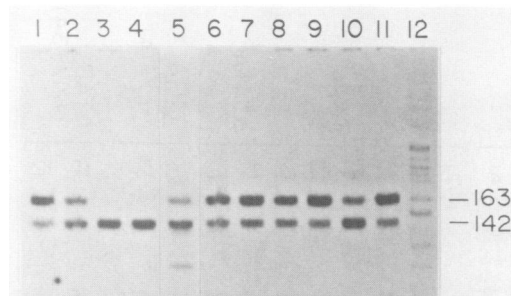


FIG. 5. In vitro transcription of HIV LTR CAT constructs. In vitro transcription reactions were performed in uninfected HeLa cell nuclear extracts using the HIV LTR constructs WT (lane 1),  $\Delta$ ENH (lane 2),  $\Delta$ SP1 (lane 3),  $\Delta$ TATA (lane 4),  $\Delta$ TAR(+11/+14) (lane 5),  $\Delta$ TAR(+31/+34) (lane 6),  $\Delta$ TAR(+19/+22) (lane 7),  $\Delta$ TAR(+40/+43) (lane 8),  $\Delta$ TAR(+19/+22)/(+40/+43) (lane 9),  $\Delta$ TAR(+11/+14)/(+40/+43) (lane 10), and WT (lane 11). Transcripts were analyzed by primer extension. Reactions contained 850 ng of each construct which resulted in a 163-bp primer-extended product and 400 ng of a truncated WT HIV LTR construct which resulted in a 142-bp primer-extended product. *Msp*I-cut pBR322 markers are shown in lane 12.

Mutation of the enhancer sequences resulted in an approximately threefold decrease in both the basal and E1A/E1B-induced levels of transcription in vitro (Fig. 6A, lanes 3 and 4). Mutations of the SP1 elements and, to a greater extent, the TATA element resulted in severe decreases in both basal and E1A/E1B-induced levels of transcription (Fig. 6A, lanes 5 to 8). It is important, however, that the TATA mutant was consistently transcribed more efficiently in extracts prepared from WT adenovirus-infected cells than in extracts prepared from *dl434*-infected cells (Fig. 6A, compare lanes 7 and 8). This result suggests that transcription factors in addition to the TATA-binding factor play a role in E1A/E1B-mediated transcriptional activation of the HIV LTR.

Mutations in the first direct repeat of the TAR region [ $\Delta$ TAR(+11/+14) and  $\Delta$ TAR(+11/+14)/(+40/+43); Fig. 6A and B, lanes 9 and 10] also significantly decreased basal and E1A/E1B-induced levels of transcription. Other mutants in the TAR region had minimal effects on in vitro transcription from the HIV LTR (Fig. 6A and B). This difference in in vitro transcription was not due to differences in the stability of mRNA in *dl434*-infected and WT adenovirus-infected cell extracts (data not shown). Furthermore, none of the TAR mutants showed any significant change in RNA stability during a 1-h incubation in uninfected HeLa cell extract (data not shown).

Thus, elements within the TAR region, in particular the UBP-1-binding site located between +5 and +12, in addition to the enhancer, SP1, and TATA regions are important for both basal and E1A/E1B-induced levels of transcription in vitro. In an effort to determine the relative importance of the individual promoter elements in E1A/E1B-mediated induction of the HIV LTR, the transcripts shown in Fig. 6 were quantitated via densitometry. Mutations in the enhancer, SP1, and the first CTCTCTGG motif of the TAR region all reduced transcription to roughly the same extent in both WT- and *dl434*-infected cell extracts, thus maintaining a constant relative fold induction (Table 3 and Fig. 6). No mutation specifically abolished E1A/E1B-mediated induction. We were unable to determine the ratio of transcription in WT- versus *dl434*-infected cell extracts for the TATA mutant because of the inability to detect specific transcripts



