Identification of *cis*-Acting Repressive Sequences within the Negative Regulatory Element of Human Immunodeficiency Virus Type 1

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The negative regulatory element of human immunodeficiency virus type 1 is a 260-nucleotide-long sequence that decreases the rate of RNA transcription initiation specified by the long terminal repeat. This region has the potential to bind several cellular transcription factors. Here it is shown that sequences which recognize the NFAT-1 and USF cellular transcription factors contribute to this negative regulatory effect. The sequences within the negative regulatory element which resemble the AP-1 site and the URS do not negatively regulate human immunodeficiency virus long terminal repeat transcription initiation.

Replication of human immunodeficiency virus type 1 (HIV-1) is controlled by both host-specified and virus regulatory proteins. The initial step in the synthesis of a new virus particle is the transcription of viral RNA from the HIV-1 proviral DNA. The initial rate of transcription is determined by interactions of cellular proteins with sequences in the long terminal repeat (LTR) near the site of the RNA initiation. Some of the sequences in the viral LTR DNA enhance the rate of RNA synthesis, whereas others present in a region called the negative regulatory element (NRE) decrease LTR-directed gene expression (6, 9, 11). The NRE reduces the expression of viral RNA and proteins. Viruses that contain deletion of this entire region replicate more rapidly in both established T cells and monocyte cell lines (6). Here we show that the two distinct *cis*-acting sequence regions within the NRE contribute to the negative influence of this region on replication of HIV-1.

To determine which subregions within the NRE might be required for the negative effect on HIV-1 LTR-directed gene expression, a series of small deletions within the NRE was made by oligonucleotide-directed site-specific mutagenesis (5). Two of the deletions, DUSF and DNFAT-1, within the NRE were selected to remove sequences known to bind to cell-specified transcription factors. The sequences between positions –173 and –159 from the transcription initiation site are similar to those recognized by the upstream stimulatory factor (USF) (Fig. 1). USF was originally defined as a protein present in HeLa cells which increased the activity of a late adenovirus promoter (1). Nuclear extracts prepared from HeLa cells as well as from the human T-cell line Jurkat contain proteins which bind to the sequences in the HIV-1 LTR located between positions –173 and –159 from the initiation site (4). These sequences share strong similarity with the negative regulatory site in the interleukin 2 receptor (IL-2R) alpha-chain promoter (12). Moreover, the purified USF protein also binds to this region of the HIV-1 LTR (M. Sawadogo, R. Roeder, C. Rosen, and W. Haseltine, unpublished observations). The sequences located between nucleotides –253 and –213 are protected by the nuclear extract

from activated Jurkat cells in DNA footprint experiments (10). Since the LTR fragment containing these sequences can efficiently compete with the NFAT-1 site in the IL-2 promoter that binds to a cellular protein called nuclear factor in activated T cells 1 (NFAT-1), these HIV-1 LTR sequences are designed as the NFAT-1-binding site. Binding of a protein to this sequence is dependent upon T-cell activation (10).

Two more deletions, DAP-1 and DURS, in the NRE were made to remove sequences similar to those that have been shown to bind other eucaryotic transcription factors (Fig. 1). Sequences located between nucleotides –346 and –317 upstream from the initiation site contain sequences which resemble the consensus binding site for AP-1 (3). The region between –185 and –175 upstream from the initiation site is similar to that which binds to the repressor of the yeast arginase gene, called the upstream repressive sequence (URS) (13). An additional mutant designated DEcoRV was made by deleting an *EcoRV* fragment (nucleotides –420 to –338 upstream of the initiation site) from the NRE (Fig. 1).

The relative ability of each of these deletion mutations to direct the expression of bacterial chloramphenicol acetyltransferase (CAT) was determined by transfection of the plasmids containing these LTR sequences located 5' to the CAT gene into a CD4-positive T-cell line, Jurkat-*tat*. The cell line used constitutively expresses the HIV-1 *tat* protein, which activates gene expression directed by the HIV-1 LTR. A plasmid in which the expression of the human growth hormone (*hgh*) gene is under the control of the murine leukemia virus SL3 LTR, SL3-*hgh*, was cotransfected with the HIV LTR-CAT gene to control for possible differences in transfection efficiency. The data in Fig. 2 show that the relative level of CAT activity increased for the promoters in which the NRE was deleted as well as for the USF deletion mutant. The level of LTR activity was not noticeably affected by deletion of the NFAT-1 sequences, deletion of the URS, or the deletion designated delRV. Deletion of the AP-1 site slightly reduced the level of CAT enzyme activity. Similar results were obtained in multiple independent experiments.

