An NF-κB Site in the 5'-Untranslated Leader Region of the Human Immunodeficiency Virus Type 1 Enhances the Viral Expression in Response to NF-κB-activating Stimuli*

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The human immunodeficiency virus, type 1 (HIV-1), is a lentivirus infecting CD4+ cells and causing AIDS, a progressive degenerative disease of the immune and central nervous system (1). The variable latency period of this disease is possibly related to cellular and environmental factors determining the levels of HIV-1 expression and replication. The expression of HIV-1 is directed by the LTR that contains the sequences for DNA- and RNA-binding cellular and viral proteins (reviewed in Ref. 2). Upstream of the transcription start site, the LTR contains three functional regions, the minimal promoter, the enhancer, and the so-called negative regulatory region. The minimal promoter encompasses the TATA box (3, 4), an LBP-1 site (5–8), and three Sp1 sites (9). The enhancer contains the binding sites for the cellular transacting factors NF-κB (10), TCF-κ1/LEF-1 (11, 12), and Ets-1 (13), which provide a signaling-specific activation of HIV-1 LTR, as well as a cell type-specific regulation of HIV-1 expression. The so-called negative regulatory region contains the binding sites for USF (14, 15), C/EBP (16), NFAT-1 (14), AP-1 (14, 17), and nuclear receptors (18), and it is still questionable whether it negatively affects the HIV-1 expression and replication (3, 14, 19, 20). The 5'-untranslated leader region of HIV-1 includes the initiator (Inr) sequences (21, 22), the inducer of short transcripts element (23, 24), and the binding sites for cellular proteins, such as LBP-1/UBP-1 (5–8), TFIIF I/JUSF (21, 25), UBP-2 (26), LBP-2 (27), TDP-43 (28), and CTF/NF-1 (5). In addition, this region overlaps the trans-activation response (TAR) element (nucleotides +19 to +42) that, as RNA hairpin, interacts with the viral trans-activator Tat and cellular RNA-binding proteins, increasing the elongation and/or initiation of HIV-1 transcription (2). Several stimuli such as cytokines (29–32), DNA-damaging agents (33, 34), and viral proteins (35–39) induce the HIV-1 expression. These different inducers mainly act through the activation of NF-κB complexes that bind to the NF-κB sites located upstream of the TATA box.

We previously showed that DNA alkylating agents induce the HIV-1 expression in human B lymphocytes (34). In the absence of Tat, the full induction of HIV-1 LTR required the integrity of both the NF-κB sites upstream of the TATA box and the +34/+37 sequence of TAR. Moreover, DNA alkylating agents rapidly induced a DNA-binding activity to the two NF-κB sites in the HIV-1 enhancer, as well to the +24/+47 sequence of TAR DNA. These results suggested that a mutation-responsive element is located within the TAR DNA region of HIV-1 (34). In the present study, we have analyzed the Tat-independent enhancer activity of TAR. For this purpose, a set of TAR mutants were used in transient expression and DNA band shift assays to identify the sequence of TAR required for the enhancement of HIV-1 expression and binding to cellular proteins in response to activating stimuli, such as DNA alkylating agents, PMA and TNF-α. Results demonstrate the presence of a NF-κB consensus in the TAR region, which is required for the full induction of HIV-1 expression by NF-κB activating stimuli. The NF-κB site encompasses nucleotides +31 to +40 of the 5'-untranslated leader region of HIV-1; it binds to p50p65

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1 The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; AIDS, acquired immunodeficiency syndrome; LTR, long terminal repeat; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor-α; CAT, chloramphenicol acetyltransferase; TDP-43, thymidine kinase; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; TAR, trans-activation response; Inr, initiator element.
NF-κB Enhancer in HIV-1 TAR DNA

