Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 1310–1314, February 1990
Microbiology

Silencing of human immunodeficiency virus long terminal repeat expression by an adenovirus E1a mutant

(trans-activation/enhancer repression/human immunodeficiency virus inhibition)

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Communicated by Ludwik Gross, November 28, 1989

ABSTRACT **Gene expression from the human immunodeficiency virus (HIV) long terminal repeat (LTR) is strongly stimulated by the viral *tat* gene. The HIV LTR is also activated by several physical and chemical agents and heterologous viral genes, including adenovirus E1a. As E1a has separable transcriptional activation and repression functions, we examined the negative regulatory effects of E1a on the expression of the HIV LTR by using a trans-dominant E1a mutant. Mutant hr5 strongly suppressed the basal activity of the LTR as well as trans-activation of the LTR by heterologous agents such as the cytomegalovirus immediate early gene or DNA-damaging agents such as mitomycin C and UV irradiation. In addition, hr5 also caused significant suppression of *tat* gene-mediated trans-activation. The suppression of HIV LTR expression by hr5 appears to be mediated, at least in part, by the repression of the HIV enhancer, as the activity of an enhancer test system composed of the human T-cell leukemia virus I LTR containing an HIV-1 enhancer substitution was severely repressed by hr5. Cotransfection of HIV-1 proviral DNA with hr5 DNA resulted in a significant reduction of HIV production.**

The levels of human immunodeficiency virus HIV replication appear to be dictated by the extent of viral gene expression controlled by the coordinate action of viral regulatory genes. Two of the regulatory genes of HIV (*tat* and *rev*) are important in a stimulatory pathway of HIV replication that augments viral gene expression (for review, see ref. 1). The *tat* gene is a potent *trans*-activator of gene expression from the HIV long terminal repeat (LTR) (2, 3). It has also been shown that a number of heterologous viral (4–9), physical, and chemical agents (10–13) activate the HIV LTR, implicating them as potential activating agents of latent viral infections.

Adenovirus E1a is a general transcriptional activator of a number of viral and cellular genes (14). E1a has been reported to trans-activate the HIV-1 LTR (7, 8). E1a also possesses a negative regulatory function that represses the activity of certain viral and cellular enhancers (15–17). Since an enhancer element appears to play an important role in the expression of HIV-1 LTR (18, 19), we examined whether the expression of HIV genes could be suppressed by negative regulatory functions of E1a.

The positive and negative regulatory effects of E1a are separable. Transcriptional activation appears to be an exclusive function of a 289-amino acid protein (289R) coded by a 13S mRNA (Fig. 1 *Upper*) and is specified by a unique 46-amino acid domain of 289R (21). The negative regulatory function, on the other hand, is mediated by 289R as well as by a 243-amino acid protein (243R) coded by a 12S mRNA (Fig. 1 *Upper*). 243R and the trans-activation-defective mutants of 289R appear to be more efficient repressors (22). It could be envisioned that such activation-defective and repression-positive mutants could be used as potential agents to suppress HIV gene expression. In our studies, we exploited a unique adenovirus 5 E1a mutant, named hr5, that not only is trans-activation defective but also exhibits trans-dominant suppression of the wild-type (wt) E1a activation function (23). We reasoned that hr5 would be a more promising agent in suppressing HIV gene expression due to its dual repression effects. The trans-dominant repression effect may have an added advantage against stray activation of the LTR by E1a-like agents. Here we report that hr5 not only silences basal HIV-1 LTR expression but also suppresses trans-activation by several agents including the *tat* gene; further, HIV viral production from proviral DNA or in chronically infected T4 cells is also inhibited by hr5 coexpression.

