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Effects of Long Terminal Repeat Mutations on Human Immunodeficiency Virus Type 1 Replication

YICHEN LU, MOLLY STENZEL, JOSEPH G. SODROSKI, AND WILLIAM A. HASELTINE*

Dana-Farber Cancer Institute, Department of Pathology, Harvard Medical School, 44 Binney Street, Boston, Massachusetts 02115

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The effects of deletions within three functional regions of the long terminal repeat of human immunodeficiency virus type 1 upon the ability of the long terminal repeat to direct production of the chloramphenicol acetyltransferase gene product and upon the ability of viruses that carry the mutations to replicate in human cell lines was investigated. The results show that the enhancer and TATAA sequences were required for efficient virus replication. Deletion of the negative regulatory element (NRE) yielded a virus that replicated more rapidly than did an otherwise isogeneic NRE-positive virus. The suppressive effect of the NRE did not depend upon the negative regulatory gene (*nef*), as both NRE-positive and NRE-negative viruses were defective for *nef*. We conclude that factors specified by the cell interact with the NRE sequences to retard human immunodeficiency virus type 1 replication.

The replication rate of human immunodeficiency virus type 1 (HIV-1) is governed by proteins specified by the virus itself as well as by factors present in uninfected cells. Viral proteins that affect the replication rate include the product of the *trans*-activator gene (*tat*) (5, 7), the product of the regulator of expression of virion proteins (*rev*) (6, 26), and the product of the negative regulatory factor gene (*nef*) (13, 28). The role of the cellular proteins in control of virus replication is inferred from the effects of disruption of sequences in the long terminal repeat (LTR) that flank the proviral DNA. Disruption of such sequences affects the rate of transcription initiation directed by the LTR in uninfected cells. The effects of changes in the LTR on transcription activity have generally been investigated by using the viral LTR to direct the synthesis of heterologous gene products. Such studies have defined an enhancer sequence that stimulates the rate of RNA initiation (16, 17, 22, 25, 29) as well as a silencer sequence, called a negative regulatory element (NRE), that decreases the rate of HIV LTR-directed gene expression (22, 25). To date, the effects of changes in these sequences on the replication of the virus itself have not been investigated. To determine whether mutations in the LTR that remove the enhancer, the silencer, or the promotor sequences affect the replication, a set of HIV-1 strains that are isogeneic except for sequences within the LTR were constructed.

The locations of four defined functional regions of the HIV-1 viral LTR are pictured in Fig. 1A. The site of the initiation of viral RNA synthesis is depicted at nucleotide number +1 (15, 17, 20). The sequence TATAA, a conserved eucaryotic promoter element, is located between nucleotides −27 and −23 (4). The enhancer sequence is located between nucleotides −109 and −79. The enhancer contains an imperfect tandem repeat sequence. The HIV-1 enhancer shows sequence similarities with other transcription enhancers, including the early region enhancer of simian virus 40 (30), the immediate-early gene enhancer of the human cytomegalovirus (2), the human immunoglobulin kappa gene enhancer (23), and a sequence in the regulatory region of the interferon response gene, the human gene 6-16 (19). The HIV-1 enhancer also contains a consensus recognition sequence for the DNA-binding proteins NF-κB (16) and H2TF1 (1). The sequences located between −420 and −156 contain the NRE. The sequence required for the response to the *tat* product is located between nucleotides +14 and +44 and is designated TAR (11, 14, 18, 21, 22). Additionally, binding sites for several other eucaryotic transcription factors have been defined, including those for SP-1 (9, 10), AP-1 (8), NFAT-1 (24), USF (3, 9), NF-1, and LBP (11) (Fig. 1A).

The effects of three deletions within the 5′ region of the HIV LTR on the replication of properties of the virus were investigated. One deletion removed the entire TATAA sequence. This deletion was made by oligonucleotide-directed mutagenesis and was designated DTATAA. An LTR devoid of the entire enhancer, deleted from nucleotides −109 to −79, was also made, as was an LTR devoid of the NRE, encompassing nucleotides −419 to −157. These mutations were designated DENH and DNRE, respectively.