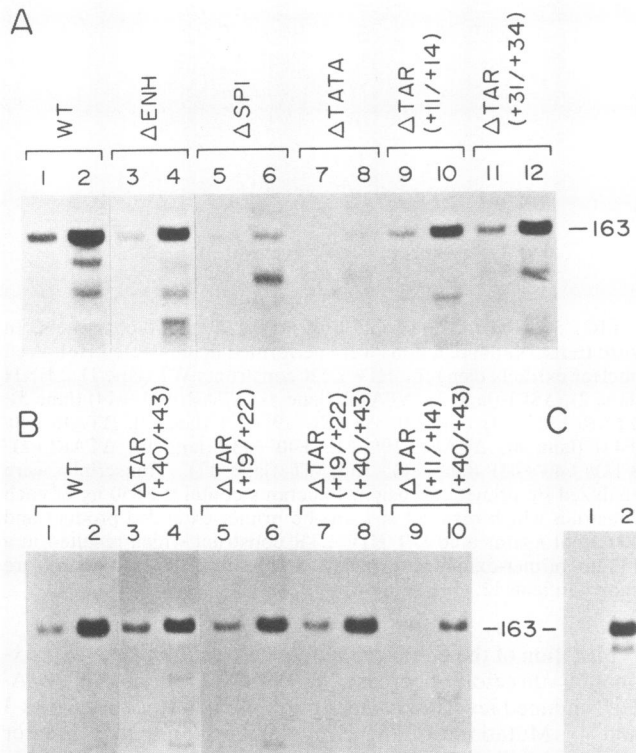


FIG. 6. In vitro transcription of HIV LTR CAT constructs in *dl434*- and WT adenovirus-infected cell extracts. (A) HIV LTR constructs for WT (lanes 1 and 2),  $\Delta$ ENH (lanes 3 and 4),  $\Delta$ SP1 (lanes 5 and 6),  $\Delta$ TATA (lanes 7 and 8),  $\Delta$ TAR(+11/+14) (lanes 9 and 10), and  $\Delta$ TAR(+31/+34) (lanes 11 and 12) were used in in vitro transcription reactions performed with either *dl434* (lanes 1, 3, 5, 7, 9, and 11)- or WT adenovirus (lanes 2, 4, 6, 8, 10, and 12)-infected HeLa cell extracts. The position of the 163-bp primer extended product is indicated. (B) HIV LTR constructs for WT (lanes 1 and 2),  $\Delta$ TAR(+40/+43) (lanes 3 and 4),  $\Delta$ TAR(+19/+22) (lanes 5 and 6),  $\Delta$ TAR(+19/+22)/(+40/+43) (lanes 7 and 8), and  $\Delta$ TAR(+11/+14)/(+40/+43) (lanes 9 and 10) were used in in vitro transcription reactions performed with either *dl434* (lanes 1, 3, 5, 7, and 9)- or WT adenovirus (lanes 2, 4, 6, 8, and 10)-infected HeLa cell extracts. The position of the 163-bp primer-extended product is indicated. (C) WT HIV LTR constructs were incubated with uninfected HeLa nuclear extract in either the presence (lane 1) or absence (lane 2) of 1  $\mu$ g of  $\alpha$ -amanitin per ml.

in *dl434*-infected cell extracts. However, as mentioned above, the  $\Delta$ TATA construct was clearly transcribed more efficiently in extracts prepared from WT adenovirus-infected cells than from *dl434*-infected cells. Together, these results indicate that the same promoter elements are required for both basal and E1A/E1B-induced levels of transcription from the HIV LTR and that any reduction in E1A/E1B-induced expression is accompanied by a proportional decrease in basal levels of HIV LTR transcription.

## DISCUSSION

In this report, we have investigated the sequence elements required for adenovirus-induced activation of the HIV LTR. Our results demonstrate that multiple regulatory domains within the HIV LTR, including the enhancer, SP1, TATA, and TAR regions, are required for both full basal and adenovirus-induced levels of transcription in vivo and in vitro. Our observation that maintenance of the stem-loop

TABLE 3. In vitro transcription analysis of HIV LTR mutants in WT adenovirus-infected and *dl434*-infected cells