MATERIALS AND METHODS

Plasmids. Plasmids pE1a (pLA1Δb; ref. 24) and pHR5 (23) have been described. Plasmids pTAT-III expressing the *tat* gene under the control of the HIV-1 LTR and the replication competent proviral DNA clone pHXB2 (25) were obtained from Flossie Wong-Staal (National Institutes of Health). pLTR-CAT expressing the bacterial chloramphenicol acetyltransferase (CAT) gene under control of the HIV-1 (SF2) LTR was a gift from P. Luciw. pLTR-I-CAT (26) expressing the CAT gene under control of the HTLV-I LTR was provided by I. Chen. pCMV-IE has been described (27). Plasmids pHR5ΔB and pHR5ΔB-X were constructed by deletion of 4 base pairs (bp) at position 608 (*Bst*XI site) or deletion of the sequences between position 608 and position 1338 (*Xba* I site), respectively (Fig. 1 *Upper*). The enhancer test plasmid pEn-1-CAT, in which the enhancer of the human T-lymphotropic virus I HTLV-I LTR was replaced with the enhancer of HIV-1, was constructed by replacing a 240-bp (*Nde* I–*Sma* I) fragment of the HTLV-I LTR with a 120-bp (*Sca* I–*Pvu* II) fragment of HIV-1 LTR in pLTR-I-CAT. Similarly, pEn-2-CAT was constructed by cloning a synthetic double-stranded oligonucleotide consisting of bases −77 to −106 of the HIV-1 core enhancer between the *Nde* I and *Sma* I sites of pLTR-I-CAT. Plasmid pΔLTR-I-CAT lacking the enhancer was constructed by deletion of the *Nde* I–*Sma* I fragment of pLTR-I-CAT.

Transfections and CAT Assays. For trans-activation and suppression assays, HeLa cells (3×10^5 cells per 60-mm dish) were transfected with 1.0 μg of pLTR-CAT and 1.0 μg of pTAT-III or 1 μg of cytomegalovirus immediate early gene

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Abbreviations: CAT, chloramphenicol acetyltransferase; HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; LTR, long terminal repeat; PMA, phorbol 12-myristate 13-acetate; wt, wild type; SV40, simian virus 40; EBP-1, enhancer binding protein 1; 289R and 243R, 289- and 243-amino acid protein respectively.

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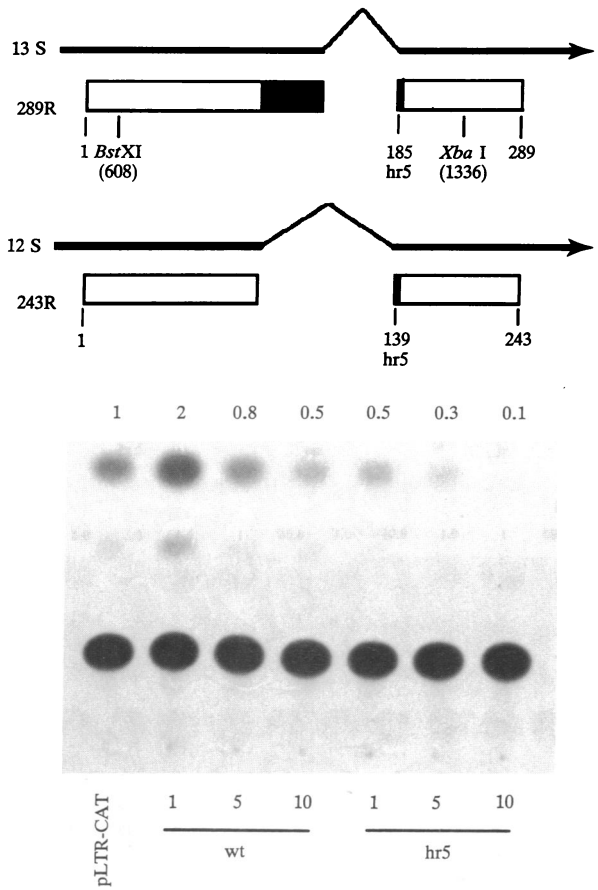

FIG. 1. Organization of Ad5 E1a region and effect of E1a on basal HIV-1 LTR expression. (*Upper*) Two major mRNA species (12 S and 13 S) and their protein products (289R and 243R) are indicated. The mutational changes of hr5 (20) are shown. The hr5 mutation introduces a single amino acid change in both the 289R (Ser-185 → Asn) and the 243R (Gly-139 → Asp). The trans-activation domain of E1a is indicated by the solid bar. (*Lower*) Effect of E1a on LTR expression is shown. HeLa cells were transfected with pLTR-CAT and indicated amounts (μg at the bottom) of pE1a or pHR5. The relative CAT activities with reference to cells expressing only LTR CAT are indicated on the top.

