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The NF-κB Binding Sites in the Human Immunodeficiency Virus Type 1 Long Terminal Repeat Are Not Required for Virus Infectivity

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Mutations were introduced into the regulatory sequences in the long terminal repeat of an infectious molecular clone of the human immunodeficiency virus. Viruses in which the NF-κB binding sites were deleted or ones in which one or two Sp1 binding sites were mutated still replicated efficiently in human T lymphocytes. A deletion of the two NF-κB sites plus the three Sp1 sites or a mutation of the *tat*-responsive region rendered the virus replication incompetent. Thus, the NF-κB sequences are not required for human immunodeficiency virus infectivity; however, a *tat*-responsive region is essential.

Replication of the human immunodeficiency virus (HIV) is regulated by virally encoded proteins, such as *tat* (3, 36), *rev* (8, 37), and *nef* (2, 23), and by cellular transcriptional factors (39), all of which modulate viral gene expression. The locations of *cis*-acting DNA regulatory sequences in the HIV long terminal repeat (LTR) that mediate the effects of the viral *tat* gene (17, 18, 32) and of the cellular transcriptional factor Sp1 (20) have been previously determined. At least three cellular proteins (11, 26, 42, 43) have been shown to bind to the enhancer sequence in the LTR, a directly repeated motif referred to as the NF-κB binding site. The role that these sequences play in activation of the LTR in in vitro assays (6, 26, 31, 42, 43) has been previously described. In the present study, we evaluated the function of these sequences during productive HIV infection of human T cells by introducing a series of mutations into an infectious molecular clone of HIV-1 containing a single LTR. These experiments demonstrated that the NF-κB binding sites in the HIV LTR are not required for production of progeny virions. In contrast, deletion of the two NF-κB sites plus the three Sp1 sites rendered the proviral DNA replication incompetent, as did a 4-base-pair (bp) deletion in the *tat* response (TAR) region.

A circularly permuted variant of the infectious molecular clone of HIV-1, pNL4-3 (1), which contained a single LTR was constructed. A complete LTR element linked to genes situated near both the 5′ and 3′ ends of the viral genome was generated by ligating the 912-bp *Hin*dIII-*Sph*I fragment (U5 sequences) to the 1,141-bp *Bam*HI-*Hin*dIII fragment (U3 + R sequences) (Fig. 1). This reconstructed LTR (Fig. 1B) was then molecularly cloned into *Bam*HI-*Sph*I-cleaved M13 mp19 DNA to facilitate the introduction of specific mutations by oligonucleotide-directed, site-specific mutagenesis (45) as modified by Kunkel (22). After confirmation of the mutagenized segments by DNA sequence analysis (33), the LTR elements were excised from the M13 vector by cleavage with *Bam*HI and *Sph*I. The wild-type (pILIC) LTR and one Sp1 mutant (pSpB) LTR segment were ligated to the 7.4-kilobase (kb) *Sph*I-*Xho*I internal HIV fragment from pNL4-3 (Fig. 1C) and then inserted into plasmid vector pIBI20, which had been cleaved with *Bam*HI and *Xho*I. All other mutagenized LTR segments were ligated to the 7.0-kb *Sph*I-*Bam*HI fragment from pNL4-3 (Fig. 1D) and cloned into pUC19 at the *Bam*HI site. The structures of the resultant single LTR provirus plasmid DNAs were confirmed by cleavage with *Bam*HI, *Xho*I, or *Hin*dIII and by double digestion with *Bam*HI plus *Sph*I. The mutagenized segments of each LTR were also resequenced in the reconstructed single LTR proviral clones before use in biological assays.