The effect of these deletions on the function of the viral LTR was measured by placement of wild-type and mutant LTRs 5′ to the bacterial chloramphenicol acetyltransferase (CAT) gene. The ability of the plasmids to direct the synthesis of the CAT enzyme was determined by transfection of cells with these plasmids. The human T-cell line Jurkat was used as the recipient. Jurkat cells express the CD4 protein that serves as a receptor for HIV-1. Jurkat cells have been previously demonstrated to support viral replication upon transfection with an HIV-1 infectious provirus (5). The ability of the altered sequence to respond to the *tat* product was determined by transfection of a Jurkat cell line that expresses constitutively the *tat* protein (Jurkat-*tat*). Treatment of Jurkat-*tat* cells with phytohemagglutinin (PHA; Sigma Chemical Co.) and the phorbol ester (PMA; Sigma Chemical Co.) activates the expression of a set of genes that are similar to those activated upon mitogen activation of fresh human T cells (16, 25, 29). The effect of mitogen activation on the activities of the wild-type and mutant LTRs was also determined.

The LTR mutations were also introduced into an infectious provirus HXBc2-C21 (5, 20). During transcription, the U3 region of the viral RNA is derived from the 3′ LTR of the provirus. This U3 region is then duplicated upon formation

* Corresponding author.

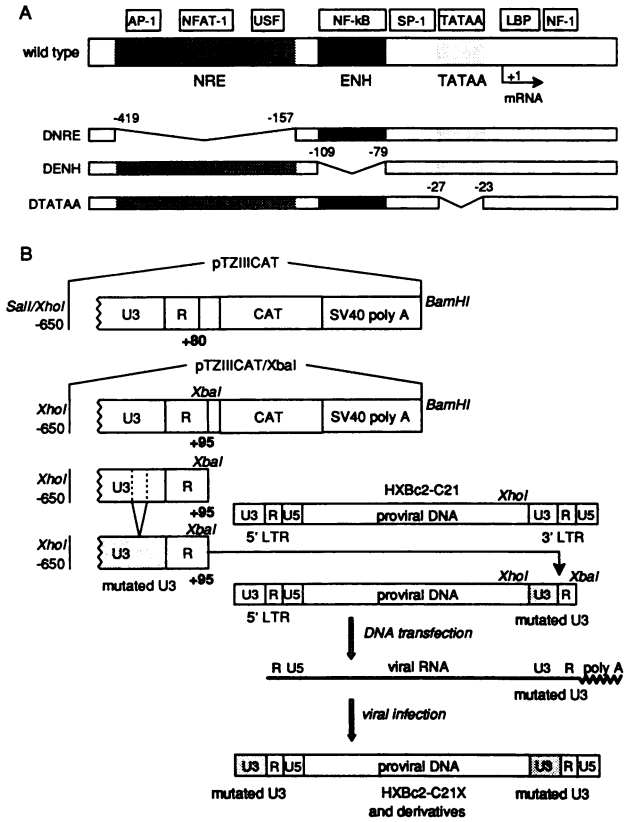

FIG. 1. (A) Recognition sequences for proteins that bind to the HIV-1 LTR are shown above the LTR diagram. The NRE, enhancer region (ENH), and TATAA sequences are shown. The position +1 indicates the site of initiation of mRNA synthesis. The locations of DNRE, DENH, and DTATAA are depicted. The light shading corresponds to nucleotides −27 to −23, the intermediate shading corresponds to nucleotides −419 to −157, and the dark shading corresponds to nucleotides −109 to −79. (B) Construction of HIV-1 LTR mutants and the mutant containing HIV-1 proviral DNA. The mutagenesis vector plasmid pTZIIICAT was made from the insertion of the *Xho*I-*Bam*HI fragment containing HIV-1 LTR and CAT gene from the plasmid pU3RIII (27) into the *Sal*I-*Bam*HI sites of pTZ19U (United States Biochemical Corp.). The constructed pTZIIICAT then had both pBR322 double-stranded plasmid DNA and M13 bacteriophage single-stranded DNA replication origins, so that the CAT expression plasmid DNA and single-stranded mutagenesis template DNA could both be made from the same vector. pTZIIICAT contains HIV-1 sequences from *Xho*I (position 8897) to *Hin*dIII (9616) (20). By using oligonucleotide-directed site-specific mutagenesis (12), the *Sal*I-*Xho*I fusion site was restored to a *Xho*I site and the HIV-1 sequence was extended to position 9634 in order to include the entire R region of the LTR, the complete poly(A) signal, and the poly(A) site (15). An additional *Xba*I site was also created at the 3′ end of the poly(A) site in the LTR. The vector was then used to make the deletion mutants in the desired regions by oligonucleotide-directed mutagenesis. The NRE deletion was made by removing the *Eco*RV (−418) to *Ava*I (−157) fragments from pTZIIICAT. The *Xho*I-*Xba*I fragment containing either the wild-type LTR (U3-R) or the mutant LTRs was then used to replace the *Xho*I-*Xba*I fragment from HXBc2-C21, which includes the 3′ U3-R-U5 as well as the flanking cellular sequences. The shading represents the mutated U3 region of the LTR.

