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Identification of a Replication-Competent Pathogenic Human Immunodeficiency Virus Type 1 with a Duplication in the TCF-1α Region but Lacking NF-κB Binding Sites

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Multiple human immunodeficiency virus type 1 (HIV-1) sequences with deletions of NF-κB binding sites at both the 5′ and 3′ long terminal repeats (LTRs) were identified in serial samples collected from an infected individual. The effect of this deletion on the level of transcription was studied by transient transfection of an LTR-driven luciferase reporter gene and by infection with a full-length recombinant HIV-1 containing a luciferase reporter (HIV_{HXBluc}). Detectable levels of gene expression were found in both systems, in the presence or absence of the viral transactivator Tat. Interestingly, a duplication of a putative TCF-1α motif was found in place of the NF-κB elements in these viruses. Higher transcriptional activity was observed with HXBLTR (NF-κB intact) than with the patient's LTR (NF-κB deleted), suggesting that the NF-κB binding sites may promote optimal levels of viral gene transcription. The ability of these viruses with NF-κB deleted to replicate and cause substantial decline in CD4 cell counts demonstrates that the NF-κB binding sites are not absolutely required for viral replication or pathogenicity in vivo. These results are consistent with the notion that the HIV-1 LTR possesses functional redundancy which allows it to interact with multiple transcription factors, thereby ensuring viral replication in a variety of cell types.

The human immunodeficiency virus type 1 (HIV-1) provirus, like all retroviruses, contains two identical long terminal repeats (LTRs) flanking the three structural genes *gag*, *pol*, and *env* that are essential for viral replication. Each LTR consists of three regions, namely, U3, R, and U5 (12, 14). HIV-1 transcription is regulated by multiple viral and cellular transcription factors that bind to distinct sequence elements in the 5′ LTR (12–14, 17, 22, 26, 35). Moreover, the initiation of HIV-1 transcription is under the control of cellular factors; the subsequent activation of HIV-1 transcription, however, is largely mediated by virus-encoded transactivators such as Tat (12–14, 26, 33, 35). Previous reports have identified several sequence elements within the LTR that are important in regulating the level of viral transcription. For example, efficient transcription of HIV-1 in T lymphocytes or monocytes/macrophages requires the presence of the TATA box motif, the binding sites for NF-κB and Sp1, and the Tat-response region (TAR) (12–14, 16, 22, 26, 33, 34). Mutagenesis of these sequence elements either individually or summarily inhibits or inactivates HIV-1 replication (12, 14, 16, 22, 25, 31, 33, 34, 36).

The HIV-1 5′ LTR contains two NF-κB binding sites located upstream of the Sp1 binding sites and the TATA promoter (12, 14). Conservation of these two binding sites among most HIV-1 isolates suggests their importance in viral replication (9, 12, 14, 24, 26, 29, 30). NF-κB binding sites interact with and respond to the rel/NF-κB family of transcription factors which can be induced by a variety of mitogens, cytokines, viruses, and other agents (3, 12–14). Members of the human rel/NF-κB family include p50, p52, p65, c-rel, and relB (3, 14, 35, 38). NF-κB exists typically as a heterodimer composed of p50 and p65 subunits (3, 38). While p50 functions as a strong DNA binding subunit, p65 is responsible for the transcriptional activity of the NF-κB complex (38). Originally, NF-κB was believed to be present only in B lymphocytes, but it was later found in a variety of cell types, including T lymphocytes and monocytes/macrophages upon activation (3).