The DNA sequences of the mutant LTRs used in these studies are shown in Fig. 2. The sequence shown for the reconstructed wild-type single-LTR infectious clone (pILIC) corresponds to that of the lymphadenopathy-associated virus strain of HIV-1 (40). The pNFA mutant LTR contains a 29-bp deletion that removes the two NF-κB binding sites; a 6-bp *Bcl*I restriction enzyme recognition sequence was inserted in their place. The pdl-NF mutation also removes the two NF-κB sites without insertion of additional DNA sequences. Two base-pair point mutations (GG to TT) that interfere with Sp1 binding (20) were introduced into the 5′ site (pSpB) or both the 5′ and middle Sp1 sites (pSpC). The pSNF LTR contains changes affecting adjacent Sp1 and NF-κB sites. The 5′ Sp1 site was mutagenized as described above; in addition, a 3-bp point mutation changing GGG to CTC was introduced into the 3′ NF-κB binding sequence, eliminating the ability of NF-κB to bind to this site (26). A large deletion that removes both NF-κB binding sites and all three Sp1 sites (pTA) was also constructed. The TAR regions of the wild-type (pILIC) and mutant (pTAR) LTRs are depicted in Fig. 2B. pTAR contains a 4-bp deletion at the *Sac*I site which disrupts the tip of the stem-loop structure and eliminates the LTR response to the HIV Tat protein (17).

The biological activities of HIV proviruses containing wild-type or mutagenized LTRs were assayed following the release of circularly permuted proviral DNA from plasmid vectors with *Bam*HI and ligation of the cleaved DNA to generate concatemers. The ligated DNA preparations (35 μg) were then transfected into 7.5×10^6 A3.01 cells (10) by a modification of the DEAE-dextran procedure (7); 2 days later, cocultivation with 2×10^6 MT4 cells (16) was initiated

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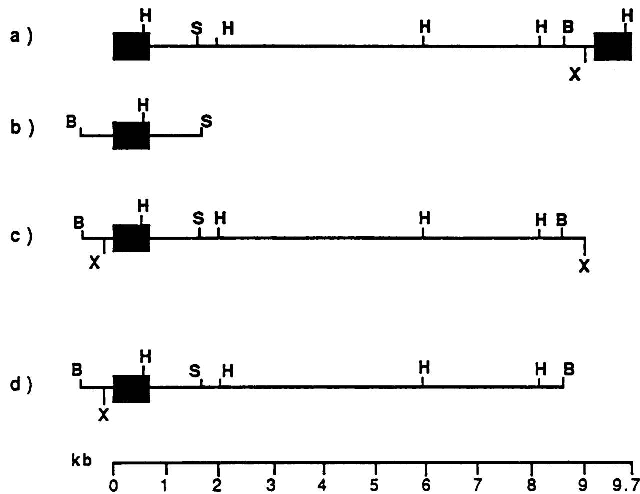

FIG. 1. HIV DNA segments used to generate LTR mutant proviruses. (a) Structure of the 9.6-kb proviral DNA of pNL4-3 (1), an infectious molecular clone of HIV. (b) The *Hin*dIII-*Sph*I fragment (map positions 0.53 to 1.44 kb) containing U5 LTR and adjacent *gag* sequences was ligated to the *Bam*HI-*Hin*dIII fragment (map positions 8.47 to 9.61 kb) containing U3 and R LTR sequences linked to the upstream *nef* gene to reconstitute a full-length LTR flanked by adjoining viral genes. This segment was molecularly cloned into M13 mp19 for site-specific mutagenesis. (c) The *Bam*HI-*Sph*I segment from the wild-type LTR or the Sp1 mutant LTR, SpB (see Fig. 2), was excised from single-LTR M13 DNA, ligated to the internal *Sph*I-*Xho*I HIV segment (1.44 to 8.89 kb), and molecularly cloned into plasmid vector pIBI20 (International Biotechnologies Inc., New Haven, Conn.), which had previously been cleaved with *Bam*HI and *Xho*I. (d) The *Bam*HI-*Sph*I segments from the five other mutant LTR M13 phage (see Fig. 2) were isolated as described above, ligated to the internal *Sph*I-*Bam*HI fragment, and molecularly cloned in pUC19 at the *Bam*HI site. Map positions are numbered with reference to the proviral DNA sequence of pNL4-3. Abbreviations: B, *Bam*HI; H, *Hin*dIII; S, *Sph*I; X, *Xho*I.

to facilitate rapid production of progeny virions. The supernatants of the transfected A3.01-MT4 cocultures were assayed for the presence of reverse transcriptase (RT) activity indicative of progeny virion production (41). Virus was detected 5 days following transfection of the wild-type provirus and 9 days following transfection of the proviruses lacking the NF-κB sites (pNFA and pdl-NF) (Fig. 2). Virus was also recovered from pSpB, pSpC, and pSNF 7 to 15 days following transfection. No virus was produced during an 8-week observation period following transfection with the provirus lacking both the NF-κB and the Sp1 sites (pTA) or with an LTR mutated in the TAR region.

