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Activation of the Human Immunodeficiency Virus Type 1 Enhancer Is Not Dependent on NFAT-1

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The function of a putative NFAT-1 site in the human immunodeficiency virus type 1 enhancer has been analyzed. Activation by the T-cell antigen receptor is minimal in Jurkat cells and is mediated by the κB sites. The putative NFAT-1 region is not required for the response to anti-CD3 or to mitogens in T-cell, B-cell, or monocyte/macrophage leukemia lines, nor is it a *cis*-acting negative regulatory element.

Transcription of human immunodeficiency virus type 1 (HIV-1) in T lymphocytes is regulated by specific cellular activation pathways stimulated by triggering of distinct cell surface receptors. κB regulatory elements of the HIV-1 enhancer are required for transcriptional activation after mitogen or cytokine stimulation (21, 22). At least two *cis*-acting regulatory elements respond to stimulation of the T-cell antigen receptor, the site which binds the nuclear factor of activated T cells (NFAT-1) in the interleukin-2 (IL-2) gene (3, 27), and the CD3-responsive site (20) of HIV-2. The NFAT-1 site in the IL-2 enhancer resembles a binding site for the *ets* proto-oncogene and is recognized by *ets* family members (29, 33). The function of these *cis*-acting enhancer elements in their respective enhancers has been demonstrated (3, 20, 27), but their role in HIV-1 gene regulation, if any, has not been precisely defined. The putative NFAT-1 binding site in HIV-1 was originally identified when a factor in stimulated Jurkat T-cell extracts, and later partially purified NFAT-1, was shown to bind the enhancer between positions −216 and −254 by DNase footprinting (1, 27) (Fig. 1A, NF_P and NF'_P). However, the region showing DNase protection is adjacent to but does not overlap the purine-rich region of sequence homology that is shared by HIV-1 and the NFAT-1 binding site of the IL-2 enhancer (PuB, Fig. 1A) (6, 27, 33). Despite this discrepancy and the lack of functional data on specific mutations of this site, NFAT-1 has been assumed to play a role in the response of the HIV-1 enhancer to T-cell stimulation (10, 27, 32). Our previous data (20, 21) demonstrated that mutation of the κB sites (Fig. 1A) of the HIV-1 enhancer abolishes enhancer activation in stimulated T cells, but the data did not exclude the possibility that HIV-1 activation was also dependent on an intact NFAT-like element. Therefore, to elucidate the function of the putative NFAT-1 site of HIV-1 and to examine the role of the T-cell receptor signal transduction pathway in HIV-1 stimulation, we have investigated the role of this element in regulation of the HIV-1 enhancer after T-cell activation.

Stimulation of T cells by phorbol esters or certain cytokines results in increased expression of HIV-1 (21, 22). In contrast to the HIV-2 enhancer, treatment of Jurkat cells with antibodies to the T-cell receptor does not stimulate the HIV-1 enhancer (20), but activation with antibodies to the T-cell receptor which have been cross-linked induces a small but measurable stimulation (13, 31). To determine whether the region with NFAT-1 homology (PuB) or the region of nuclear factor protection (NF_P) mediates changes in gene expression following T-cell activation, we performed a mutational analysis of the HIV-1 enhancer. Site-specific mutations (21) were introduced into plasmids containing the HIV-1 enhancer linked to the chloramphenicol acetyltransferase (CAT) gene (HIV-1-CAT; Fig. 1A) (25), and these plasmids (5 μg) were transfected into 10^7 Jurkat T leukemia cells by using DEAE-dextran (24). At 20 h following transfection, cells were incubated with phorbol myristate acetate (PMA; 16 nM) and phytohemagglutinin (PHA; 2 μg/ml) for an additional 20 h. This treatment increased CAT activity 11-fold (Fig. 1B). Mutation of either the PuB or protected sites did not significantly alter the response to PMA and PHA, although slightly lower basal activity was observed in the NF_P mutant (Fig. 1B). Recently, a second site in the 3′ portion of the NFAT-1 footprint has been shown to bind partially purified NFAT-1 (4). A mutation was introduced into this site (NF'_P, Fig. 1A), and in separate experiments, the response of the mutant plasmid to PMA and PHA was compared with that of the wild type in Jurkat cells. No significant effect of this mutation was seen on either basal or induced activity (Fig. 1C). In contrast, mutation of the two κB sites eliminated stimulation by PMA and PHA (Fig. 1B), consistent with previous studies (21).