and various concentrations of E1a plasmids. Total plasmid DNA concentration was adjusted with pUC13 carrier DNA and total DNA concentration was adjusted to 20 μg with salmon sperm DNA. DNA transfections were carried out by the calcium phosphate method as described by Herrmann *et al.* (28). To determine the effect of DNA-damaging agents, HeLa cells were transfected with 1 μg of pLTR-CAT and 24 hr later were treated with mitomycin C (10 μg/ml) for 24 hr or with short-wavelength UV irradiation (10 J/m^2). To study the effect of phorbol ester, HeLa cells were transfected with 2 μg of pEn-2-CAT and treated with phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) from 24 to 48 hr after transfection. Jurkat cells (8×10^6 cells) were transfected by the DEAE-dextran method (29) with 1 μg of pLTR-CAT, 1 μg of pTAT, and 1 μg of pE1a or pHR5. The relative CAT activity was determined by quantitating the radioactivity in the TLC plates (30). The percent of conversion of [^{14}C]chloramphenicol to acetylated forms was calculated and then the relative activity was derived.

Assay of HIV Viral Production. For transient assays on HIV-1 production, COS-7 or HeLa cells (5×10^5 cells per 25-cm^2 flask) were transfected with 4 μg of proviral DNA of HIV-1 (pHXB2) and 12 μg of various E1a plasmids along with pUC13 carrier DNA. HIV viral production was assayed 4 days after transfection by measuring the amount of p24 antigen in the culture supernatant by an ELISA (commercially available kits from Abbott).

RESULTS

Suppression of Basal LTR Expression. The hr5 mutation causes a single amino acid substitution in 289R and 243R (Fig. 1). This mutant is defective in trans-activation of adenovirus 5 early promoters and in addition causes trans-dominant suppression of wt E1a-mediated trans-activation (23). The effect of wt E1a or mutant hr5 was studied using plasmids that express 289R and 243R. To study the effect on basal LTR expression, a reporter plasmid expressing the bacterial CAT gene from the HIV-1 (SF2) LTR (pLTR-CAT) was cotransfected with various concentrations of plasmids expressing wt E1a (pLA1Δb, referred to here as pE1a) or the mutant hr5 (pHR5) on HeLa cells and CAT activity was determined (Fig. 1 *Lower*). To detect low levels of basal activity, the CAT enzyme reaction was carried out for a relatively longer time compared to samples from cells expressing the *tat* gene. Cotransfection of pLTR-CAT and pE1a caused a 2-fold trans-activation of LTR-CAT expression at low E1a concentration (1 μg), in agreement with a previous report (7). However, at higher concentrations, E1a suppressed the basal activity of the LTR significantly. At an E1a concentration of 10 μg, there was an ≈50% reduction in the basal activity. On the other hand, cotransfection of pLTR-CAT and pHR5 did not cause any trans-activation of LTR-CAT but instead suppressed basal activity even at the lower concentration (1 μg). About 90% of the LTR activity was suppressed at a concentration of 10 μg. In this experiment and in other subsequent experiments, two mutant derivatives of pHR5, pHR5ΔB-X, lacking most of the E1a coding region between nucleotide 608 (*Bst*XI) and 1338 (*Xba* I), or pHR5ΔB, carrying a 4-bp deletion at position 608 (*Bst*XI), did not have any significant effect on LTR expression (results not shown). This argues against promoter/enhancer competition for common cellular factors as being responsible for hr5-mediated repression. These results indicate that wt E1a has both positive and negative regulatory effects on HIV LTR expression but mutant hr5 exhibits only an inhibitory effect.

