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Repression In Vitro, by Human Adenovirus E1A Protein Domains, of Basal or Tat-Activated Transcription of the Human Immunodeficiency Virus Type 1 Long Terminal Repeat

CHAO-ZHONG SONG, PAUL M. LOEWENSTEIN, AND MAURICE GREEN*

Institute for Molecular Virology, Saint Louis University School of Medicine, St. Louis, Missouri 63110

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Human adenovirus E1A proteins can repress the expression of several viral and cellular genes. By using a cell-free transcription system, we demonstrated that the gene product of the E1A 12S mRNA, the 243-residue protein E1A243R, inhibits basal transcription from the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR). The HIV-1 transactivator protein Tat greatly stimulates transcription from the viral promoter in vitro. However, E1A243R can repress Tat-activated transcription in vitro. Strong repression of both basal and Tat-activated transcriptions requires only E1A N-terminal amino acid residues 1 to 80. Deletion analysis showed that E1A N-terminal amino acids 4 to 25 are essential for repression, whereas amino acid residues 30 to 49 and 70 to 80 are dispensable. Transcriptional repression by E1A in the cell-free transcription system is promoter specific, since under identical conditions, transcription of the adenovirus major late promoter and the Rous sarcoma virus LTR promoter was unaffected. The repression of transcription by small E1A peptides in vitro provides an assay for investigation of molecular mechanisms governing E1A-mediated repression of both basal and Tat-activated transcriptions of the HIV-1 LTR promoter.

The adenovirus (Ad) E1A gene encodes two major proteins; one is 243 residues long (E1A243R), and the other is 289 residues long (E1A289R). These proteins exert pleiotropic effects on gene expression and on host cell growth. It is of particular interest that E1A243R can repress the expression of a set of cellular genes. The biological functions of the cellular genes repressed by E1A coupled with the fact that E1A sequences required for repression are also required for E1A-mediated alterations in cellular DNA synthesis, cellular transformation, cell differentiation, and metastasis suggest a close relationship between E1A-mediated transcriptional regulation and cell growth control (for reviews, see references 4, 5, and 35).

The expression of several viral genes is also inhibited by E1A, including Ad early genes (10), those of simian virus 40 (6, 31, 42), murine polyomavirus (6, 41), and human immunodeficiency virus type 1 (HIV-1) (43, 44). HIV-1 is the cause of AIDS (3, 14, 28), and the expression of HIV-1 genes is modulated by several factors that regulate the activity of the HIV long terminal repeat (LTR) promoter (for reviews, see references 7 and 39). The HIV LTR is composed of *cis*-regulatory sequences which include a TATA box, DNA binding sites for Sp1 and NF-κB, and the downstream Tat-responsive RNA element TAR, among others (19, 26, 33). HIV gene expression and replication are dependent upon two *trans*-acting viral regulatory proteins, Tat and Rev, which are unique in that they bind to RNA targets, TAR and the Rev-responsive element RRE, respectively. Tat functions primarily as a transcriptional activator, and Rev acts mainly at the posttranscriptional level to increase the cytoplasmic accumulation of incompletely spliced viral RNA (9, 17, 32, 34, 39). HIV LTR expression is also stimulated by heterologous viral genes (20, 29, 30, 45). Previous studies have revealed opposite effects of the two major E1A gene products on HIV LTR expression. E1A289R has been reported to transactivate the HIV LTR, whereas E1A243R exhibits inhibitory activity in transient transfection assays (27, 30, 43, 44).

Despite important progress in understanding E1A transcriptional activation (for reviews, see references 4, 22, and 35), the molecular mechanism of E1A-mediated transcriptional repression remains a mystery. This is due in part to the lack of common target sequences in E1A-repressible promoters and the need for a suitable biochemical system to analyze transcription repression by E1A. To understand how E1A inhibits HIV LTR expression, we have developed a cell-free transcription repression assay and demonstrated that small E1A polypeptides repress both Tat-independent and Tat-activated transcriptions of the HIV-1 LTR efficiently in vitro.

MATERIALS AND METHODS

Plasmids. HIVLTR(−533)CAT (pBennCAT) (16), pHIV-TAR(+) (23), MLPCAT (10), and RSVCAT were used as templates for in vitro transcription. Ad type 5 E1A243R and mutants E1A1-80, E1A1-80Δ4-25, E1A1-80Δ30-49, and E1A1-80Δ70-80 were constructed as described elsewhere (37).