The mutant LTRs were introduced into an infectious provirus clone, HXB2-C21X (2, 6), derived from the HIV-1

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FIG. 1. Construction of the HIV-1 LTR containing deletions of possible *cis*-acting repressive sequences. The NRE deletion (DNRE) was made by deleting an *EcoRV* (–420) and an *AvaI* (–157) fragment. DEcoRV was made by deleting the *EcoRV* fragment (positions –420 to –338). DUSF was made by oligo-directed site-specific mutagenesis to remove the sequence between –173 and –159. The sequence (TTCATCACTGG) in this site is similar to the IL-2R NRE site (TTCATCCCAGG) (12). DNFAT-1 was made by deleting the sequence between –253 and –213. The sequence between –346 and –317 was deleted in DAP-1, and DURS lacks the sequence between –185 and –175 (CAGCCGCTA), which has similarity to the URS (CAGCCGCCCA) in the yeast arginase gene.

IIIB isolate (8). The genotype of HXB2-C21X is *gag*⁺ *pol*⁺ *vif*⁺ *vpr* negative *tat*⁺ *rev*⁺ *vpu* negative *nef* negative. The strategy of introduction of a mutant LTR containing a deletion in the U3 region into both the 5' and 3' LTRs was previously described (6).

The rate of replication of each of the mutant viruses was determined either by directly introducing the proviral DNA

through DEAE-dextran transfection into susceptible Jurkat cells or by using viruses derived by transfection to infect the Jurkat cells. In the transfection assays, individual transfection efficiencies were compared with one another by cotransfection with SL3-*hgh*. The level of *hgh* was measured 3 days posttransfection. The culture medium was then changed every day thereafter for reverse transcriptase (RT) assays. The viable cells in each cultures were also monitored. Both the USF deletion virus and the NFAT-1 deletion virus replicated more rapidly in Jurkat cells than did the parental HXB2-C21X virus (Fig. 3B). The virus carrying a deletion in the AP-1 site showed no significant difference in growth compared with the parental virus. The viable-cell numbers of these transfected cultures correlated with the amount of reverse transcriptase. Greater RT production resulted in a more rapid and extensive decline in the number of viable cells (Fig. 3A). The rate of replication of mutant and parental viruses was also determined in experiments in which Jurkat cells were infected with these viruses. For this purpose the parental and mutant proviruses were transfected into Jurkat cells. Cells which survived the infection were found to express the viruses constitutively. Supernatant fractions from these cultures were harvested, and the amount of reverse transcriptase activity in each supernatant fluid was determined. Equivalent amounts of virus, as judged by the supernatant reverse transcriptase activity, were used to initiate infection of a fresh population of Jurkat cells. The level of extracellular reverse transcriptase activity in culture supernatant was determined daily (Fig. 4). The results show that cells infected with the NRE deletion mutant yielded more reverse transcriptase activity than did cells infected with the parental virus from days 9 through 12. The amount of reverse transcriptase activity produced by cells infected with the mutant in which the USF site was deleted also

FIG. 2. Transient expression assays for the LTR promoter function. Ten micrograms of the plasmid pTZIIICAT (6) or the mutant DNA was transfected into 3×10^6 Jurkat-*tat* cells. Forty-eight hours after the transfection, the cell extract was collected and assayed for CAT activity by the methods previously described (9). The CAT activities were all normalized with the amount of protein used in the assays and to the activity of the wild-type LTR. The averages of the results from three experiments are presented. One representative set of data from an *hgh* assay in which the amount of *hgh* in 50 μ l of cell extract was measured with a radioimmunoassay kit from Nichols Institute is also presented.

FIG. 3. Proviral DNA (10 μ g) and SL3-*hgh* DNA (5 μ g) were used to transfect 5×10^6 Jurkat cells. Three days after the transfection, the amount of *hgh* in the culture supernatant was measured with a radioimmunoassay kit (Nichols Institute). The results in counts per minute were as follows: mock transfection, 200; HXB2-C21X, 2,300; DUSF, 2,200; DNFAT-1, 2,000; and DAP-1, 2,200. The culture media were changed daily to detect RT activity by a method described previously (2). The results are presented in panel B. The numbers of viable cells in the transfected cultures are presented in panel A. Symbols: ●, HXB2-C21X; □, DNFAT-1; ○, DUSF; △, DAP-1; ▲, mock transfection.

increased throughout this period. No increase in the relative level of reverse transcriptase activity was evident from cells infected with the delURS or delRV mutants. The virus in which the NFAT-1 site was deleted yielded more reverse transcriptase activity from day 7 to day 12 postinfection than did the wild-type virus. These experiments were repeated at least twice for each mutant, with similar results. It is notable that the different growth rates of mutant viruses appeared to affect peak levels of RT activity as infectious viruses, whereas such differences were manifested as a delayed onset of virus production in which infection was initiated by transfection with the provirus.