Virus stocks were prepared from the cell-free supernatants of some of the transfected cocultures and used to infect human peripheral blood lymphocytes (PBLs) or two different human T-cell lines. Cells were exposed to equivalent amounts (RT activity) of virus and monitored for production of progeny viral particles. The peak of virus production in MT4 cells infected with the wild-type or the SpB (single Sp1 mutant) inoculum occurred on days 5 and 6 (Fig. 3A). Virions containing no NF-κB sites (NFA) replicated efficiently in MT4 cells, although the onset and peak of production were delayed 2 to 3 days. A similar delay was observed for the SpC and SNF mutants. In all cases, the mutant viruses were cytopathic in MT4 cells, inducing syncytia and cell death. The NF-κB deletion mutant virus also replicated efficiently in phytohemagglutinin-stimulated human PBLs (Fig. 3B); in these cells, both the NFA mutant and the wild-type viruses induced syncytium formation and exhibited peak RT activity at 8 days following infection. Interestingly, all virions containing a mutagenized Sp1 site(s) exhibited delayed replication (RT peak at day 12) and reduced levels of virus production in PBLs. Virus particles lacking both NF-κB sites also replicated well in A3.01 cells, although the NF-κB deletion resulted in a 2-day delay in the RT peak compared with wild-type HIV-1 (Fig. 3C). Virions containing a mutation in the 5′ Sp1 site exhibited a further delay in kinetics of replication. No progeny were detected in A3.01 cells infected with virus containing mutations in two Sp1 sites (SpC) or adjacent NF-κB and Sp1 sites (SNF) at up to 24 days postinfection (Fig. 3C).

Since all of the viral stocks used in these studies were derived from cocultures of A3.01 and MT4 cells (which contain copies of human T-cell lymphotropic virus type I proviral DNA; 16), it became important to ascertain whether the replication of the HIV-1 LTR mutants, particularly the NFA mutant, reflected the presence of contaminating human T-cell lymphotropic virus type I. DNA and RNA were therefore prepared from A3.01 cells or PBLs, infected with the viral stocks used for experiments shown in Fig. 3, and examined by Southern or Northern (RNA) blot hybridization for human T-cell lymphotropic virus type I-reactive sequences. No hybridization to the human T-cell lymphotropic virus type I probe was detected in these samples, although strong reactivity was observed in the DNA and RNA samples isolated from MT4 cells (E. Vicenzi and A. Rabson; data not shown).

To confirm that the defect in the pTA virus was due to impaired LTR function in directing HIV gene expression, we analyzed the activity of the pTA LTR and other LTRs containing U3 mutations in transient transfection assays by using plasmids in which the LTRs were ligated to the chloramphenicol acetyltransferase (CAT) gene. The abilities of these mutant LTRs to respond to *tat*-mediated transacti-

TABLE 1. Effects of *tat* cotransfection on the activity of wild-type and mutant HIV-LTR CAT plasmids

Plasmid	SW480 cells		MT4 cells	
	% Acetylation[a]	Fold activation[b]	% Acetylation[a]	Fold activation[b]
Mock	0.3		0.09	
Mock + *tat*	0.25	0.83	0.09	1.0
pILIC-CAT	1.3		0.08	
pILIC-CAT + *tat*	75.5	58	7.3	87
pNFA-CAT	1.1		0.09	
pNFA-CAT + *tat*	34.1	31	3.2	35.6
pSNF-CAT	1.3		0.08	
pSNF-CAT + *tat*	57.3	44	1.3	16.9
pSpC-CAT	0.26		0.09	
pSpC-CAT + *tat*	0.47	1.8	0.8	8.7
pTA-CAT	0.19		0.09	
pTA-CAT + *tat*	0.21	0.90	0.07	0.78

[a] Percent acetylation of [^{14}C]chloramphenicol was determined by scintillation counting of unacetylated and acetylated forms resolved by thin-layer chromatography.

[b] Fold activation of chloramphenicol acetylation induced by *tat* cotransfection was determined as the ratio of percentages acetylated in the presence or absence of *tat*.