Regulation of HIV-1 LTR by NF-κB and other cellular and viral factors is quite complex. Duplication of NF-κB binding sites can enhance both LTR-driven reporter gene activity and viral replication (11, 25, 31, 36), whereas its deletion can impair viral replicative capacity in a variety of cell types such as peripheral blood mononuclear cells (PBMCs), CD4 lymphocytes, monocytic cell line U937, and lymphoblastoid T-cell lines CEM and 11.8 (1, 12, 14, 21, 23, 35, 42). In contrast, other reports have demonstrated that mutated HIV-1 missing one or both NF-κB binding sites is still capable of efficient replication in stimulated PBMCs, although a 2- to 4-day delay in peak virus production was observed in CEM and MT-4 cells (11, 25, 36, 40). Moreover, a prerequisite for HIV-1 with an NF-κB binding site deleted to replicate is the presence of three Sp1 binding sites (25). Elimination of the Sp1 binding sites can reduce HIV-1 replication to below detectable levels in all cell types tested (25, 36). The conflicting observations on the role of NF-κB binding site in HIV-1 replication could be partially explained by differences in HIV-1 constructs, cell types, and exogenous stimulating factors used in the assay systems (1, 11, 23, 25, 31, 36, 42), as was reported by Antoni et al. (2). Initiation and completion of HIV-1 replication therefore are not merely dependent on the presence of specific sequence elements in the LTR, but are also dependent on the availability and the relative abundance of transcriptional factors present in the target cells. The high degree of functional redundancy observed in the HIV-1 LTR may enable viral replication to occur in various cell types (12, 14, 36). The observation that a great variety of cell types are productively infected by HIV-1 in vitro and in vivo further supports this notion (7, 26).

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TABLE 1. General clinical and virologic characterization of serial samples from the study subject

Date of sample collection (mo/yr)	No. of CD4 counts/μl of blood	No. of proviral DNA copies/ 10^6 PBMCs	Infectious virus titer of PBMCs/plasma[a]	Replicative capacity[b]		Syncytium formation in MT-2 cells[b]
				Macrophages	H9 cells	
5/84	699	593	92/<1	+	−	−
6/85	1,153	121	32/<1	++	−	−
3/86	751	275	64/<1	++	−	−
6/87	660	641	118/<1	++	−	−
9/88	645	109	32/<1	++	−	−
9/90	696	23	3.2/<1	++	−	−
3/91	762	231	46/<1	++	−	−
5/91	454	296	59/<1	++	−	−

[a] Fifty percent tissue culture infective dose measured per 10^6 PBMCs or per milliliter of plasma.
[b] Results have been published elsewhere (8) and are included here solely for clarity.

To date, much of our knowledge about HIV-1 LTR regulation comes from in vitro studies with LTR-driven reporter genes or full-length viruses with mutations in certain elements (1, 12, 14, 16, 23, 25, 34, 36). Although these studies provide substantial insight into the regulatory mechanism of HIV-1 LTR, less is known about the effect of these mutations on the degree of HIV-1 replication or pathogenicity in vivo. Efforts have been made to evaluate the relative importance of NF-κB and Sp1 binding sites in the replication of HIV-2 and simian immunodeficiency virus SIVmac. Results of a study of pathogenicity in pig-tailed macaques suggest that duplication of the NF-κB binding site within the LTR is not responsible for the rapid, severe syndrome associated with SIVsmmPBj6.6 infection (10, 32). However, a chimera in which the SIVsmmPBj6.6 LTR was replaced with an SIVsmH4 LTR caused disease in only 50% of inoculated macaques (32). Further studies are needed to determine whether this chimera may be somewhat attenuated because of the presence of only a single NF-κB in the LTR (10, 32). While Bellas et al. (4) found that the NF-κB binding site has a substantial effect on SIVmac replication in primary macrophages, Ilyinskii and Desrosiers showed that even complete elimination of the NF-κB and the Sp1 binding sites has only a modest effect on SIVmac replication in rhesus PBMCs (20). Markovitz et al. suggested that the NF-κB binding site plays a significant role in the response of the HIV-2 LTR to extracellular stimulation (28). Ideally, the replicative capacity of these mutated SIVmac and HIV-2 should be studied with appropriate animal models, since in vivo results would have a greater relevance for understanding the replicative and pathogenic consequences of these LTR mutations. However, even if mutated SIVmac and HIV-2 were studied with animal models, the findings may not be relevant for the HIV-1 LTR, which differs considerably in its organization and function from SIVmac and HIV-2 (10, 20, 28, 32, 37).