Preparation of recombinant proteins. The Tat protein used for in vitro Tat transactivation was purified as previously described (36). The E1A proteins used in this study are shown schematically in Fig. 1A. The expression of histidine-tailed E1A243R, E1A1-80, and deletion mutants in *Escherichia coli* M15 and purification by Ni-nitrilotriacetic acid affinity chromatography were performed as described elsewhere (37). The purity of the recombinant proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining (Fig. 1B). Quantitation of recombinant proteins was performed with a Bio-Rad protein assay kit.

In vitro transcription. Nuclear extract preparation and Tat transactivation reactions were performed as previously described (36). E1A repression reactions (25 μl) contained 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9); 4 mM creatine phosphate; 0.1 mM EDTA; 10 mM $MgCl_2$; 0.7 mM dithiothreitol; 60 mM KCl; 8% glycerol; 500 μM each ATP, CTP, GTP, and UTP; 100 to 500 ng of template DNA; 20 U of placental RNase inhibitor (Boehringer Mannheim); and 40 μg of nuclear extract proteins. Purified recombinant E1A243R, E1A1-80, and deletion mutant proteins in phosphate-buffered

* Corresponding author. Phone: (314) 577-8401. Fax: (314) 577-8419. Electronic mail address: green@SLUVCA.SLU.EDU.

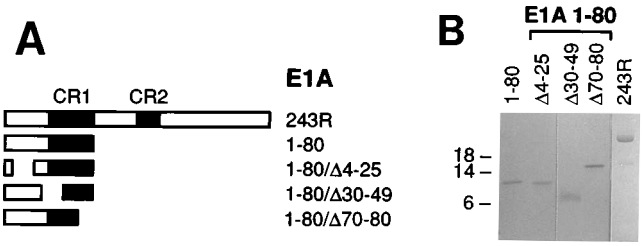

FIG. 1. Recombinant E1A and E1A mutant polypeptides used in the in vitro transcription repression assay. (A) Schematic representation of E1A243R and deletion mutants. (B) Evaluation of the purity of the E1A polypeptides by SDS-PAGE. Purified recombinant E1A243R, E1A1-80, and E1A1-80 deletion polypeptides were analyzed by SDS-PAGE and stained with Coomassie blue. E1A243R was run on a 12% acrylamide gel, and E1A1-80 and derivatives were run on a 15% acrylamide gel. The numbers to the left of the gel are molecular sizes in kilodaltons. CR1 and CR2, conserved regions 1 and 2, respectively.

saline were added to the transcription reaction where indicated. The primer extension reaction and runoff assays for transcript detection were performed as previously described (36).

RESULTS

The Ad E1A243R protein specifically represses transcription of the HIV LTR promoter in vitro. The Ad E1A 12S gene product has been reported to inhibit expression of the HIV-1 LTR promoter in transient transfection assays (43, 44). To investigate the molecular mechanism of E1A repression of the HIV LTR, we used an in vitro transcription reaction for expression of the HIV LTR that is able to respond to activation by addition of the purified HIV-1 Tat transactivator protein (36). The E1A243R protein was tested for the ability to repress basal in vitro transcription of the HIV-1 LTR promoter, i.e., in the absence of Tat. As shown in Fig. 2A, E1A243R inhibited HIV LTR transcription in a dose-dependent manner.

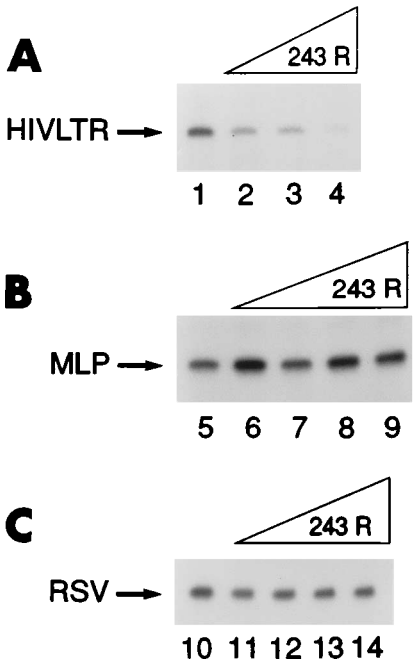

FIG. 2. Specific repression in vitro of the HIV LTR promoter by purified recombinant E1A243R. (A) HIVLTR(−533)CAT, (B) MLPCAT, and (C) RSV CAT were used as templates for the in vitro transcription reaction. Transcripts were detected by primer extension as described in Materials and Methods. Lanes 2 to 4 received 300, 600, and 1,200 ng of the E1A243R protein, respectively. Lanes 6 to 9 and 11 to 14 received 300, 600, 1,200, and 1,800 ng of the E1A243R protein, respectively.