Construct	In vitro transcription activity <sup>a</sup>		Induction (fold)
	<i>dl434</i>	WT ad	
WT	1.0	6.4	6.4
$\Delta$ ENH	0.35	2.5	7.1
$\Delta$ SP1	0.052	0.26	5.0
$\Delta$ TATA	ND	0.052	
$\Delta$ TAR(+11/+14)	0.28	2.0	7.1
$\Delta$ TAR(+31/+34)	0.51	3.7	7.3
$\Delta$ TAR(+40/+43)	1.3	5.0	3.8
$\Delta$ TAR(+19/+22)	1.1	5.0	4.5
$\Delta$ TAR(+19/+22)/(+40/+43)	1.1	5.7	5.2
$\Delta$ TAR(+11/+14)/(+40/+43)	0.22	1.5	6.8

<sup>a</sup> Specific transcripts produced in in vitro reactions using extracts prepared from either *dl434*- or WT adenovirus-infected cells were quantitated by densitometry. The intensity of the specific transcript resulting from the WT LTR construct in *dl434*-infected extracts was assigned a value of 1.0. Values for the WT LTR construct represent the average of the data presented in Fig. 6A and B. ad, Adenovirus; ND, not detectable.

structure within the TAR region was not essential for efficient transactivation of the HIV LTR by adenovirus suggests that adenovirus-mediated transcriptional induction occurs via a mechanism distinct from that of *tat*-induced activation. Furthermore, we have shown that both the 19- and 55-kDa E1B proteins are important in addition to the E1A proteins for full transactivation of the HIV LTR. It was recently shown that, in the absence of functional E1A products, the 19-kDa E1B protein can stimulate adenovirus early promoters to a small degree during infection (61). Studies with the 55-kDa E1B adenovirus mutant have suggested that this virus is defective for viral mRNA transport and subsequent viral protein expression, perhaps explaining its failure to completely activate the HIV LTR (4). The mechanisms of E1B-induced gene activation and its role on E1A expression have not yet been elucidated.

Previous studies have shown that viral immediate-early proteins such as ICP0 and E1A can stimulate transcription from the HIV LTR (47, 53). Nabel et al. (47) suggested that E1A-mediated induction of the HIV LTR required the TATA element but that ICP0 could function in the absence of TATA. Studies with several viral and cellular promoters have indicated that the TATA element is essential for E1A-mediated induction of transcription (25, 58, 65). In vitro studies using the adenovirus major late promoter have indicated that the TATA region is required for induction by both E1A (39) and pseudorabies immediate-early proteins (1). These data suggest that E1A might stimulate transcription through interactions with the TATA-binding factor. However, other promoters which either lack a TATA element (e.g., adenovirus early region 2 gene) (30, 45) or which contain mutations in the TATA motif (e.g., adenovirus early region 3 gene) (18, 38) have been shown to be E1A inducible. In addition, genes transcribed by RNA polymerase III have also been shown to be activated by E1A (for a review, see reference 5a). Thus, multiple promoter elements appear to contribute to induction of transcription by the E1A protein. The present study shows that, with the HIV LTR, the same sequence elements which confer basal expression are necessary for E1A/E1B-induced levels of transcription. Mutations that reduce E1A/E1B-induced expression also result in a proportional decrease in basal levels of transcription, thereby maintaining a constant relative fold induction. Consistent with our results, Williams et al. (62) recently found

that transactivation of the human hsp70 promoter is mediated through a functional basal transcription complex.

Our data suggest that E1A/E1B-mediated induction occurs via a mechanism distinct from that of *tat*-induced transcriptional activation. Efficient *tat* induction has been shown to be dependent on the presence of the enhancer, SP1, and TATA sequences (19). However, *tat* induction also requires the presence of a stem-loop structure in the downstream TAR region of the HIV LTR. Mutations which destabilize the upper portion of the stem or alter the loop sequences have been shown to severely reduce *tat*-induced expression of the HIV LTR, as measured by transient-expression assays (12, 31). Compensatory mutations which reform the stem structure restore *tat*-induced HIV expression to nearly WT levels (12). The results of our S1 nuclease analysis of TAR mutants demonstrate that E1A/E1B-induced transcription from the HIV LTR is not strongly dependent on maintenance of the stem-loop structure in the TAR region. This observation, in conjunction with previous studies indicating that the effects of the E1A and *tat* proteins are additive for activation of the HIV LTR (47, 53), suggests that the E1A/E1B proteins exert their effect in a manner distinct from that of *tat*. A mechanism by which E1A/E1B-mediated activation of the TAR region occurs by interaction with TAR-binding proteins such as UBP-1 (64), UBP-2 (17), LBP (33), and CTF-NF-1 (33), rather than through interaction with RNA secondary structure, seems likely.