**MATERIALS AND METHODS**

Plasmids—The plasmid pHIVCAT0, carrying the HIV-1 LTR from positions −644 to +78 upstream of the initiation of transcription of the HIV-1 genome, and the derivative base pair substitution TAR mutants pHIVCAT1, pHIVCAT2, pHIVCAT3, pHIVCAT4, pHIVCAT5, pHIVCAT6, pHIVCAT7, pHIVCAT9, and pHIVCAT11 (40) were obtained from Dr. R. W. Davis, Stanford University, Stanford, CA. The TAR mutants +39/+43 and +45/+49 (5), here referred as pHIVCAT12 and pHIVCAT13, carrying 5-base pair substitutions of TAR, were obtained from Dr. K. A. J. Jones, The Salk Institute, La Jolla, CA. The pTAR (41), here referred as HIVCAT14, carrying the +34/+37 base pair deletion of TAR, and pSVTat (41) were obtained from Dr. A. Rabson, Center for Advanced Biotechnology and Medicine, Piscataway, NJ. The TAR mutations are listed in Fig. 1A. The pTARTK and pmTARTK plasmids were generated by ligating the synthetic −24 to +47 TAR and mTAR1 oligonucleotides, respectively, to pHLCAT2 plasmid (42) linearized by SalI digestion and filled in. In the resulting plasmids, the TAR oligonucleotide is inserted upstream of the herpes simplex virus tk minimal promoter fused to the cat gene. The correct orientation and of TAR fragment was checked by sequencing. The pCD23, pCD52, and pCD54 plasmids (19) were obtained from Dr. S. J. Josephs through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The derivative pCD23ATAG and pCD52TAR were constructed by ligating the mTAR1 oligonucleotide to pCD23 and pCD52, respectively, linearized by XbaI and filled in. pCD plasmids are shown in Fig. 6A. The RcoCMVp50 and RcoCMVp65 plasmids (43) were obtained from Dr. N. Rice, Frederick Cancer Research and Development Center, Frederick, MD.

Cells, Transfections, and Chemical Treatment—MC3 cells (34) and NTera-2 cells (44) were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (Flow Laboratories, Italy), 3 mM glutamine, and 10 mM Hepes buffer (pH 7.2) (Life Technologies, Inc., Italy). Cells were transfected by electroporation as described previously (34). Briefly, cells (3 × 10⁴) were washed by electroporation in exponential growth phase were washed and resuspended in 0.3 ml of RPMI 1640 plus 20% fetal calf serum in presence of the reporter plasmid DNA (5–10 μg). Cells were subjected to two electrical pulses (0.2 kV, 960 microfarads) using a Bio-Rad apparatus, recovered, cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, and 2 days later collected for CAT assay. To measure transfection efficiency, pRSV-β-gal (2 μg) was co-transfected, and β-galactosidase assays were performed as described previously (34). For chemical treatments, 12 h after transfection cells were divided in equivalent aliquots to be treated or left untreated. Two days post-treatment the cells were harvested, washed with phosphate-buffered saline, and collected for CAT assay. At least four independent experiments with different plasmid preparations were performed to evaluate the transient expression of the cat gene. The chemicals used were mitomycin C (Koywa, Japan), PMA (Sigma), and human recombinant TNF-α (Boehringer Mannheim, Germany).

Assay of CAT Activity—Cell extracts were prepared by three cycles of freeze-thawing in 0.2 ml of 0.25 M Tris, pH 7.8, and the CAT assay was performed as described previously (34). Briefly, each CAT assay contained 1–50 μg of proteins, 20 μl of 4 mM acetyl coenzyme A (Boehringer Mannheim, Germany), 1 μl (0.5 μg) of [14C]chloramphenicol (5), and 1 ml of 0.25 M Tris, pH 7.8. The cell extracts were used at protein concentrations ensuring linear conversion of substrate in each reaction. Reactions were incubated at 37 °C for 3 h, extracted with ethyl acetate, dried, and spotted on Polygram Sil G silica gel plates (Macherey-Nagel, Germany). Plates were run in a TLC tank containing chloroform/methanol (95:5). After a 16-h autoradiography, the TLC plates were cut, and samples were counted in a Packard LS5000TD scintillation counter. The percent acetylation of [14C]chloramphenicol was determined by scintillation counting the unacylated and the acetylated forms resolved by thin layer chromatography. The NF-κB complex and enhances the HIV-1 expression either in cooperation with or in absence of the NF-κB enhancer upstream of the TATA box.