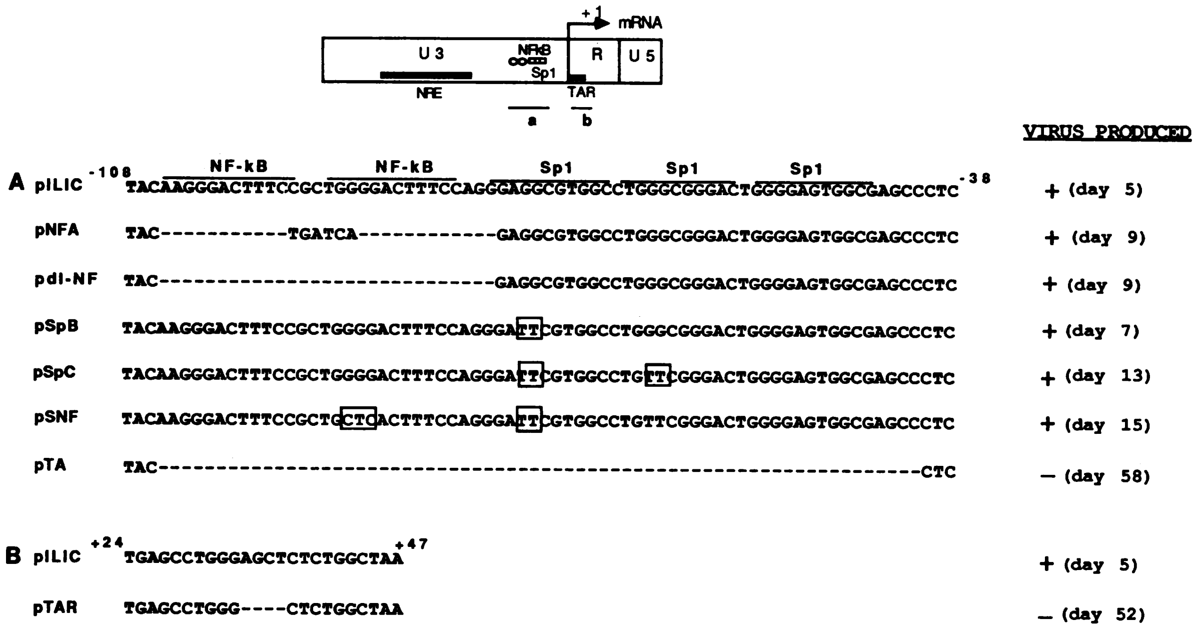

FIG. 2. Mutations introduced into the HIV-1 LTR and recovery of virus following transfection of human T cells. The structure of the LTR is shown schematically at the top; the positions of important *cis*-acting regulatory sequences are labeled, and the locations of mutagenized segments (a and b) are indicated. Production of virus following transfection into A3.01 cells and cocultivation with MT4 cells is indicated at the right; the date following transfection on which viral RT activity was first detected is shown in parentheses. (A) The nucleotide sequence of the LTR from positions −108 to −38 (numbered with respect of the start of RNA transcription [25, 31]) is shown for the wild-type single LTR (pILIC) as well as the mutant LTRs containing changes in the NF-κB and Spl sites. Deleted nucleotides are indicated by broken lines. Base substitutions are boxed. (B) The nucleotide sequence of a portion of the TAR region (+24 to +47 with respect to the RNA start site) is shown for the wild-type virus (pILIC) and for the TAR deletion mutant pTAR. Circularly permuted single-LTR proviruses containing the indicated mutations were reconstructed and concatemerized as described in the text. Concatemerized viral DNA was transfected into A3.01 cells, and 2 days after transfection, the A3.01 cells were cocultivated with MT4 cells. Culture supernatants were assayed for RT activity.