of the new provirus. For this reason, we replaced the 3′ LTR of HXBc2-C21 with the altered U3-R sequences (Fig. 1B). The 5′ end of the provirus produced after one round of replication of such a provirus contains the mutant in the 5′ as well as the 3′ LTR. The wild-type proviral DNA (HXBc2-C21X) is a derivative of HXBc-C21 in which the 3′ U3-R-U5 (LTR) sequences are replaced by U3-R sequences. The precise strategy used for these constructions is shown in Fig. 1, and details are provided in the figure legend.

TABLE 1. Transient expression assays for the LTR promoter function

Treatment[a]	Promoter activity[b] with virus:			
	Wild type	DNRE	DENH	DTATAA
Jurkat	1.00	1.15	0.13	0.04
Jurkat-*tat*	108	615	35	2
Jurkat-*tat* + PMA + PHA	292	1599	74	2.8

[a] A 10-μg sample of the plasmid pTZIIICAT or the mutant DNA was transfected into 3 × 10^6 Jurkat or Jurkat-*tat* cells. At 48 h after transfection, the cell extract was collected and assayed for CAT activity by using the methods previously described (22). In the cases of activation, the mitogens (PMA, 50 ng/ml; PHA, 1 μg/ml) were added to the cultures 16 h before harvesting the cells.

[b] The promoter activities were relative to the wild-type LTR promoter activity in the same set of transfections.

To obtain stocks of infectious viruses and to obtain an initial measurement of the ability of mutant viruses to replicate, Jurkat cells were transfected with the wild-type and mutant proviruses. When possible, virus particles were also harvested from the supernatant of the transfected culture. The supernatant served as a source of the virus that was used to initiate infection. The ability of the virus to infect myeloid cell line U937 was also investigated.

Activities of the mutant LTRs. As anticipated, the level of CAT activity directed by the HIV-1 LTR was 100 times greater in Jurkat-*tat* cells than in Jurkat cells (Table 1). The amount of activity detected in Jurkat-*tat* cells was increased further by treatment of the cells with PHA and PMA.

Deletion of the TATAA sequence dramatically reduced the activity of the viral LTR. The level of the CAT activity detected upon transfection of Jurkat cells was reduced to the background activity for the CAT assay. However, a small but detectable level of CAT enzyme activity was directed by the DTATAA LTR mutant in the Jurkat-*tat* cells. The amount of CAT activity directed in the Jurkat-*tat* cells was increased substantially by treatment with PHA and PMA (Table 1). Deletion of the enhancer, nucleotides −109 to −79, reduced the LTR-directed CAT activity in Jurkat cells almost 10-fold. This low level of CAT activity was dramatically increased in Jurkat-*tat* cells. The level of CAT enzyme activity in Jurkat-*tat* cells was one-third that of the wild-type LTR. The level of activity of the DENH mutant in Jurkat-*tat* cells was also increased about twofold by mitogen activation.

Deletion of the NRE region slightly increased the level of CAT enzyme activity detected upon transfection of Jurkat cells. However, the level of CAT enzyme activity directed by the NRE mutant in Jurkat-*tat* cells was more than five times greater than that observed for the wild-type LTR. This high level of activity was increased still further upon mitogen stimulation of the Jurkat-*tat* cells (Table 1).

Replication properties of the virus mutant in the LTR. To obtain viral stocks for further analysis, Jurkat cells were transfected with either HXBc2-C21X proviral DNA or DNA from proviruses that contain mutant 3′ LTRs. Initiation of RNA synthesis from the transfected provirus is anticipated to occur correctly within the intact 5′ LTR and to produce virus particles that carry the mutant U3 region. The Jurkat cells have been previously demonstrated to support replication of HIV-1 posttransfection (5).