The characterization of a novel NF-κB enhancer in the HIV-1 LTR provides additional information to understand the NF-κB-mediated regulation of HIV-1 transcription.
CAT activity was expressed as the percent acetylated chloramphenicol per μg of protein per 3 h.

Electrophoretic Mobility Shift Assays—Nuclear extracts and gel retardation assays were performed as described previously (34). Briefly, cells were harvested, washed twice in cold phosphate-buffered saline, and resuspended in lysing buffer (10 mM Heps, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.2% v/v Nonidet P-40) for 5 min. Nuclei were collected by centrifugation (500 × g, 5 min), rinsed with Nonidet P-40-free lysing buffer, and resuspended in 150 μl of buffer containing 250 mM Tris-HCl, pH 7.8, 20% glycerol, 60 mM KCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride. Nuclei were then subjected to three cycles of freezing and thawing. The suspension was cleared by centrifugation (7000 × g, 15 min), and aliquots were immediately tested in gel retardation assay or stored in liquid phase N2 until use. TAR oligonucleotide probes used are represented in Fig. 2A. HIV-1 NF-κB oligonucleotides were 5′-CAAGGAGCTTCCGCTGGGAGCTTCACGG-3′ and Sp1 oligonucleotide was 5′-GGCGGGTGGCCTGGCGGACTGGGAGTGGCGC-3′. The TAR probe was annealed to its complementary strand and end-labeled with γ-32P ATP (Amerham Int., Buckinghamshire, UK) using polynucleotide kinase (New England Biolabs, Beverly, MA). Equal amounts (5 μg) of cell extracts were incubated in a 20-μl reaction mixture containing 10% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 2 μg of poly(d(G-C)) (Boehringer Mannheim, Germany) for 5 min on ice. One μl of γ-32P-labeled double-stranded probe (0.2 ng, 4–6 × 10^5 cpm) was then added with or without a 25–200-fold molar excess of competitor oligonucleotide. The reactions were incubated at room temperature for 15 min and run on a 6% acrylamide/bisacrylamide (30:1) gel in 22.5 mM Tris borate, 0.5 mM EDTA. Gels were dried and autoradiographed.

DNA Affinity Chromatography and Immunoblotting—Nuclear extracts (100 μg) obtained from MC3 cells treated for 1 h with mitomycin C (10 μM) were incubated with 100 ng of streptavidin-conjugated paramagnetic beads (Dynabeads M-280 Streptavidin, Dynal, Norway) bound to the biotinylated double-stranded oligonucleotide according to the manufacturer’s instructions in a 20-μl binding solution containing 10% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% v/v Nonidet P-40, and 10 μg of poly(d(G-C)) for 30 min at room temperature. As negative control, equal amounts of extracts were also incubated with unconjugated beads. Binding proteins were extensively washed with the binding solution and eluted with 20 μl of elution buffer containing 125 mM Tris-HCl, 1% 2-mercaptoethanol, 10% glycerol, and 2% SDS at 100 °C for 5 min. The eluate was separated by electrophoresis in 10% SDS-polyacrylamide gel. The immunoblotting was performed with rabbit polyclonal antibodies raised against the N-terminal of human p50 or C-terminal of human p65 followed by enhanced chemiluminescence detection (Amersham, United Kingdom). The rabbit anti-p50 and anti-p65 antiseraum (43) were kindly provided by Dr. N. Rice, Frederick Cancer Research and Development Center, Frederick, MD.