Suppression of Tat-Mediated Trans-Activation. To determine whether the trans-activation of LTR by the *tat* gene could be compromised by the negative regulatory effects of E1a, we examined the effects of coexpression of wt E1a or hr5 (Fig. 2*A*). HeLa cells were cotransfected with pLTR-CAT, pTAT-III expressing the *tat* gene, and pE1a or pHR5. As expected, transfection of HeLa cells with pLTR-CAT alone resulted in low levels of basal CAT activity whereas cotransfection with pTAT-III induced a large increase in CAT activity. Cotransfection of pTAT-III and the E1a (wt) plasmid caused a small additive increase in trans-activation at low E1a concentration (1 μg). However, transfection of increasing amounts of E1a resulted in a gradual decrease in trans-activation. About 50% inhibition of Tat activity could be seen at a Tat/E1a molar ratio of 1:10. On the other hand, cotransfection of pTAT-III and pHR5 caused ≈60% reduction in trans-activation, even at a low DNA concentration (1 μg) and the inhibition was much more pronounced at higher DNA concentrations. The effect of E1a and hr5 on Tat-mediated trans-activation was also examined in a T4 cell line, Jurkat (Fig. 2*B*). As for HeLa cells, hr5 induced ≈60% inhibition of trans-activation at a low (1 μg) DNA concentration. These results indicate that the trans-activation of HIV LTR by Tat could be efficiently inhibited by hr5.

Suppression of LTR Activation Mediated by Heterologous Agents. Various heterologous viral, physical, and chemical agents have been shown to activate the expression of the HIV-1 LTR. We have examined the effect of E1a or hr5 on the activation of LTR by the cytomegalovirus immediate

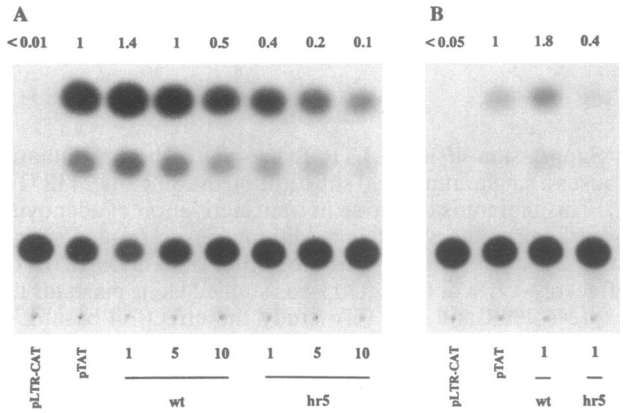

FIG. 2. Effect of E1a on Tat-mediated trans-activation of HIV-1 LTR. HeLa (*A*) or Jurkat (*B*) cells were transfected with pLTR-CAT, pTAT-III, and indicated amounts (μg at the bottom) of pE1a or pHR5. The CAT activity in cells expressing E1a or hr5 in relation to cells expressing the *tat* gene is indicated at the top.

early gene and the DNA-damaging agents, mitomycin C and UV irradiation (Fig. 3). In HeLa cells transfected with pLTR-CAT and pCMV-IE (Fig. 3*A*), there was ≈30-fold stimulation in the expression of LTR-CAT, as shown by Bohan *et al.* (27). Inclusion of wt E1a augmented the extent of trans-activation ≈2-fold at low DNA concentration (1 μg); however, at a higher DNA concentration (e.g., 10 μg), there was detectable inhibition. In contrast, inclusion of hr5 did not enhance trans-activation, but instead caused inhibition even at low concentrations. It is known that the HIV-1 LTR can be activated by DNA damaging agents such as UV irradiation or mitomycin C (11). To determine whether the activation of LTR expression mediated by these two agents could also be inhibited by E1a or hr5, HeLa cells transfected with pLTR-CAT and pE1a or pHR5 were treated with UV irradiation or mitomycin C. As seen in Fig. 3*B*, both wt E1a or hr5 induced large inhibition of UV- or mitomycin C-induced activation of LTR. These observations indicate that hr5 may have a general trans-acting silencing effect on HIV LTR expression. It should be noted that wt E1a and hr5 had a stronger inhibitory effect on trans-activation by the DNA damaging agents than trans-activation by the cytomegalovirus immediate early gene. This differential effect may be the reflection of the different mechanisms by which these agents activate the LTR.