During the course of studying HIV-1 LTR sequence variation in HIV-1-infected individuals, we came upon one subject whose HIV-1 LTR sequences lack both NF-κB binding sites, while retaining the Sp1 binding sites, TATA box motif, and TAR (see below). Taking advantage of this unique situation in which a natural deletion of both NF-κB binding sites was found, systematic studies of the HIV-1 species present in this subject were performed to elucidate some of the in vivo regulatory mechanisms of the HIV-1 LTR that would otherwise be difficult to determine by in vitro studies. Therefore, serial samples collected from this individual were subjected to virologic and immunologic characterizations. Quantitative viral culture, viral phenotypic analysis, quantitative proviral DNA measurement, PCR amplification of LTRs and direct sequencing, and in vitro functional analysis of the 5′ LTRs were performed with samples collected serially. Our results suggested the following conclusions. First, NF-κB binding sites within the LTR do not appear to be absolutely required for HIV-1 replication or pathogenicity in this patient. Second, compared to the wild type, the LTR with NF-κB deleted demonstrated less transcriptional activity in the context of the luciferase reporter gene system and the whole HIV-1 molecular clone HIV_{HXB}. Thus, NF-κB binding sites appear to play a role in efficiently transcribing HIV-1. Detailed descriptions and the implications of these studies are presented and discussed below.

The study subject, patient D from our previous study (8), is a homosexual male who became seropositive for HIV-1 between February and May of 1980. Eight sequential PBMC samples were collected between May of 1984 and May of 1991. During this period, he did not receive any antiviral therapy for HIV-1 and remained clinically well, with the exception of recurrent genital herpes simplex infections. His CD4 cell counts remained relatively stable within the normal range until 1991, when they dropped to 454 cells per μl. The cell count declined to below 200 per μl by 1994 (18), thus meeting the Centers for Disease Control definition of AIDS (5). The general clinical and virologic characteristics of this individual are summarized in Table 1. HIV-1 was successfully isolated from all of the PBMC samples collected. The infectious titer of HIV-1 ranged from 3.2 to 118 50% tissue culture infective doses per million cells, as quantified by a standard limiting dilution assay (19). The quantity of HIV-1 proviral DNA copies in PBMCs correlates significantly ($P < 0.001$) with the infectious titer. Furthermore, the amount of HIV-1 measured by these two techniques lies at the low end compared with that found in patients with moderate rates of disease progression (19, 39). In addition, sequential isolates from this individual were able to replicate in both normal donor PBMCs and macrophages, but failed to infect H9 or MT-2 cells or to induce syncytium formation (8). Plasma cultures were uniformly negative for infectious virus (<1 50% tissue culture infective dose per milliliter) in all of the samples tested. Unfortunately, the patient has been lost to follow up, and no samples after 1991 are available.

In order to study the LTR sequences in this individual, genomic DNA was first extracted by a standard proteinase K-phenol protocol (39) from serial PBMC samples pre- and post viral culture. For each sample, approximately 1 μg of genomic DNA was subjected to limiting dilution (39) before proceeding to two rounds of PCR. Nested primers were used for both the 5′ and 3′ LTRs to increase the sensitivity and the specificity of the amplification. Both rounds of PCR consisted of 25 cycles, with each cycle involving three steps: 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1.5 min at 72°C for elongation. A final extension of 7 min at 72°C was

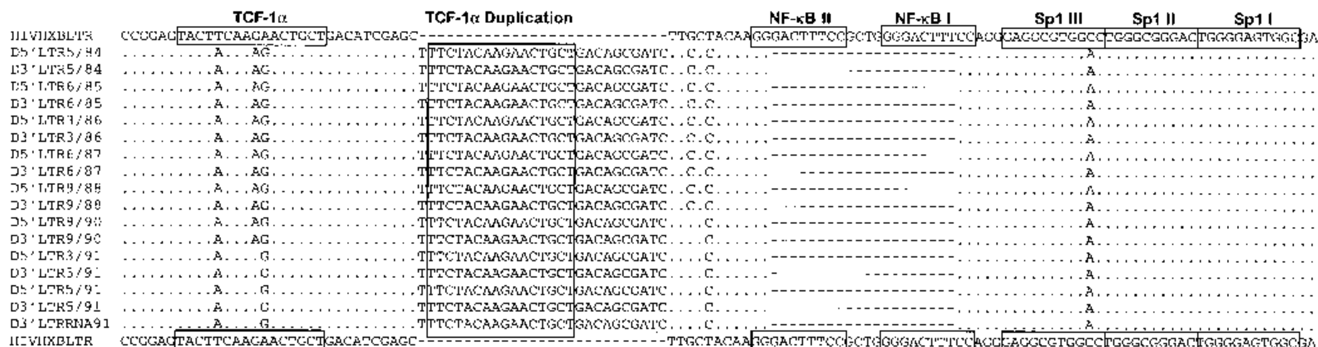


FIG. 1. The 5′ and 3′ LTR consensus sequences derived from the study subject over time, aligned against HIV_{HXBLTR}. Dots indicate identical sequences, and dashes indicate gaps introduced to preserve alignment. The locations of binding sites for TCF-1α, NF-κB, and Sp1 are shown in boxes, according to the findings of Gaynor (14).