RESULTS

An NF-κB-binding Site Is Present in the 5′-Untranslated Leader Region of HIV-1—In the absence of Tat, the TAR region functionally cooperates with the NF-κB enhancer upstream of the TATA box of HIV-1 to confer the maximal induction of HIV-1 gene expression by DNA alkylating agents (34). To identify the mutagen-responsive sequence within TAR, the pHIV-CAT0 plasmid, carrying the HIV-1 LTR fused to the cat reporter gene, and the derivative plasmids, carrying base pair substitutions within the region +28 to +51, were used in transient expression assays (Fig. 1A). For this purpose, MC3 cells, an Epstein-Barr virus-immortalized B cell line (34), were transfected with these plasmids and treated with the DNA alkylating agent mitomycin C. MC3 cells constitutively express a low level of NF-κB activity that is rapidly increased by NF-κB activating stimuli (45). Moreover, they are representative of in vivo target cells for HIV-1 infection (46, 47). As shown in Fig. 1, A and B, base pair substitutions of the GGAGCTC sequence extending from +31 to +37 (pHIVCAT 3, 4, 5, 6, 9, 11) significantly reduced the activation by mitomycin C, whereas mutations flanking this sequence (pHIVCAT 1, 2, 7, 12, 13) were irrelevant. These findings confirm the results previously obtained with pHIV14, a mutant in which the +34/+37 sequence had been deleted (Fig. 1, A and B) (34), and they indicate that the mutagen-responsive element maps from +31 to +37. This tract overlaps the potential NF-κB site GGAGCTCCTC located from nucleotides +31 to +40 (Fig. 1A). Moreover, the +31/+37 sequence is contained in the TAR region from +19 to +42 that generates the upper stem-loop RNA structure required for an efficient transactivation by Tat (Fig. 1, A and C) (2, 26, 40). These evidences suggest a dual role of the TAR sequence from +31 to +37 in both the mutagen-mediated and the Tat-mediated transactivation of HIV-1 LTR.

DNA alkylating agents induced a binding activity to the double-stranded DNA oligonucleotide representing the +24/+47 TAR sequence (34). This sequence contains a CTF/NF-1-binding site (5), as well as a potential NF-κB site (Fig. 2A). To identify the minimal sequence required for the binding activity to TAR DNA, EMSA was performed by using mutant TAR oligonucleotides to compete the TAR DNA-binding activity. The mutant oligonucleotides contained deletions or base pair sub-
Next, we tested whether NF-κB site located from 24/47 to the binding of CTF/NF-κB since it was still observed when the 47 or 200-fold molar excess of the indicated unlabeled oligonucleotides. 100-, and 200-fold molar excess of the indicated unlabeled oligonucleotides including either the wild-type and to the HIV-1 NF-κB enhancer. Competitions were performed with 25-, 50-, 100-, and 200-fold molar excess of the indicated unlabeled oligonucleotides.

The binding activities to the +24/+47 TAR oligonucleotide and to the HIV-1 NF-κB oligonucleotide are reciprocally competed. Nuclear extracts (5 μg) from MC3 cells treated with mitomycin C (10 μg/ml for 1 h) were incubated with a double-stranded 32P-labeled oligonucleotide representing the +24/+47 TAR sequence or the HIV-1 NF-κB enhancer. Competitions were performed with 25-, 50-, 100-, and 200-fold molar excess of the indicated unlabeled oligonucleotides.