Effect on HIV Production. To determine whether the negative regulatory effects of E1a on the HIV-1 LTR could be exploited to inhibit HIV replication, the effect on HIV production was assessed in transient assays. In these assays, pE1a or pHR5 were cotransfected with replication-competent proviral DNA of HIV-1 (pHXB2) in COS cells or HeLa cells and the extent of HIV production was quantitated by assaying the culture medium for levels of p24 (gag) antigen production (Fig. 4). In both COS and HeLa cells, wt E1a and hr5 inhibited HIV production significantly in a 4-day period; however, inhibition by hr5 was more efficient.

Enhancer Repression. To determine the possible mechanism of inhibition of HIV-1 LTR activity by hr5, we tested its effect on the HIV-1 enhancer. For this purpose, an enhancer test plasmid (pEn-1-CAT; Fig. 5*B*) was constructed by replacing the HTLV-I enhancer (33, 34) with HIV-1 LTR sequences located between bp −17 and −120 (Fig. 5*A*). For the construction of the enhancer test plasmid, we used a reporter plasmid (pLTR-I-CAT) expressing the CAT gene under the transcriptional control of the HTLV-I LTR (26). The −17 to −120 region of the HIV-1 LTR functions as an enhancer in an orientation-independent manner (31, 35). As seen in Fig. 6*A*, pΔLTR-I-CAT lacking the HTLV-I enhancer

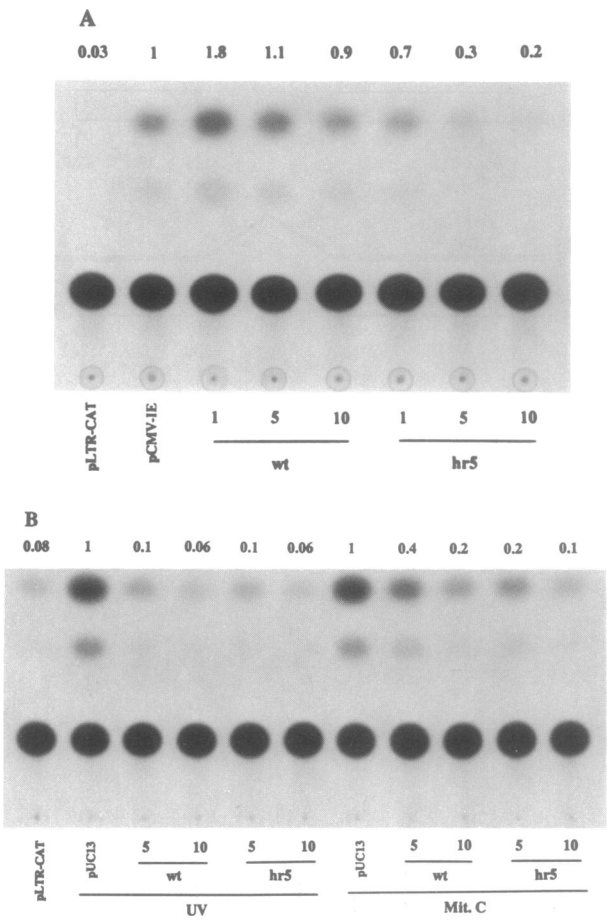

FIG. 3. Effect of E1a on activation of HIV-1 LTR by heterologous agents. (*A*) HeLa cells were transfected with pLTR-CAT, pCMV-IE, and indicated amounts (μg at the bottom) of pE1a or pHR5. (*B*) HeLa cells were transfected with pLTR-CAT alone or with pE1a or pHR5 and treated with short-wavelength UV irradiation or with mitomycin C (Mit. C). CAT activity was determined as in Fig. 1 and is shown at the top of each section.

did not direct any significant CAT activity. Similarly, there was also no detectable CAT expression when pΔLTR-I-CAT was cotransfected with the *tax* gene of HTLV-II or wt E1a (results not shown). Substitution of the HTLV-I enhancer with the HIV-1 enhancer (pEn-1-CAT) resulted in a large increase in CAT expression. Cotransfection of pEn-1-CAT and pE1a at a ratio of 1:2 decreased CAT expression by ≈50%, whereas cotransfection of pEn-1-CAT and pHR5 at the same ratio caused a more pronounced reduction (80%) in