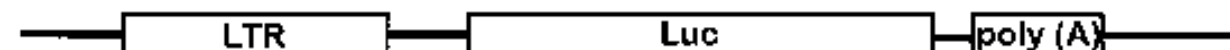
performed at the end of the 25 cycles. A *Pfu* polymerase (Stratagene) with proofreading activity was used in conjunction with *Taq* (Boehringer Mannheim) polymerase to improve the accuracy of the amplification while maintaining the high processivity of *Taq*. The nucleotide sequences of the outer and inner primer pairs for both the 5′ and 3′ LTRs are listed below; the coordinates shown in parentheses denote their positions in the HIVNL43 sequences (+, sense; −, antisense): 5′ LTR outer, 5′-CACACACAAGGCTACTTCCCTGATTGGC AGA (+57); 5′-TCTGATAATGCTGAAAACATGGG (−1319); 5′ LTR inner, 5′-CAAGGCTACTTCCCTGATTG GCAGAACTACACACCAGG (+63); 5′-AATGATCTAAG TTCTTCTGATCCTGT (−1022); 3′ LTR outer, 5′-ATGGG TGGCAAGTGGTCAA (+8787); 5′-TGCTAGAGATTTTC CAC (−9709); 3′ LTR inner, 5′-TTTCCAGTCAGACCTCA GGTACC (+8988); 5′-GTCTGAGGGATCTCTAGTTACC AGAGTC (−9680). Direct sequencing of the PCR products was performed by the method of Winship et al. (41). Viral RNA sequences in the culture supernatant of the last sample were also determined as previously described (43). Briefly, viral RNA was extracted by guanidinium thiocyanate-phenol solution. cDNA was then synthesized at 42°C for 30 min with avian myeloblastosis virus reverse transcriptase (Promega). The nested PCR amplification and sequencing of cDNA products were then carried out as stated above. Appropriate negative controls were included throughout the entire course of study to monitor the possibility of cross-contamination.

Figure 1 shows the consensus sequence of the 5′ and 3′ LTRs obtained from each of the serial samples. Both NF-κB binding sites had apparently been deleted in all of the samples tested from both the 5′ and 3′ LTRs, suggesting that the loss of the NF-κB binding sites in this individual is a genuine phenomenon. Three Sp1 binding sites, the TATA box motif, and TAR regions are well conserved among the sequences obtained. Interestingly, compared with the HIV_{HXB} LTR, a 27-nucleotide insertion was found upstream from the deleted NF-κB binding sites in all of the sequences (Fig. 1). Some of the 27 nucleotides are, in fact, a duplication of the upstream region, previously designated as TCF-1α sites (12, 14). Duplication of TCF-1α in HIV-1 LTR is not an uncommon finding, because similar observations have been made in the studies of naturally occurring LTR variants in both asymptomatic as well as symptomatic patients (24, 30). In addition, enhanced activity of the LTR in directing both viral and heterologous gene expression has been observed with the duplication of TCF-1α (15). It is therefore plausible to suspect that the duplication of TCF-1α in this individual may compensate for some of the lost enhancer activity due to the deletion of the NF-κB binding sites.

The degree of sequence diversity of the 5′ LTR varies from year to year. The intrayear diversities were 1.7% in 1985, 2.3% in 1988, 1.3% in 1990, and 0.5% in 1991; thus, no clear pattern of change can be observed. However, by comparing the interyear diversities, it is quite clear that the viral population had been diverging away from the early viral species. The genetic distance between 1985 and 1988 is 2.3%, that between 1985 and 1989 is 2.8%, and that between 1985 and 1991 is 3.3%. The consistent increase in genetic distance over time towards the early viral species suggests that viral evolution continues despite the asymptomatic stage of HIV-1 infection, and there is no truly latent period for viral variation in vivo.