NF-κB/Rel Proteins Bind to the Mutagen-responsive Element of TAR—Next, we tested whether NF-κB/Rel proteins could bind to the NF-κB site within the TAR region. For this purpose, nuclear extracts from MC3 cells treated with mitomycin C were incubated with streptavidin-conjugated beads bound to biotinylated oligonucleotides including either the wild-type +24/+47 TAR or the mutants mTAR1 and mTAR4, lacking the NF-κB consensus (Fig. 2A). The retained proteins were eluted and analyzed by immunoblotting using antisera raised against p50 and p65 Rel proteins. Both p50 and p65 proteins were specifically retained by the wild-type TAR oligonucleotide, whereas they were not recovered from mTAR1, mTAR4, or from an unrelated oligonucleotide (Fig. 4A). The presence of p50 and p65 proteins in the TAR binding complex was further analyzed in EMSA by the use of antisera raised against p50 or p65 Rel proteins. As shown in Fig. 4B, both antisera inhibited the TAR DNA-binding activity, whereas they were ineffective in the presence of the relative antagonist peptides. The observation of inhibition rather than supershift by anti-p50 and anti-p65 antisera was peculiar of DNA band shift using as a probe the TAR oligonucleotide. In fact, the same extracts were incubated with beads alone or bound to oligonucleotides representing the NF-κB or Sp1 sequences upstream of the TATA box of HIV-1. Immunoblotting analysis of eluted proteins was sequentially performed with anti-p50 and anti-p65 antisera followed by enhanced chemiluminescence detection. B, inhibition of TAR DNA binding activity by anti-p50 and anti-p65 antisera. Nuclear extracts (5 μg) were incubated with 32P-labeled +24/+47 TAR oligonucleotide in presence or absence of anti-p50 or anti-p65 antisera (2 μl). Competitions with the antagonist peptides were performed by preincubating each antisera with the relative competitor peptide (150 ng/ml) for 20 min. The protein-TAR DNA oligonucleotide complexes were analyzed by EMSA. Competitor TAR oligonucleotide was 100-fold molar excess with respect to the 32P-labeled TAR oligonucleotide.
TAR was moved upstream of the TATA box and acted exclusively at the DNA level. For this purpose, a single copy of the +24/+47 TAR oligonucleotide was inserted in direct or inverse orientation upstream of the herpes simplex virus tk minimal promoter fused to the cat reporter gene to generate the pTARTK-sense and pTARTK-antisense plasmids, respectively. MC3 cells were transiently transfected with these plasmids and treated with mitomycin C. As shown in Table I, deletion of the TAR NF-κB site was responsible for the enhancer activity, the TAR1 oligonucleotide deleted of the NF-κB site was inserted upstream of the tk promoter to generate the pmTARITK plasmid. As shown in Table I, the deletion of the NF-κB consensus abolished the induction of CAT activity by mitomycin C, thus confirming the requirement of an integral NF-κB-binding site for responsiveness to the genotoxic treatment.

To verify whether proteins of the NF-κB/Rel family directly trans-activate in vivo the expression of pTARTK plasmids, NTera-2 cells, which constitutively show very low or no NF-κB activity (44), were transiently transfected with pBLCAT2, pTARTK-sense, or pmTARITK plasmids together with plasmids expressing p50 and p65. The TAR-driven CAT activity increased up to 5-fold by cotransfecting p50 and p65 (Fig. 5). This increase was not observed in pBLCAT2, lacking the TAR insert, and pmTAR1, deleted of the NF-κB site in the TAR insert (Fig. 5). Moreover, the CAT activity increased up to 20-fold with p2TARTK, a plasmid that carries two copies of the TAR oligonucleotide (Fig. 5). This induction was comparable to the one observed with pDR (Fig. 5), a plasmid that carries the two tandem NF-κB sites of HIV-1 enhancer upstream of the tk promoter (48). These results indicate that the +24/+47 TAR region contains a functional NF-κB site that is required both for the induction by the NF-κB activator mitomycin C and for the trans-activation by p50/p65 NF-κB complexes.