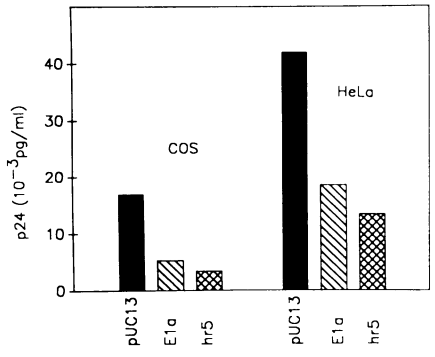

FIG. 4. Effect of E1a on HIV production. COS-7 or HeLa cells were transfected with 4 μg of pHXB2 and 12 μg of pE1a or pHR5. The amount of p24 antigen present in the culture fluid was quantitated by ELISA 4 days after transfection.

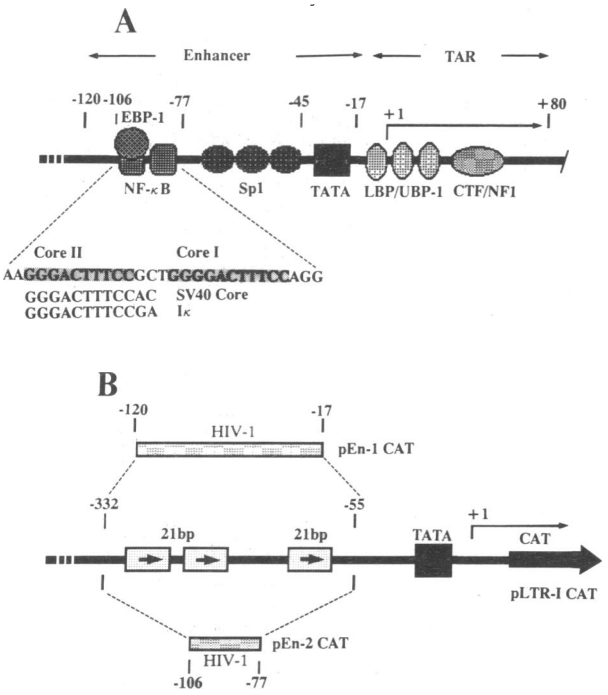

FIG. 5. Organization of HIV-1 LTR and enhancer test plasmids. (*A*) In the HIV-1 LTR, the binding sites for various transcription factors are indicated. The enhancer region (bp −17 to −120) and the tar element (bp −17 to +80) are shown (31). The scheme for factor binding is a modification of that described by Selby *et al.* (32). Under the HIV core enhancer sequences, the SV40 core enhancer and immunoglobulin κ enhancer sequences are shown. (*B*) In the enhancer test plasmids, the HTLV-I enhancer sequences (33, 34) located between bp −332 (*Sma* I) and −55 (*Nde* I) were replaced with various HIV-1 enhancer sequences in a reporter plasmid, pLTR-I-CAT (26): pEn-1-CAT, bp −17 to −120 or pEn-2-CAT, bp −77 to −106.

CAT expression (Fig. 6*A*). These results indicate that hr5 exerts a significant inhibitory effect on the activity of the enhancer region located between bp −17 and −120.

The HIV-1 enhancer region contains two 10-bp repeats (core enhancers I and II) homologous to the simian virus 40 (SV40) core enhancer and immunoglobulin κ enhancer (NF-κB) (ref. 31; Fig. 5*A*). To determine whether hr5 interferes with the activity of the core enhancer region, an enhancer test plasmid, pEn-2-CAT, was constructed in a manner similar to pEn-1-CAT (Fig. 5*B*) by replacing the HTLV-I enhancer region with a synthetic core enhancer region (bp −77 to −106). HeLa cells were transfected with pEn-2-CAT in the presence or absence of hr5 and CAT activity was measured (Fig. 6*B*). The basal activity of pEn-2-CAT was less by a factor of 20–30 than that of pEn-1-CAT, suggesting additional sequences located within the region of bp −17 to −120 play a role in the constitutive expression of pEn-1-CAT. Nonetheless, hr5 also repressed the basal activity of pEn-2-CAT, suggesting that hr5 inhibits the constitutive activity of the core enhancer elements.