The impact of the NF-κB deletion on the level of transcriptional activity was evaluated in the context of a luciferase reporter gene system (Fig. 2) (6). The LTR sequences obtained from the last sample were cloned into the upstream region of the luciferase reporter gene in plasmid pGL-2 (Promega), and the resultant constructs are named DLTRluc. The LTR sequence from HIV_{HXB} was also cloned into the same reporter vector for comparison and named HXBLTRluc. Five, 1, 0.2, and 0.04 μg of each construct were used in the transfection of 293 cells, a human embryonic kidney cell line, in the presence (0.1 μg) or absence of a Tat expression vector (pCMVTAT). The cells were incubated at 37°C and harvested 48 h posttrans-

1. HXBLTRluc or DLTRluc



2. HIVHXBluc or HIVDluc

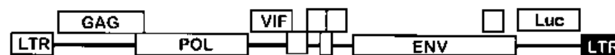


FIG. 2. Schematic illustration of the constructs used in evaluating the LTR transcriptional activities, both in the context of a luciferase (Luc) reporter vector (upper panel) and the whole infectious virus (lower panel). The 3′ LTR that has been replaced with the LTR with NF-κB deleted is highlighted.

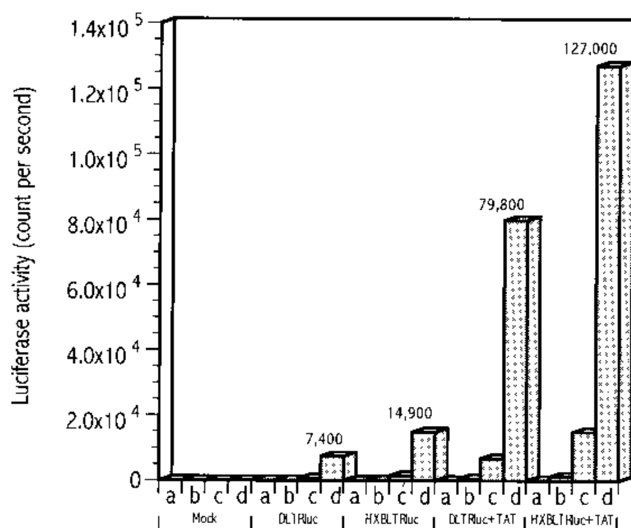


FIG. 3. Comparison of transcriptional activities of the LTR with NF-κB deleted (DLTRluc) and the LTR with NF-κB intact (HXBLTRluc) in the presence and absence of transactivator Tat. Luciferase activities were measured 48 h posttransfection of 293 cells. A total of 0.04, 0.2, 1, and 5 μg of each construct was used in the transfection, indicated by the letters a, b, c, and d, respectively.

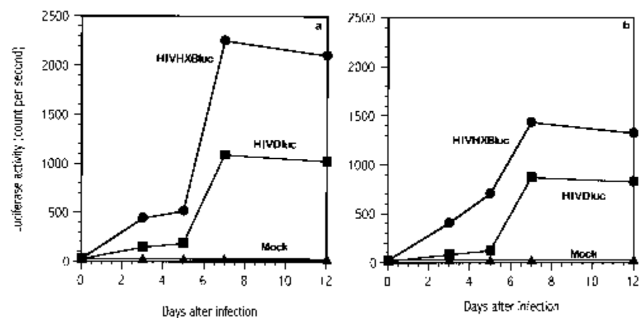


FIG. 4. Comparison of transcriptional activities of the LTR with NF-κB deleted (HIV_{Dluc}) and the LTR with NF-κB intact (HIV_{HXBluc}) in the context of the whole infectious virus. Luciferase activities were measured after infection of 5×10^6 PHA-stimulated donor PMBCs (a) and Jurkat T cells (b) on days 0, 3, 5, 7, and 12.

fection for luciferase assays. Harvested cells were lysed with 200 μl of 1× luciferase lysis buffer (Promega). Twenty microliters of the lysates was then assayed for photon emission with a luminometer (Packard). The final readings of luciferase activity were corrected for both the transfection efficiency and the protein concentration in the cell lysates. Transfection efficiencies were controlled by cotransfecting a β-galactosidase expression vector, pSV-β-galactosidase (Promega). The protein content in the cell lysates was determined by the Bio-Rad protein assay system (Bio-Rad). All transfections were performed at least in duplicate.