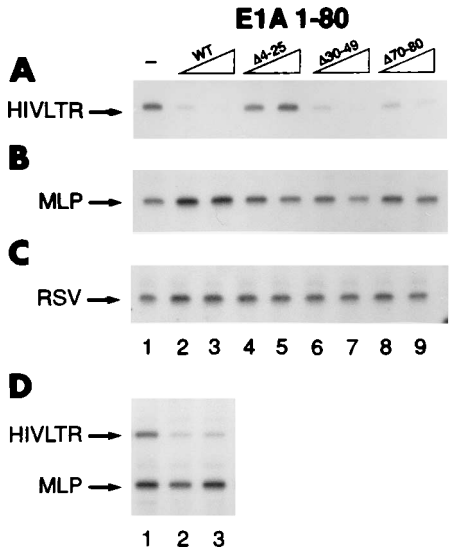

FIG. 3. Specific repression of the HIV LTR promoter by purified recombinant E1A1-80 and deletion polypeptides. (A) HIVLTR(−533)CAT, (B) MLP CAT, and (C) RSVCAT were used as templates in the in vitro transcription reaction, and RNA products were analyzed by primer extension. Lanes 2 and 3 contained 200 and 400 ng of E1A1-80, respectively. Lanes 4 and 5 contained 200 and 400 ng of E1A1-80Δ4-25. Lanes 6 and 7 contained 200 and 400 ng of E1A1-80Δ30-49. Lanes 8 and 9 contained 200 and 400 ng of E1A1-80Δ70-80. (D) HIVLTRCAT and MLPCAT templates were included in the same transcription reaction and analyzed by primer extension. Lanes 2 and 3 contained 200 and 400 ng, respectively, of E1A1-80. WT, wild type.

To rule out the possibility of nonspecific inhibition under in vitro transcription conditions, the major late promoter (MLP), which is substantially unaffected by the E1A243R protein in vivo (10, 13), was used as a control template for in vitro transcription. No inhibition of the MLP was observed with the same and higher levels of E1A243R (Fig. 2B, lanes 6 to 9) that inhibited the HIV LTR (Fig. 2A). To further confirm the specificity of repression of the HIV LTR by E1A, a second control promoter, RSVCAT, was tested. As observed with the MLP, the Rous sarcoma virus promoter was not affected by E1A243R (see Fig. 4C, lanes 11 to 14). From these results, we conclude that the full-length E1A243R protein specifically inhibits the transcription of the HIV-1 LTR in vitro.

E1A N-terminal residues 1 to 80 are sufficient for repression of HIV LTR transcription. To define the minimal E1A protein regions required for repression of the HIV LTR, we did a mutational analysis of E1A polypeptides. From previous studies, it appears that conserved region 1 (amino acids ~40 to 80) (25) and the relatively nonconserved N-terminal region are essential and can be sufficient for mediation of transcriptional repression by E1A, although several reports indicate that other regions are needed under certain conditions (for a review, see reference 4). Therefore, we first prepared and tested the ability of the E1A1-80 polypeptide to repress HIV LTR activity in vitro. As shown in Fig. 3A, E1A1-80 efficiently inhibited HIV LTR transcription in a dose-dependent manner (compare lane 1 with lanes 2 and 3). The dose-dependent relationship was evidenced further by the observation that addition of increasing amounts of nuclear extract to the E1A-repressed reaction mixture overcame E1A repression, presumably because of the addition of a factor(s) present in the added nuclear extract that interacts with E1A (Fig. 4).

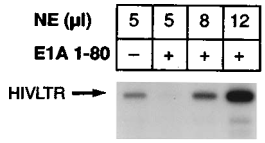

FIG. 4. Repression of HIV LTR transcription in vitro by E1A1-80 is reversed by additional nuclear extract (NE) in a dose-dependent manner. HIVLTR (−533)CAT was used as the template in the in vitro transcription reaction, and RNA products were analyzed by primer extension. The leftmost lane contained no E1A1-80, and the other lanes contained 400 ng of E1A1-80. The volume of nuclear extract used in the reaction is indicated above each lane.