It is known that the HIV-1 LTR can be stimulated by phorbol esters (10, 12) by activation of the inducible factor, NF-κB (32, 36–38). We also tested the effect of hr5 on phorbol ester-induced expression of the core enhancer region (Fig. 6*C*). HeLa cells were transfected with pEn-2-CAT in the presence or absence of pHR5 and subsequently treated with the phorbol ester, phorbol 12-myristate 13-acetate (PMA). In contrast to the uninduced level of expression of pEn-2-CAT, PMA treatment resulted in high levels of CAT expression, suggesting that the core enhancer region alone can mediate efficient induced expression of the enhancer test

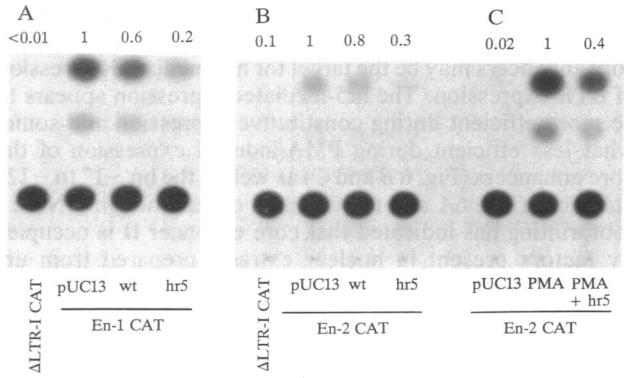

FIG. 6. Effect of E1a on HIV-1 enhancers. HeLa cells were transfected with pΔLTR-I-CAT or the enhancer test plasmids, pEn-1-CAT (*A*) or pEn-2-CAT (*B* and *C*) in the absence or presence of pE1a or pHR5. (*C*) Transfected cells were treated with PMA. (*B*) The CAT enzyme reaction was carried out for a period 10 times longer than in *C*. CAT activity was determined as in Fig. 1 and shown at the top.

plasmid. Coexpression of En-2-CAT and hr5 also resulted in >50% reduction in PMA-induced expression. These results suggest that the core enhancers may be the target of hr5-mediated repression.

DISCUSSION

We have shown that adenovirus E1a has a negative regulatory effect on HIV-1 LTR expression. A trans-dominant mutant of E1a (hr5) appears to have a strong trans-acting silencer activity against the HIV-1 LTR. Mutant hr5 not only compromised the potent trans-activation effect of the *tat* gene but also effectively inhibited trans-activation of the LTR by heterologous agents such as cytomegalovirus immediate early gene, mitomycin C, or UV irradiation. From our transient assays on the production of HIV from replication competent proviral DNA, it appears that the strong negative regulatory effect of hr5 could be exploited to inhibit HIV replication.

The LTR-silencing activity of hr5 appears to be, at least in part, mediated by repressing the activity of the HIV enhancer. However, it should be emphasized that the role of other elements of the LTR on hr5-mediated repression cannot be ruled out. It appears unlikely that hr5 interferes with the functions of canonical promoter elements such as the TATA region or the Sp1 or nuclear factor (NF)-1 binding sites, as hr5 does not appear to inhibit the activity of the HSV-1 *tk* promoter (39) that contains these promoter elements (A.M. and G.C., unpublished data). It has been shown by Glenn and Ricciardi (23) that hr5 does not interfere with the trans-activation of adenovirus early promoters by pseudorabies virus immediate early gene. This strengthens our conclusion that the primary target of hr5-mediated repression is the core enhancer of HIV-1 LTR since the pseudorabies immediate early gene-activated adenovirus promoters do not contain the core enhancers.

Our results indicate that hr5 negates the trans-activation mediated by diverse activating agents. Since the enhancer element appears to be crucial for the activation of the LTR, it appears that the observed effect on the trans-activation may reflect enhancer repression rather than an effect on the various activators.