Figure 3 shows the average luciferase activity driven by the LTR with NF-κB deleted (DLTRluc) and the intact HXBLTR (HXBLTRluc) in the presence and absence of Tat. Detectable levels of luciferase activity were found in cells transfected with either DLTRluc alone or with DLTRluc and Tat (Fig. 3), although the latter demonstrated a substantially higher activity. In addition, the transcriptional activity of HXBLTRluc is higher than that of DLTRluc ($P < 0.05$), in the presence or absence of Tat. This observation is in agreement with previous reports in which higher transcriptional activity was found to correlate with more NF-κB binding sites on the LTR (11, 36).

The patient's LTR sequences were also introduced into the infectious provirus HIV_{HXBluc} to study the effect of the NF-κB deletion on the level of viral transcription (Fig. 2). Provirus HIV_{HXBluc} was constructed by insertion of the *Photinus pyralis* luciferase gene in *nef* (6). It is known that the U3 region of the viral RNA is derived from the 3′ LTR of the provirus. This U3 region is then duplicated upon formation of the new provirus (26, 27). We therefore replaced the 3′ LTR of HIV_{HXBluc} with the LTR with NF-κB deleted and named the provirus construct HIV_{Dluc} (Fig. 2). The new provirus produced after one round of HIV_{Dluc} replication will contain the NF-κB deletions in its 5′ and 3′ LTR. The level of luciferase activity measured in the infected target cells will directly correlate with the transcriptional capacity of the corresponding 5′ LTR, thus enabling us to study the effect of the NF-κB deletion in the context of the whole virus. In brief, the LTR with NF-κB deleted was cloned between the *Xho*I and *Asc*I sites of HIV_{HXBluc}. The *Xho*I site is a naturally occurring site, whereas the *Asc*I site was introduced at the end of the 3′ LTR by site-directed mutagenesis. Twenty micrograms of provirus constructs was used to transfect 293 cells. Forty-eight hours posttransfection, culture supernatant was harvested and filtered. The virus concentration in the culture supernatant was standardized by measuring the $p24^{gag}$ concentration (Abbott Laboratories) before use in the subsequent infection assay. Five million phytohemagglutinin (PHA)-stimulated donor PBMCs or Jurkat T cells were inoculated with 5 ng of $p24^{gag}$ of the virus. After a 2-h incubation at 37°C, the cells were washed extensively and resuspended in growth medium supplemented with 10% fetal calf serum and 10 U of interleukin 2 per ml. Infected PBMCs and Jurkat T cells were harvested for the luciferase assay on days 0, 3, 5, 7, and 12. Cell lysis and measurement of luciferase activity were carried out as described above.

Figure 4 shows the level of luciferase activity detected in HIV_{HXBluc}- or HIV_{Dluc}-infected PBMCs and Jurkat T cells at days 0, 3, 5, 7, and 12, respectively. In HIV_{Dluc}-infected PBMCs and Jurkat T cells, viral transcription continues independent of the NF-κB binding sites. Peak levels of transcription were recorded approximately 7 days postinfection, with a moderate decline in the ensuing period, although a generally higher level of transcription was found in PHA-stimulated PBMCs than in Jurkat T cells (Fig. 4). In addition, luciferase activity in both HIV_{HXBluc}-infected PBMCs and Jurkat T cells registers almost twice that in HIV_{Dluc}-infected PBMCs and Jurkat T cells in all samples detected (Fig. 4). This finding suggests that HXBLTR has a higher transcriptional capacity than DLTR in both PBMCs and T cells ($P < 0.05$). These results clearly demonstrate that NF-κB binding sites are not indispensable in the course of viral replication during the infection of primary cells. The higher transcriptional activity found in HIV_{HXBluc} with respect to HIV_{Dluc} also suggests that NF-κB binding sites are required for optimal levels of viral gene transcription. This observation is in agreement with those of previous reports in which increased numbers of NF-κB binding sites were shown to correlate with higher levels of transcriptional activity by the LTR and increased viral replication in almost all cell types studied (11, 36).