To further map the sequences responsible for repression in E1A1-80, E1A1-80Δ4-25, E1A1-80Δ30-49, and E1A1-80Δ70-80 were tested for the ability to repress HIV LTR transcription in vitro. As shown in Fig. 3A, deletion of residues 4 to 25 completely abrogated the inhibitory function of E1A1-80. However, E1A1-80Δ30-49 and E1A1-80Δ70-80 repressed transcription of the HIV LTR as efficiently as did wild-type E1A1-80. Control templates MLPCAT and RSVCAT were not inhibited by either of these mutants (Fig. 3B and C). As additional evidence of true promoter specificity, the HIV-LTRCAT and MLPCAT templates were mixed together and analyzed for E1A repression by primer extension. E1A1-80 levels that blocked HIV LTR transcription did not affect MLP transcription (Fig. 3D). To summarize, E1A1-80, which includes nonconserved residues 1 to 39 and conserved region 1, is sufficient for repression of gene expression from the HIV LTR in vitro. Repression is highly specific because both the MLP and the Rous sarcoma virus promoter were not affected. Although the E1A extreme N-terminal sequence between residues 4 and 25 is absolutely required for the E1A repression function, sequences within the regions that contain residues 30 to 49 and 70 to 80 are nonessential.

Effect of E1A on HIV-1 Tat transactivation of the viral LTR. The results described above show that E1A strongly and specifically represses basal transcription from the HIV LTR promoter in vitro. However, transcription from the viral LTR is stimulated up to 1,000-fold by the HIV-1 Tat transactivator (for reviews, see references 9 and 39). Therefore, it was of interest to determine whether E1A could repress Tat-activated transcription. For these experiments, an in vitro transcription system which responds to the addition of purified recombinant Tat was used (21, 36). Tat (100 ng) greatly stimulated transcription from the HIV LTR, as measured by a runoff transcription assay (Fig. 5, compare lanes 1 and 2). E1A243R was able to repress Tat-activated transcription in a dose-dependent manner (Fig. 4, lanes 3 to 6).

It was of further interest to determine whether the E1A1-80 polypeptide can also repress Tat transactivation. As shown in Fig. 6, E1A1-80 was indeed able to block Tat transactivation effectively (compare lanes 3 and 4 with lane 2). When the

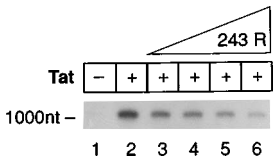

FIG. 5. Repression of Tat-activated transcription of the HIV LTR promoter by the E1A243R protein. A runoff transcription assay for Tat transactivation was performed as described in Materials and Methods (36). Lanes 2 to 6 contained 100 ng of purified recombinant HIV-1 Tat1-86. Lanes 3 to 6 contained 300, 600, 1,200, and 1,800 ng of the E1A243R protein. nt, nucleotides.

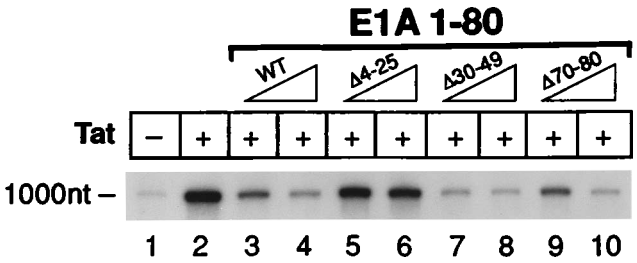

FIG. 6. Repression of Tat-activated transcription of the HIV LTR promoter by E1A1-80 and deletion polypeptides. Lanes 2 to 10 contained 100 ng of purified Tat1-86. Lanes 3 and 4 contained 200 and 400 ng of E1A1-80, respectively. Lanes 5 and 6 contained 200 and 400 ng of E1A1-80Δ4-25. Lanes 7 and 8 contained 200 and 400 ng of E1A1-80Δ30-49, respectively. Lanes 9 and 10 contained 200 and 400 ng of E1A1-80Δ70-80, respectively. WT, wild type. nt, nucleotides.

E1A1-80 deletion mutants were tested, the same pattern of sequence requirements for inhibition as for Tat-independent transcription was observed (Fig. 6). The inhibition of Tat transactivation by E1A1-80, E1A1-80Δ30-49, and E1A1-80Δ70-80 showed a dose-dependent pattern. Although inclusion of 400 ng of E1A1-80, E1A1-80Δ30-49, and E1A1-80Δ70-80 repressed Tat transactivation strongly, no significant inhibition was observed when up to 600 ng of E1A1-80Δ4-25 was added. In conclusion, the above-described results demonstrate that E1A1-80 can specifically repress both Tat-independent and Tat-activated transcription of the HIV-1 LTR promoter.