The mechanism of enhancer repression mediated by the E1a proteins has not been investigated in detail. Although E1a is known to repress the activity of a number of enhancers, this process appears to be somewhat specific as similar core enhancer elements are shared by the various E1a-repressed enhancers such as SV40, polyoma B, IgG, and

insulin (40). However, the exact target sequence has not been identified experimentally. Our results indicate that the HIV core enhancers may be the target for hr5-mediated repression of LTR expression. The hr5-mediated repression appears to be more efficient during constitutive expression and somewhat less efficient during PMA-induced expression of the core enhancers (Fig. 6 *B* and *C*) as well as the bp −17 to −120 enhancer (Fig. 6*A* and unpublished observations). DNase I footprinting has indicated that core enhancer II is occupied by factors present in nuclear extracts prepared from untreated HeLa cells (41). On the other hand, phorbol ester treatment resulted in factor binding to both enhancers (42, 43), with a preference for core enhancer I (44). These observations suggest that core enhancer II may be primarily a constitutive enhancer while core enhancers I and perhaps, to a lesser extent, core enhancer II may contribute to phorbol ester-induced expression. Since hr5-induced repression appears to be efficient during uninduced expression and somewhat less pronounced during PMA-induced expression, it is possible that core enhancer II may be the primary target of hr5. Core enhancer II appears to interact with nuclear factors, NF-κB (37) and enhancer binding protein 1 (EBP-1) (45). Core enhancer I, on the other hand, appears to interact with NF-κB with higher affinity. It should be noted that a nuclear factor, also designated EPB-1, interacts with the SV40 core enhancer and sequences related to the NF-κB binding site (46). These observations raise the possibility that hr5 may directly or indirectly interfere with the activity of factors such as EBP-1, NF-κB, or other related factors. A specific inhibitor of NF-κB (IκB) has also been identified (47). It may be possible that hr5 protein may directly act like an inhibitor of enhancer binding factors or may indirectly enhance the activity of cellular inhibitors. The rather efficient repression of HIV-1 enhancer by hr5 provides an opportunity to further unravel the mechanism of enhancer repression.

Since E1a has been reported to trans-activate the HIV LTR (6, 7), it is believed that adenovirus infection may activate HIV replication in latently infected cells. However, this prediction has not been tested experimentally. Our results also indicate that wt E1a can activate the LTR to some extent. However, in light of the inhibitory effect of E1a observed at higher concentrations (Fig. 1) and the inhibition of HIV production from replication-competent proviral DNA, the role of adenovirus as an activating agent of HIV replication is not certain.

The important outcome of our studies is that hr5 could be used as a potential inhibitor of HIV replication. hr5 may not only interfere with the stimulatory pathway of HIV gene expression mediated by the *tat* gene but also could effectively eliminate the activation of LTR expression mediated by other viral and physical agents. Thus, hr5 appears to be an example of a heterologous virus (i.e., hr5) that could be beneficially exploited to inhibit the replication of a pathogenic virus. A higher level of suppression of HIV viral production than observed in transient assays may be possible by infection with hr5 virus particles. Since adenoviruses are natural passengers of human lymphoid cells (see ref. 48), appropriately designed vectors expressing hr5 could be used as a potential therapeutic agent to suppress HIV-mediated pathogenesis. Mutant hr5 is a nontransforming defective virus, thus minimizing the possibility of it becoming a virulent pathogen in immunodeficient individuals. Since the structure and function of adenovirus genes are well studied, it is possible to redesign this mutant in a manner more suited for therapeutic use.

We thank Irvin Chen, Paul Luciw, Rob Ricciardi, Flossie Wong-Staal, and AIDS Research and Reference Reagent Program for various plasmids and cells. We thank Chung Park for her valuable help in the p24 antigen assays. A.M.V. is on leave from Instituto Adolfo Lutz, Sao Paulo, and was supported by the Conselho Nacional de Pesquisas (Brazil). This work was supported by Grants CA-31719 and CA-33616 from the National Institutes of Health.

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