The TAR NF-κB Enhancer Acts Either Cooperatively with or Independently of the NF-κB Enhancer Upstream of the TATA Box of HIV-1 LTR—Next, we investigated the role of TAR NF-κB in the context of HIV-1 LTR in response to different NF-κB-activating stimuli. For this purpose, MC3 cells were transfected with the wild-type HIV-1 LTR upstream of the cat gene (pCD23) or with the derivative mutant plasmids carrying a deletion of the TAR NF-κB site (pCD23 TAR), or a deletion of the two NF-κB site and the 5' Sp1 sites upstream of the TATA box (pCD52), or deletions of the NF-κB and 5' Sp1 sites upstream of the TATA box and of the TAR NF-κB site (pCD52 TAR), or deletions of the two NF-κB sites and three Sp1 sites upstream of the TATA box (pCD54) (Fig. 6A). Then the transfected cells were treated with PMA, TNF-α, or mitomycin C, which activate NF-κB (45, 49). The CAT activity driven by the wild-type HIV-1 LTR was significantly induced by the chemical treatments (Fig. 6B, pCD23). This activation was reduced by deletion of the NF-κB sites upstream of the TATA box or deletion of the TAR NF-κB site (Fig. 6B, pCD52 and pCD23 TAR, respectively), and it was abolished by deletion of both the NF-κB sites upstream of the TATA box and the TAR NF-κB site (Fig. 6B, pCD52 TAR). Moreover, the TAR NF-κB-directed activation of HIV-1 minimal promoter was abolished by deletion of the Sp1 sites upstream of the TATA box (Fig. 6B, pCD54). These results indicate that the TAR NF-κB site cooperates with the upstream NF-κB enhancer to induce the maximal activation of HIV-1 expression, and it can exert a residual enhancer activity in the absence of the two NF-κB sites upstream of the TATA box. However, the TAR NF-κB activity requires the presence of the Sp1 sites upstream of the TATA box. This suggests a functional cooperation between the Sp1 and the TAR NF-κB complex from their positions upstream and downstream of the TATA box, respectively.

The TAR NF-κB-driven activation of HIV-1 LTR was further investigated in response to p50 and p65 Rel proteins by transient expression assays. For this purpose, NTera-2 cells were transfected with pCD23, pCD23 TAR, pCD52, and pCD52 TAR plasmids alone or in combination with p50- and p65-expressing vectors. The CAT activity driven by HIV-1 LTR containing the NF-κB sites upstream of the TATA box and the TAR NF-κB site was significantly increased by p65 alone or in combination with p50, whereas it was not affected by p50 alone (pCD23 in Fig. 7). A similar responsiveness was observed when the HIV-1 LTR was deleted of the TAR NF-κB site (Fig. 7,
The HIV-1 LTR deleted of the NF-κB enhancer upstream of the TATA box was still activated by increasing doses of p65 in combination with p50, whereas it was uninhibited by p65 or p50 alone (Fig. 7, pCD52). The deletion of both the upstream NF-κB enhancer and the TAR NF-κB site abrogated the activation of HIV-1 LTR by NF-κB proteins (Fig. 7, pCD52ΔTAR), thus indicating that the examined NF-κB elements were strictly required to confer responsiveness of HIV-1 LTR to NF-κB complex. These results indicated that the TAR NF-κB site was specifically induced by p50z/p65 NF-κB heterodimers, whereas the NF-κB enhancer upstream of the TATA box was responsive to both p50z/p65 heterodimers and p65 homodimers. This is consistent with the ability of p50/p65 complex to bind to the TAR NF-κB site (Fig. 4B).

**DISCUSSION**

The 5'-untranslated leader region of HIV-1 interacts with several cellular trans-acting factors, whose role in the regulation of HIV-1 gene expression is not completely understood (2). We now describe the presence of an NF-κB responsive element in the TAR region that is required for the maximal induction of HIV-1 gene expression in response to NF-κB activating stimuli. The NF-κB site GGGAGCTCTC spans from nucleotide 131 to 140 (Fig. 1A), and it was initially identified by transient expression assays of plasmids carrying the wild-type or mutant HIV-1 LTR fused to the cat gene in pCD plasmids. The CAT activity was evaluated 48 h after treatment and expressed as percent specific acetylation of [14C]chloramphenicol per 50 μg of protein per 3 h (%AC-CM). A representative experiment of four independent experiments giving similar results is shown.