The results of S1 analysis of HIV LTR mutants analyzed *in vivo* correlate well with data obtained from *in vitro* transcriptional analysis. This is seen by comparing the E1A/E1B-mediated activation *in vivo* for the various mutants (Table 2) and the *in vitro* assays performed with WT adenovirus-infected extracts (Table 3). The enhancer region, the TATA element, and the three SP1-binding sites were all shown to be required for full basal and E1A/E1B-induced levels of transcription *in vitro* as well as *in vivo*. The SP1 mutations alter the pattern of binding over the TATA and TAR regions and affect *tat*-induced activation (27). These results and previous deletion analysis (19) suggest a role for this region in stabilizing TATA factor binding. Similar interactions of TATA-binding factors with factors binding to upstream regions have been described previously (27, 56).

The importance of elements within the TAR region was also reflected in *in vitro* transcription assays. In particular, mutations that disrupted the first direct repeat located between sequences +5 and +12 significantly reduced basal and E1A/E1B-induced levels of HIV LTR transcription. These results are in agreement with previous data which had indicated a role for the TAR region in basal levels of HIV LTR transcription *in vitro* (33). Recently, a protein, UBP-1, has been purified which appears to bind to both the CTC TCTGG direct repeat located between +5 and +12 and the TATA element (64). Since assays of other promoters have revealed a stronger dependence on the TATA element *in vitro* than *in vivo* compared with other regulatory elements (38), the results presented here could be explained if the proximal elements of the TAR region function in concert with the SP1-binding sites to stabilize TATA factor binding. We point out that mutation of the loop sequences, which resulted in a dramatic decrease in E1A/E1B-induced levels of steady-state RNA *in vivo*, had no significant effect in our *in vitro* analysis. This apparent discrepancy may reflect a role for the loop region at a posttranscriptional level. Characterization of RNA- and DNA-binding proteins which interact with the loop sequences will be required to determine the role of the loop region in HIV gene expression.

Our finding that multiple promoter elements contribute to both basal and E1A/E1B-induced levels of HIV LTR expression *in vitro* as well as *in vivo* is consistent with a model in which the E1A/E1B proteins stimulate the formation and/or stability of active transcription complexes. In the HIV LTR, this complex likely contains both upstream (enhancer, SP1, TATA) and downstream (UBP-1 and UBP-2) cellular DNA-binding proteins. The ability to correlate *in vivo* effects in the HIV LTR with *in vitro* assays should provide a mechanism to dissect those factors required for E1A/E1B-mediated transcriptional induction. Studies using purified HIV LTR-binding proteins such as SP1, EBP-1, UBP-1, and UBP-2 will be important in determining the various levels of control required for the *in vitro* activation of the HIV LTR. These studies should provide a better understanding of the mechanisms of HIV transcriptional regulation and provide the ability to distinguish between promoter elements required for transactivation by the E1A/E1B and *tat* proteins.

#### ACKNOWLEDGMENTS

We thank Charles Leavitt for preparation of the manuscript and the laboratory of Arnie Berk for providing the adenovirus mutants used in this study.

This work was supported by grants from the California Universitywide AIDS Task Force and by Public Health Service grants AI-25288, CA30981, and AI-18272 from the National Institutes of Health. R.G. was supported by grant JFRA-146 from the American Cancer Society, A.D. was supported by a Faculty Research Award from the American Cancer Society, S.K. was supported by Public Health Service award GM-07185 from the National Institutes of Health, and J.G. was supported by Public Health Service grant GMO-80942 from the National Institutes of Health to the UCLA Medical Scientist Training Program.

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