When cells transfected with the HIV-1 enhancer were stimulated by cross-linking of antibodies to the T-cell receptor, minimal activation (ca. threefold) was seen, and this limited effect was abolished when the κB sites were mutated (Fig. 1D). Mutation of the PuB or NF_P element did not affect CAT activity (Fig. 1D). In contrast, similar stimulation of cells transfected with the HIV-2 enhancer resulted in ~30-fold induction, highlighting the differential responsiveness of these two viral enhancers. Similar results were observed for HIV-1 and these mutants following activation of Jurkat cells by PMA or PHA alone or in the mouse T-cell line EL4 (data not shown) (20, 21). In the experiments with EL4 cells, 25- to 100-fold induction of the IL-2 enhancer was seen, and site-specific mutation of the NFAT-1 site in the IL-2 enhancer reduced stimulation 10- to 25-fold (12). These results show that functionally active NFAT-1 was made in these cells but did not stimulate the HIV-1 enhancer.

Cells of the monocyte/macrophage lineage are also an important reservoir for HIV infection in vivo (5, 7, 8, 16). In

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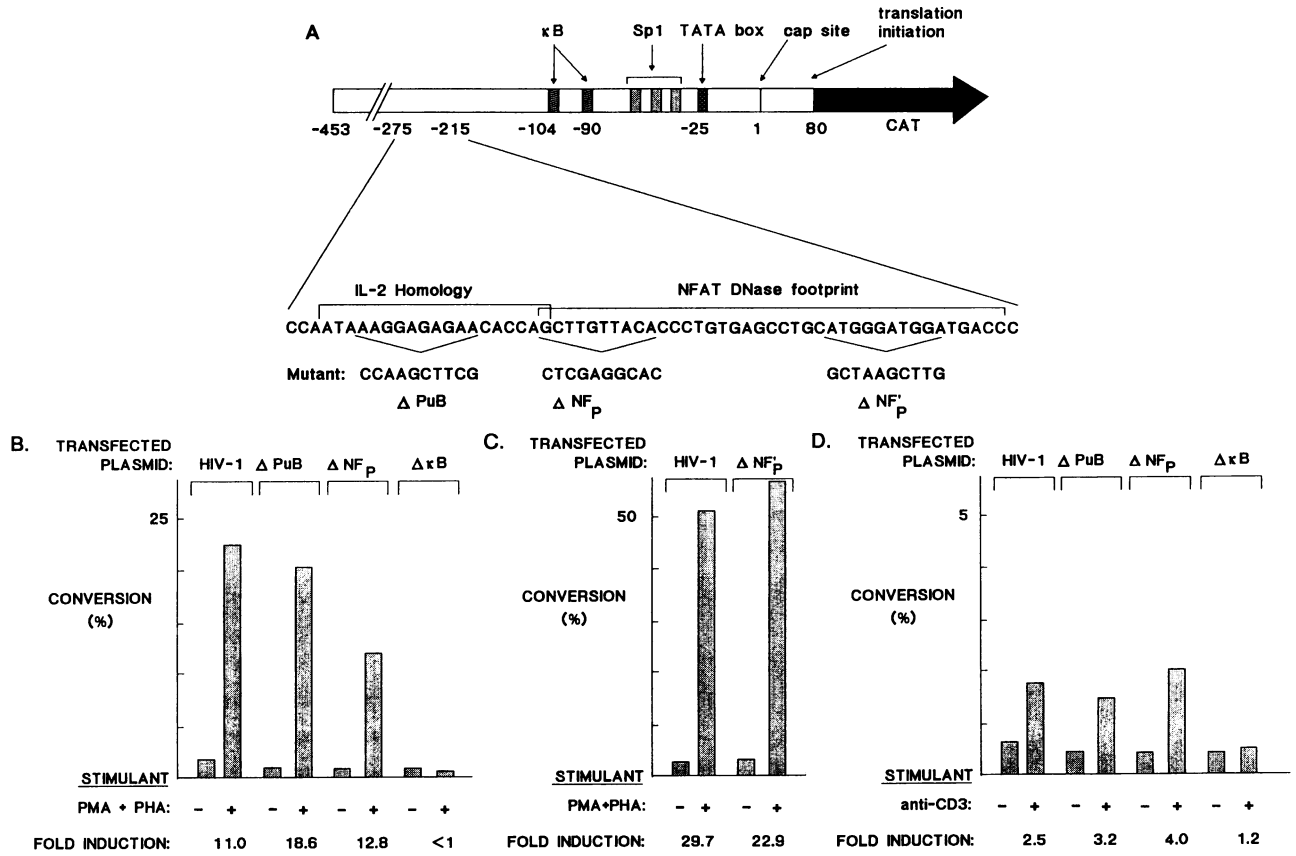