vation was of particular interest in light of the apparent requirement for TAR region-*tat* interaction for viral replication (pTAR; see above and reference 5). The *Xho*I-*Hin*dIII segments containing the U3 and R regions of a number of the mutant viruses were ligated to a *Hin*dIII-*Bam*HI segment containing the gene for CAT (14) and molecularly cloned in plasmid pIBI20 at the *Xho*I and *Bam*HI sites. LTR-CAT plasmids (5 μg) were transfected alone into SW480 colon carcinoma cells (1) by calcium phosphate precipitation (15) or were cotransfected with 5 μg of the *tat*-expressing plasmid pAR (12). MT4 cells were transfected by a modified DEAE protocol (7); the *tat*-expressing plasmid pSVtat12 (19) was used in cotransfections. The wild-type LTR (pILIC-CAT) and the LTR mutants pNFA-CAT, pSNF-CAT, and pSpC-CAT all responded to *tat* transactivation (Table 1); however, deletion of all of the Spl and NF-κB sites (pTA-CAT) resulted in low basal activity and loss of *tat* responsiveness in both cell types. These results indicate that the replication incompetence of the pTA provirus was due to impairment of HIV gene expression and not to loss of sequences that may play a role in other aspects of the viral life cycle.

While previous studies have examined the functions that the NF-κB sites mediate during activation of the HIV LTR by mitogens and phorbol esters (6, 21, 26, 35, 38, 43), cytokines (7a, 28, 29), and other viruses (4, 13, 27), the role of these sequences during productive viral infection has not been intensively evaluated. The results of our experiments indicate that while NF-κB sites may influence the rate of viral replication in some cell types, they are not required for HIV replication in human T cells.

Sequence analysis of polymerase chain reaction-amplified DNA from the infected cocultures used to generate wild-type and mutagenized virus stocks confirmed that the NF-κB deletion was retained in the intracellular proviral DNA. No NF-κB binding sites were detected in NFA virus-producing cells. In a separate experiment, NFA virus stocks were produced by direct transfection of A3.01 cells (no cocultivation with MT4 cells). Sequence analysis of the polymerase chain reaction-amplified proviral DNA present in T cells infected with this virus stock also confirmed the presence of the original NF-κB deletion.

The finding that the NF-κB deletion virus replicated with wild-type kinetics in phytohemagglutinin-stimulated human PBLs is somewhat surprising. Previous studies have shown that HIV does not replicate efficiently in unstimulated PBLs; treatment with phytohemagglutinin was required for viral growth (9, 24, 44). Our studies indicate that NF-κB binding to the HIV LTR is not required for HIV replication in stimulated PBLs. This suggests that other transcriptional factors, such as NFAT-1 (34) or the putative repressor of HIV gene expression rpt-1 (30), play a role in regulating HIV replication in normal human lymphocytes.

The replication rate of HIV containing no NF-κB sites varied in the different cell types examined. In both PBLs and A3.01 cells, the NFA virus replicated with kinetics very similar to those of the wild type; however, in MT4 cells, production of progeny NFA virions was delayed. We cannot explain these differences, but several possibilities can be entertained. On the basis of gel retardation assays, we have obtained evidence that MT4 cells contain relatively high levels of NF-κB binding activity (E. Duh, G. Englund, and