The HIV-1 LTR deleted of the NF-κB enhancer upstream of the TATA box was still activated by increasing doses of p65 in combination with p50, whereas it was uninhibited by p65 or p50 alone (Fig. 7, pCD52). The deletion of both the upstream NF-κB enhancer and the TAR NF-κB site abrogated the activation of HIV-1 LTR by NF-κB proteins (Fig. 7, pCD52ΔTAR), thus indicating that the examined NF-κB elements were strictly required to confer responsiveness of HIV-1 LTR to NF-κB complex. These results indicated that the TAR NF-κB site was specifically induced by p50z/p65 NF-κB heterodimers, whereas the NF-κB enhancer upstream of the TATA box was responsive to both p50z/p65 heterodimers and p65 homodimers. This is consistent with the ability of p50/p65 complex to bind to the TAR NF-κB site (Fig. 4B).

**Fig. 7.** Activation of wild-type and mutant HIV-1 LTR by p50 and p65 Rel proteins. NTera-2 cells (5 × 10⁶) were electroporated with the indicated plasmids (10 μg) alone or together with p50- and/or p65-expressing plasmids at the indicated dose. Transfection efficiency was monitored by cotransfecting pRSV-β-gal (2 μg) and measuring the β-galactosidase activity. The CAT activity was evaluated 48 h later and expressed as percent specific acetylation of [14C]chloramphenicol per 50 μg of protein per 3 h (%AC-CM). A representative experiment of four independent experiments giving similar results is shown.

The deletion of both the upstream NF-κB enhancer and the TAR NF-κB site abrogated the activation of HIV-1 LTR by NF-κB proteins (Fig. 7, pCD52ΔTAR), thus indicating that the examined NF-κB elements were strictly required to confer responsiveness of HIV-1 LTR to NF-κB complex. These results indicated that the TAR NF-κB site was specifically induced by p50z/p65 NF-κB heterodimers, whereas the NF-κB enhancer upstream of the TATA box was responsive to both p50z/p65 heterodimers and p65 homodimers. This is consistent with the ability of p50/p65 complex to bind to the TAR NF-κB site (Fig. 4B).
stream of the TATA box and the TAR NF-κB site. A still significant activation was observed when the NF-κB enhancer upstream of the TATA sequence was deleted, thus indicating a possible role for TAR NF-κB in the HIV-1 LTR activation. This was confirmed by the lack of activation when, in addition to the upstream NF-κB enhancer, the TAR NF-κB site was also deleted. These results indicate that the TAR NF-κB site cooperates with the NF-κB sites upstream of the TATA box in the NF-κB-mediated induction of HIV-1 LTR, and it can still provide a significant activation of HIV-1LTR in the absence of the upstream NF-κB enhancer. The TAR NF-κB activity requires the presence of the Sp1 sites upstream of the TATA box to trans-activate the HIV-1 gene expression. A similar requirement for Sp1 sites was previously shown for the enhancer activity of NF-κB sites upstream of the TAR (17, 50, 51). Interestingly, the TAR NF-κB activity is mediated by the binding of p50/p65 heterodimers and not by p50 or p65 homodimers. In contrast, the NF-κB enhancer upstream of the TATA box is responsive to both p50/p65 heterodimers and p65 homodimers. A physical interaction between the p65 Rel protein and the transcription factors TATA-binding protein and TFIIB was described (52, 53). Thus, the NF-κB TAR site could allow the p50/p65 NF-κB complex to reside downstream of the TATA box and, from this position, to interact with the initiation complex in order to increase the rate of transcription from the HIV-1 minimal promoter in concert with the Sp1 trans-acting factors.