To determine whether viruses could be made from the altered proviruses, the amount of cell-free reverse transcriptase released from cell supernatant viruses was measured 9 days posttransfection. Plasmid DNA (10 μg) containing the proviral genome was used to transfect 3×10^6 Jurkat cells by the DEAE-dextran method as previously described (5). The culture medium (RPMI supplemented with 10% fetal calf serum) was changed every 3 days, and the culture supernatant was harvested and filtered through a 0.22-μm-pore-size filter 9 days after the transfection. The filtered supernatant was then added to an equal volume of fetal calf serum. Approximately 1 ml of the mixture was then tested for reverse transcriptase activity, and the remainder was frozen at −70°C. Mock-infected cells (control) yielded 600 cpm of reverse transcriptase activity per ml. No reverse transcriptase activity was detected in cells that had been transfected with the provirus deleted for the TATAA sequence (600 cpm/ml). The enhancer-deleted virus yielded only one-fifth the amount of reverse transcriptase activity (51,500 cpm/ml) as did the HXBc2-C21X virus (242,500 cpm/ml). About five times more reverse transcriptase activity was recovered from Jurkat cells that had been transfected with the provirus deleted for the NRE sequence (1,372,900 cpm/ml).

Aliquots of the viral supernatant for the initial experiments were used for infection of fresh Jurkat cell cultures and for infection of U937 cells. The supernatant containing HXBc2-C21X, DENH, and DNRE viruses was diluted so that all three virus stocks contained an equivalent amount of reverse transcriptase activity. The amount of virus produced by each infected culture was determined by measurement of the concentration of extracellular p24 capsid antigen. Under these conditions, the cell-free virus produced by HXBc2-C21X and DNRE viruses reproducibly yielded productive viral infections. Virus infection was not initiated in several attempts using the DENH virus stock.

A comparison of the yield of the virus particles from the cultures of Jurkat cells infected with HXBc2-C21X and DNRE viruses is shown in Fig. 2A. The kinetics of infection of the two viruses was roughly similar, with virus concentration reaching a peak in both cultures on days 9 to 10 following infection and dropping rapidly thereafter. The amount of virus released from cultures infected with the DNRE virus was consistently found to be about three to five times greater than the amount released from cultures infected with the wild-type HXBc2-C21X virus, an effect observed in three independent experiments. The drop in virus production that occurred in both cultures reflects dramatic reduction in cell numbers late in infection, a consequence of the cytopathic effects of replication of both viruses. HXBc2-C21X and DNRE viruses both established productive infections in U937 cells. However, the marked cytopathic effects that were evident upon infection of the Jurkat cell culture were not evident upon viral infection of these cells. Consequently, virus production was measured later, 14 days postinfection. The amount of virus produced from the U937 cultures continually treated with PHA and PMA or with alpha interferon (Sigma) as well as the amount produced from untreated U937 was measured.

The level of HXBc2-C21X virus produced by the U937 cells was about 1/10 the amount released from Jurkat cells at the peak of virus production. However, at least 10 times more virus was released from U937 cells infected with the DNRE mutant than from cells infected with HXBc2-C21X. The amount of virus produced by the DNRE mutant in U937 cells was greater than the amount produced by HXBc2-C21X infection of Jurkat cells at the peak of infection. The amount of DNRE virus produced in U937 cells 14 days postinfection was similar to the amount produced at peak infection by the DNRE-deleted virus in Jurkat cells. Treatment of U937 with a combination of PHA and PMA or with alpha interferon, inducers of U937 differentiation, had no demonstrable effect on virus production.