In order to confirm that the observed differences in transcriptional activity are indeed due to mutations in the TCF-1α and NF-κB regions, PCR amplification and sequencing of the entire 5′ LTR were carried out after one round of infection of both PHA-stimulated PBMCs and Jurkat T cells. Figure 5 shows the actual 5′ DLTR nucleotide sequence compared with the 5′ HXBLTR nucleotide sequence. It is clear that the du-

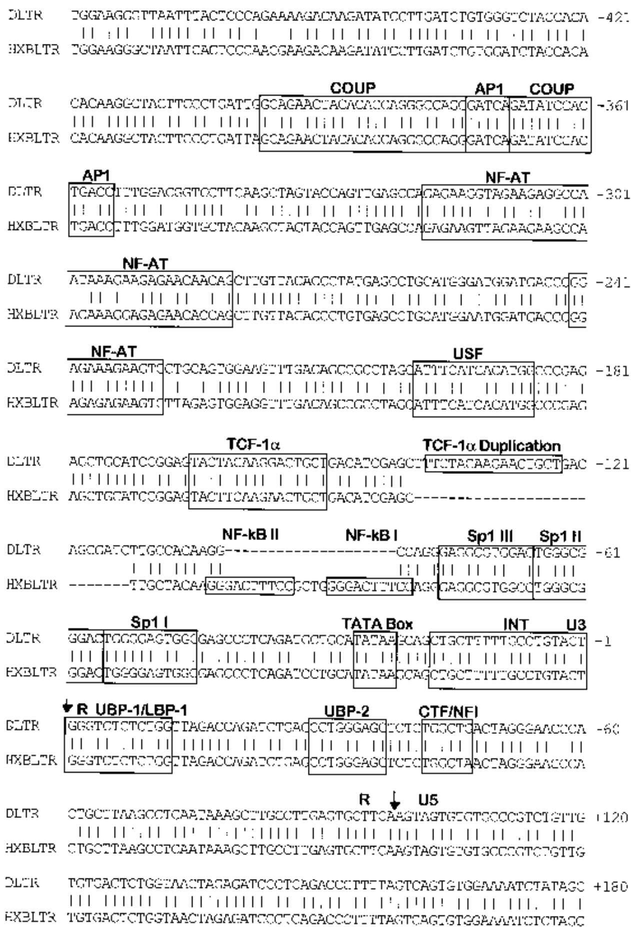


FIG. 5. Comparison of the nucleotide sequence of the LTR with NF-κB deleted (DLTR) with that of the LTR with NF-κB intact (HXBLTR) after one round of infection. Regions that are bound by the cellular transcription factors are shown in boxes, and their locations are indicated according to the findings of Gaynor (14). Vertical lines indicate identical sequences, and dashes indicate gaps introduced to preserve alignment.

plication of TCF-1α region and the deleted NF-κB binding sites are present in the U3 region of 5′ DLTR. The sequence variation in other regions of 5′ DLTR, however, is quite minimal, which is reflected by the high degree of conservation in almost all of the elements that are functionally important (Fig. 5).

In summary, a naturally occurring HIV-1 strain with NF-κB binding sites deleted was identified in all available samples collected from an HIV-1-infected individual. The ability of this virus to replicate and force a decline in $CD4^+$ lymphocyte counts clearly indicated that a virus lacking NF-κB binding sites can cause disease in humans. While many possible explanations may exist, functional redundancy of the LTR is likely to be the most plausible one (36). Multiple sequence elements present in the HIV-1 LTR permit their interactions with multiple transcription factors individually or in particular combinations, depending on the availability and relative abundance of the factors present in the infected target cells. “Safety in numbers” would not be a far-fetched description of the strategy that the HIV-1 LTR uses to ensure its replication in a great variety of cell types. Furthermore, although dispensable, NF-κB binding sites are found to enhance the capacity of the HIV-1 LTR in directing transcription of both viral and heterologous genes, suggesting that the presence of NF-κB binding sites in the vast majority of viral isolates may function to promote optimal levels of viral gene transcription. Finally, a duplication of a putative TCF-1α motif was also found in place of the NF-κB elements in these viruses, but the biological impact of this duplication warrants further investigation.

Nucleotide sequence accession number. The sequences obtained were given GenBank accession numbers U80224 and U80225.

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