The TAR NF-κB site is included in a region of HIV-1 LTR that contains a complex array of putative regulatory elements. In fact, the TAR NF-κB sequence overlaps the upper stem-loop sequence of TAR RNA that binds to cellular proteins cooperating with the viral trans-activator Tat (reviewed in Ref. 2). In addition, the TAR NF-κB site partially overlaps the binding site for a cellular factor called UBP-2, not yet purified and functionally characterized (26). The putative initiator element Inr2 was identified at positions +29 to +42 (21), which encompass the TAR NF-κB site. In synergy with the upstream Inr1 element, Inr2 conferred full promoter activity by interacting with the USF protein (21). Mutations of Inr2 abrogating the NF-κB sequence affected neither the initiation start site position nor the basal and USF-mediated transcription of HIV-1 (5, 21). The TAR NF-κB site is flanked by the inducers of short transcripts element that is located at positions −5/−26 and +40/−59 and mediates the synthesis of short transcripts of HIV-1 (24). Mutations of TAR NF-κB site reduced the production of full-length rather than short-length RNAs (24), thus excluding the involvement of the NF-κB sequence in the inducer of short transcripts activity.

The presence of a NF-κB enhancer in TAR may provide an additional explanation for the conflicting results concerning the ability to replicate of HIV-1 proviral strains deleted of the NF-κB enhancer upstream of the TATA box (41, 54–56). In primary cells, such as phytohemagglutinin-stimulated PBLs, NF-κB-deleted proviral strains were shown either to replicate similarly to the wild-type strain (41, 54) or to be unable to replicate (56). Indeed, the TAR NF-κB site is conserved in the mutant HIV-1 provirus lacking the NF-κB enhancer upstream of the TATA box, and it could play a role in the viral transcription and replication in response to efficient activation of NF-κB. Accordingly, the reduced transcription and replication of the +33/+34 TAR mutant virus (57), lacking the TAR NF-κB site, could be attributed not only to the absence of Tat-mediated trans-activation but also to the inability of NF-κB complex to bind to the TAR NF-κB enhancer. The difficulty to show a biological role for TAR DNA in HIV-1 replication depends on the additional regulatory role of TAR as a stem-loop RNA structure responsive to Tat. In fact, TAR mutations abrogating the NF-κB site also affect the Tat-mediated activation of HIV-1 LTR (Fig. 1) (reviewed in Ref. 2). For this purpose, the role of TAR DNA needs to be examined in Tat-defective HIV-1 strains where the HIV-1 gene expression depends exclusively on cellular transacting factors. Indeed, Tat-defective HIV-1 can be expressed and replicated in T-cell lines and primary mononuclear cells in response to a NF-κB-activating stimulus, such as TNF-α (58). This suggests that, in the absence of Tat, NF-κB may still provide a sufficient HIV-1 gene expression and replication.

The identification of an additional NF-κB enhancer in TAR points to the relevance of NF-κB transacting factors in the HIV-1 gene regulation. The TAR NF-κB sequence is well conserved in different primary isolates of HIV-1 (59) and in the same HIV-1 strain through several rounds of viral replication (60). Moreover, a putative NF-κB site can be identified at positions +37/+46 of HIV-2 TAR DNA (61), and similar to TAR NF-κB of HIV-1, it overlaps the upper stem-loop sequence in the hairpin of HIV-2 TAR RNA. These evidences suggest a natural selection in favor of viral genomes containing NF-κB-binding sites in the TAR region. The NF-κB activity is induced in response to different stimuli such as cytokines and DNA damaging agents (49). Indeed, all these treatments increase the cellular production of free radicals, which may represent a sort of a second messenger of NF-κB activation (62). In the realm of hypothesis, HIV-1 genome could have acquired regulatory regions, such as NF-κB sites, which provide an efficient viral expression and replication in order to escape from cells damaged by free radicals, possibly destined to death. An analogous mechanism is used by the bacteriophage lambda to escape from Escherichia coli in SOS signaling (63). In this view, efforts should be developed to inhibit the NF-κB activation in order to suppress the HIV-1 expression and replication.

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