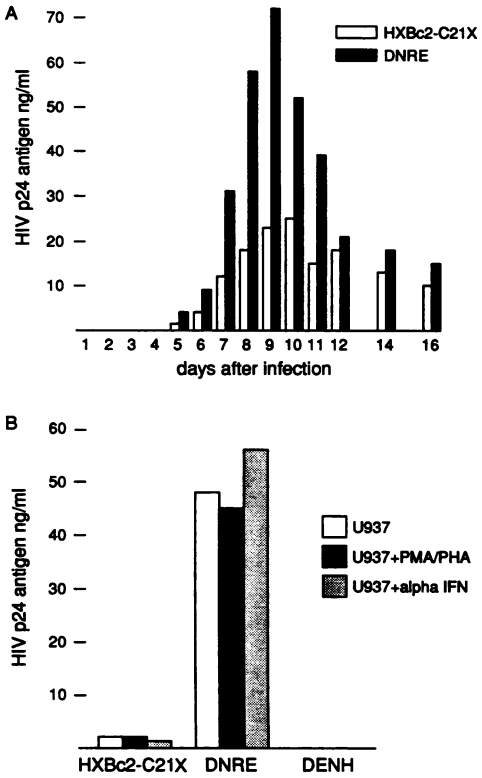

FIG. 2. (A) Comparison of the growth rates of the NRE deletion virus and the wild-type virus in infected Jurkat cells as judged by the amount of p24 protein released into the culture supernatant using the p24 radioimmunoassay (Dupont, NEN Research Products) kit. Jurkat cells (2×10^6) were infected by the filtered material containing 0.5×10^6 cpm of reverse transcriptase activity as detected by the method previously described (5). The culture medium was changed daily after the infection, and the presence of the viral p24 antigen in the supernatant was tested. (B) Detection of the p24 antigen in supernatants of U937 cells infected by the HXBc2-C21X, DNRE, and DENH viruses. U937 cells (3×10^6) were infected by the filtered material containing 1×10^5 cpm of reverse transcriptase equivalents of virus. The culture medium was changed twice a week. The viral p24 antigen in the medium was detected 14 days after infection. In the cases of activation, 50 ng of PMA per ml, 1 μg of PHA per ml, or 300 IU of alpha interferon per ml was added 1 day before the antigen detection.

These experiments demonstrate that sequences which affect the level of activity of the HIV-1 LTR as defined by using the viral promoter to direct heterologous gene expression also affect replication rates of the virus itself. The most striking observation reported here is that deletion of the NRE resulted in a substantial overproduction of virus particles in a lymphoid and a myeloid cell line. We conclude that the NRE of the HIV-1 LTR plays an important role in limiting the rate of virus replication in these two cell types.

The suppressive effect of the NRE on virus replication observed in these experiments was independent of the negative regulatory factor encoded by the viral *nef* gene. The

HXBc2-C21X provirus used here as the wild-type control is defective in *nef*, a consequence of a premature termination codon that prevents expression of a functional *nef* product (13, 28). The 5- to 10-fold increase in replication of NRE-deleted virus cannot be attributed to interactions with the *nef* product. Evidently, both *cis* and *trans* regulatory elements specified by the HIV-1 genome contribute to slowed replication of the intact virus.

The NRE contains a number of sequences that are similar to those which bind well-characterized transcription factors (Fig. 1A). These sequences include the consensus binding sites for some cellular proteins that have been shown to increase transcription initiation rates for certain cellular and viral promoters. Specifically, sequences expected to bind to the transcription factors AP-1, NFAT-1, and USF are located between nucleotides −346 and −317, −254 and −216, and −173 and −159, respectively. Our preliminary experiments demonstrate that the sequences between nucleotides −338 and −156 bind the purified cellular protein NF-1 (R. Rupp, A. E. Sippel, Y. Lu, and W. A. Haseltine, unpublished observations). The sequences between nucleotides −338 and −156 have also been shown to bind USF (M. Sawadogo, R. G. Roeder, C. A. Rosen, Y. Lu, and W. A. Haseltine, unpublished observations). It is possible that binding of these proteins far upstream of the site of RNA initiation decreases rather than increases the rate of RNA initiation, perhaps by forming a nearby and competing false-initiation complex. The potential role of each of the transcription factor-binding sequences in negative regulation of HIV-1 replication is currently under investigation.

The TATAA sequences appear to be required for virus replication. It is likely that the transcription directed by TATAA-deficient provirus is simply too low to permit synthesis of substantial amounts of the virus regulatory proteins that are needed to initiate productive HIV-1 infection.

The failure to observe infection initiated by the enhancer-defective virus deserves comment. Direct transfection of Jurkat cells by the DENH virus did yield a low but detectable level of virus. Detection of virus in this assay almost certainly depends upon spread of the virus in this culture, because a virus incapable of spread, such as a TATAA-defective virus, was not detected in this experiment even though all of the original proviruses contain an intact 5′ LTR. Spread of the infection in the Jurkat CD4+ cell culture occurred both by cell-to-cell transmission as well as by de novo infection by cell-free virus. Evidently, de novo infection by the DENH virus is inefficient and cannot be detected under the experimental conditions used here.

Two sequences that respond to T-cell activation have been identified in the HIV-1 LTR, the NF-κB recognition sequence found in the viral enhancer and the NFAT-1 sequence that is present in the NRE. The observation that the LTR deleted for either one of these sequences still responds to PHA and PMA treatment in Jurkat-*tat* cells indicates that both of the activation sequences contribute to stimulation of viral RNA synthesis by these mitogens. Note also that none of the changes in the U3 region eliminated the response of the LTR to the viral *tat* transactivator protein, consistent with previous observations that sequences 3′ to the site of RNA initiation determine the response to *tat*. Continued study of the role of cellular transcription factors in the regulation of replication of HIV-1 should clarify our understanding of the controlled nature of naturally occurring viral infections.

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