The experiments described here extend our current understanding of the sequences within the HIV LTR which negatively regulate the rate of viral RNA initiation. The NRE region contains consensus binding sequences for numerous cellular transcription factors. Deletion of two of these, one which specifies binding of a protein activated during activation of T cells (the NFAT-1-binding site) and another which has a binding site previously demonstrated by the USF transcription factor, resulted in production of viruses which replicated more rapidly than did the parental virus in T-cell cultures. Deletion of several other proposed

FIG. 4. Growth rate comparison between HXB21-X and the LTR mutations. Ten micrograms of plasmid DNA containing the wild-type (HXB21-X) (○), the NRE deletion (DNRE) (□), the *EcoRV* fragment deletion (DEcoRV) (▲), the AP-1 deletion (DAP-1) (●), the NFAT-1 deletion (DNFAT-1) (□), the URS deletion (DURS) (■), or the USF deletion (DUSF) (△) proviral genome was used to transfect 3×10^6 Jurkat cells by the DEAE-dextran method as previously described (2). The culture medium, RPMI 1640 supplemented with 10% fetal calf serum, was changed every 3 days, and the supernatants of the cultures were harvested and filtered through 0.22- μ m-pore-size filters 9 days after the transfection. The filtered supernatant was then added to an equal volume of fetal calf serum. Approximately 1 ml of this mixture was tested for RT activity, and the remainder was frozen at -70°C . Jurkat cells (2×10^6) were then infected with the filtered material containing 0.5×10^6 cpm of reverse transcriptase by the method previously described (2). The culture medium was changed daily after the infection, and the reverse transcriptase activity in the supernatant was measured. The averages of two samples are presented.

binding sites within the NRE region of the LTR had no apparent effect on transcription initiation.

It is curious that one of the sequences which had a negative effect on virus replication, NFAT-1, did not appear to negatively regulate the rate of expression of a heterologous gene driven by the viral LTR. The NFAT-1 deletion LTR, however, did have better promoter activity when the host cells were stimulated by phorbol ester (Y. Lu, N. Touzjian, M. Stenzel, T. Dorfman, J. Sodroski, and W. Haseltine, submitted for publication). It is possible that NFAT-1 deletion has an effect on the LTR only when the host cells are activated. It is also possible that the negative effects of the NFAT-1 sequences on virus replication are due to processes other than viral transcription.

It is remarkable that the two sequences which contribute to the negative regulation of the HIV-1 LTR, the NFAT-1-binding site and the USF-binding site, have also been

identified in the sequence 5' to the site of RNA initiation of the IL-2 gene and the IL-2R alpha-chain gene (10, 12). The USF site in the IL-2R alpha-chain promoter has been demonstrated to be the site for a negative regulatory factor, and the same factor also binds to the USF site in the HIV-1 LTR (12). The NFAT-1 site responds to T-cell activation signals by recognition of a transcription factor activated upon T-cell stimulation (12). It is possible that the binding of NFAT-1 to the HIV-1 LTR has a negative effect by competing with the downstream enhancer site, which functions better when it is alone.

The USF transcription factor has a positive effect on the rate of initiation on the adenovirus 2 major late promoter. The contrasting suppressive effect of this sequence on the HIV LTR may be a consequence of either the opposite orientation of the binding sequence or the increased distance of this sequence from the site of transcription initiation (173 nucleotides for HIV compared with 55 nucleotides for adenovirus 2) or due to recognition by a different protein in Jurkat cells. Sequences surrounding the USF region may also influence the function of a protein that binds to this region.

A general principle of redundant regulatory elements is emerging in the analysis of HIV-1 gene function. This report demonstrates that at least two sequences contribute to the negative effect of the NRE region of the LTR on RNA initiation. The effects of these sequences appear to be independent of one another, as the negative regulatory effect is observed in the absence of either the NFAT-1 or the USF sequence. We have recently demonstrated that the increased rate of HIV-1 transcription initiation observed in T cells in response to mitogenic stimulation is also a consequence of recognition of cellular proteins by two different sequences within the HIV-1 LTR, the NFAT-1 sequence described here and a sequence which binds the NF- κ B transcription factors (7). These two sequences also act independently of one another. We have also found that HIV-1 encodes a second positive *trans* activator, the product of *vpr*, in addition to the *trans* activator originally described, the product of the *tat* gene. The *vpr* product greatly accelerates the rate of the viral protein expression early in infection. Overlapping regulatory functions might ensure replication of virus populations that sustain a high rate of mutation during the course of replication.

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