DISCUSSION

To understand how E1A represses transcription from the HIV LTR, we developed an in vitro transcription system in which the HIV LTR is activated by the HIV-1 Tat protein (36) and is repressed by specific Ad E1A polypeptides. By using this assay, we showed that both Tat-independent and Tat-activated transcriptions of the HIV-1 LTR are repressed by the E1A243R protein in vitro. The observed transcriptional repression by E1A is not indiscriminate, since transcription of the MLP and the Rous sarcoma virus promoter is relatively unaffected. The results presented in this study support the conclusion that specific protein-protein interactions are involved in E1A repression. First, the ability of E1A to repress transcription of the HIV LTR promoter in a nuclear extract of uninfected cells demonstrates that no E1A-inducible cellular gene product is required for repression. Second, the dose-dependent nature of repression in vitro suggests that the added E1A protein interacts with a cellular factor(s) in the nuclear transcription extract. The general nature of these conclusions is supported by the fact that other E1A-repressible promoters, including those of simian virus 40, interstitial collagenase, and rat insulin II, are repressed by E1A polypeptides under in vitro, as well as in vivo, conditions in which protein synthesis does not occur (37).

By using deletion mutant E1A proteins, we delineated the regions of E1A that are necessary and sufficient for mediation of repression in vitro. Deletion mutant polypeptides E1A1-80Δ30-49 and E1A1-80Δ70-80 showed repression efficiencies similar to that of wild-type E1A1-80 (Fig. 3). However, deletion of the N-terminal region between amino acids 4 and 25 completely impaired the ability of E1A1-80 to repress transcription. Although the primary structure of the E1A N-terminal sequence is not conserved among different E1A serotypes (40), it has been suggested that there is conservation of a predicted α-helical structure containing the conserved three-amino-acid sequence ILE (15). The only cellular protein iden-

tified to date that depends upon amino-terminal residues ~1 to 25 of E1A for in vivo association is the 300-kDa cellular protein p300 (for a review, see reference 24). p300 has recently been cloned, and functional analysis has revealed that p300 lacking an intact E1A-binding site can overcome E1A repression of the simian virus 40 promoter in transient expression analysis (11). On the basis of the high degree of homology between p300 and CREB-binding protein CBP (8), it was proposed that p300 and CBP belong to a conserved family of coactivators (1). Our results are consistent with the possibility that p300 or members of the p300 family are involved in E1A repression. E1A1-80Δ4-25 is defective in our in vitro repression assay, whereas E1A1-80Δ30-49 and E1A1-80Δ70-80 are active. Similar sequence requirements were reported for coimmunoprecipitation of E1A with p300 and for E1A repression by transient expression analysis (2, 12, 18, 38, 46). Although deletion of amino acid residues 30 to 49 or 70 to 80 in the E1A243R background has been reported to reduce the capacity to bind to p300 somewhat (2), our results with the same two mutants in the E1A1-80 polypeptide background showed nearly wild-type efficiency in transcriptional repression. These quantitative differences between the efficiency of in vivo binding and in vitro transcription repression can be attributed to the inherent difference between the natures of these two assays.

With an enhancer test system, it was found that HIV LTR sequences between −106 and −77, which contain binding sites for NF-κB and enhancer-binding protein 1, can mediate E1A inhibition of transcription (43). However, as noted by the investigators, the role of other HIV LTR promoter elements on the inhibitory ability of E1A cannot be ruled out. This is supported by the finding that Tat-independent transcription of the HIV LTR promoter truncated to −83, which contains only three SP1-binding sites upstream of the TATA box, is also repressed efficiently by E1A1-80 (our unpublished data). Thus, it seems likely that E1A repression of the HIV LTR can be mediated by sequence elements other than NF-κB binding sites. We have recently found that the core HIV LTR promoter (−31 to +80) is repressed by E1A1-80, implying that the ultimate target of E1A repression, at least with HIV LTR, is the general transcription machinery (unpublished data). Further, by E1A1-80 peptide affinity depletion and reconstitution experiments, we found evidence that p300 and general transcription factor TFIID are specifically involved in E1A repression (unpublished data). With the in vitro transcription repression system, we are further studying these cellular targets and the molecular mechanism(s) involved in E1A repression of the HIV LTR promoter. What is learned by studying the HIV LTR may be germane to the E1A repression involved in diverse functions, including cell differentiation and metastasis (for a review, see reference 24). Finally, the specificity and efficiency of E1A1-80 repression of HIV LTR expression may conceivably be used to explore the potential use of E1A peptides to inhibit HIV-1 replication.

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