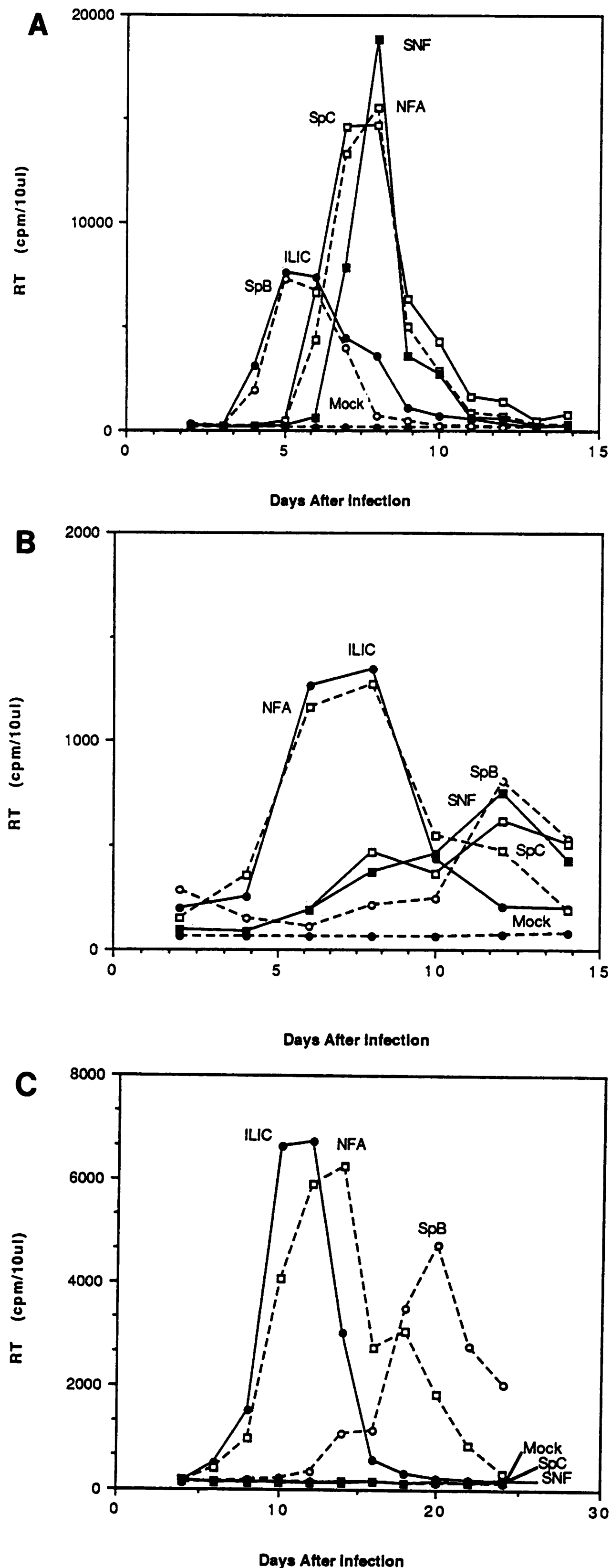

FIG. 3. Replication of wild-type and LTR mutant derivatives of HIV-1 in human T cells. Supernatants of transfected A3.01-MT4 cocultures were filtered through 22-μm-pore-size filters, and the RT activity per milliliter was determined. T-cell cultures in 24-well dishes (Costar, Cambridge, Mass.) were inoculated with equivalent amounts of RT activity for wild-type and mutant viruses, and production of progeny virions was monitored by RT assay. (A) Infection of MT4 cells. Cells (2×10^5) were infected with 5×10^3 cpm of RT activity of each viral stock. (B) Infection of PBLs. Phytohemagglutinin-stimulated human PBLs (2.5×10^5) were infected with 2×10^5 cpm of RT activity and maintained in 10% interleukin 2 (Electro-Nucleonics, Columbia, Md.). (C) Infection of A3.01 cells. Cells (2×10^5) were infected with 2×10^5 cpm of RT activity. In these experiments, one tissue culture infective dose of wild-type virus was equivalent to approximately 10 cpm in A3.01 cells.

A. Rabson, unpublished data). The very rapid replication of many HIV-1 isolates in MT4 cells may very well reflect this abundance of NF-κB; virus lacking NF-κB sites might be expected to replicate more slowly in these cells. In contrast, A3.01 cells contain relatively low levels of NF-κB binding activity (E. Duh, G. Poli, and A. Rabson, manuscript in preparation); viral replication in A3.01 cells might, therefore, be more dependent on other cellular regulatory factors, such as Spl or the recently described NFAT-1 (34). Our results also address the role of Spl in productive viral infection. Although virions containing alterations in one or two Spl sites efficiently infected MT4 cells, their replication was significantly reduced in PBLs. Furthermore, replication of SpB (one altered Spl site) was markedly delayed in A3.01 cells; SpC (mutations in two Spl sites) and SNF (mutations in adjacent NF-κB and Spl sites) failed to replicate in these cells during a 4-week observation period. These results suggest that in A3.01 cells with only low levels of NF-κB, HIV-1 replication is very dependent on the interaction of Spl with the viral LTR.

If NF-κB binding sites are not required for HIV replication, what functions might they serve in the HIV life cycle? In T cells that contain high levels of NF-κB activity (e.g., MT4 cells or activated T lymphocytes), NF-κB binding sequences may act as enhancer elements augmenting the levels of viral RNA synthesis and increasing viral replication. In infected cells which express little or no HIV, induction of NF-κB or other factors that bind to the same sequence, such as HIVen86a (11) or EBP-1 (42), may activate the LTR and thereby modulate HIV latency. The ensuing augmentation of HIV RNA expression could lead to production of *tat* and *rev* and result in high levels of progeny virion production and, ultimately, cell death.

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