FIG. 1. Site-specific mutations of the HIV-1 LTR and their effect on expression in stimulated Jurkat cells. (A) Schematic representation of the relevant enhancer region of HIV-1. Altered bases within the mutant plasmids used in this study are shown below the wild-type sequence. The region of homology to the IL-2 enhancer (6), PuB, and the sites of DNase protection (NF_P and NF'_P [27]) are indicated. (B) The roles of PuB, NF_P, and κB sites in the HIV-1 enhancer were determined by transfection of the indicated wild-type and mutant plasmids into Jurkat T cells. Cell extracts were prepared, and CAT activity was determined by standard methods (9). Transfection efficiencies were normalized to protein concentration. Values for percent conversion of chloramphenicol to acetylated forms shown are the average of two independent transfections and representative of a total of three to six independent transfections. (C) Effect of site-specific mutation of the NF'_P site on the HIV-1 enhancer. Values shown are the average of two independent transfections. (D) The role of *cis*-acting regulatory sequences in the stimulation of the HIV-1 enhancer by antibodies to the T-cell receptor was determined. The anti-CD3 (OKT3) monoclonal antibody (1:1,000 dilution of mouse ascites fluid) was immobilized on the tissue culture plate with a 10-μg/ml dilution of goat anti-mouse immunoglobulin G (Kirkegaard & Perry). Values shown are the average of two independent transfections and are representative of four independent transfections.

mature cells, NF-κB binding activity is readily detected and is not further induced by treatment with PMA (11). In these cells, another transcription factor, PU.1, has been shown recently to bind purine-rich sequences similar to the purine-rich binding site in the NFAT-1 element of the IL-2 enhancer (15, 23), which somewhat resembles the HIV-1 PuB site. To determine whether the PuB and NF_P sites functioned differently in macrophage leukemia lines, a mature monocyte leukemia line, THP-1, was transfected with the HIV-1-CAT or mutant plasmids. Consistent with its high constitutive levels of NF-κB (11), THP-1 cells transfected with HIV-1 CAT showed no increase in CAT activity after PMA treatment, and the κB mutant displayed markedly lower basal expression (11) (Fig. 2A). Both the PuB and NF_P mutants showed no difference in CAT activity after mitogen treatment, and the mutant plasmids showed activity similar to that of the wild-type enhancer (Fig. 2A). To examine the role of these elements in B cells, which also express PU.1, these plasmids were introduced into an Epstein-Barr virus-transformed B-cell leukemia line, clone 13. PuB and NF_P mutant plasmids again showed expression similar to the wild type (Fig. 2B). These experiments suggest that the PuB and NF_P elements are not required for induction of the HIV-1 enhancer in T, B, or monocytic leukemia cells and suggests that, while a protein may bind to the NF_P element in vitro, this interaction does not appear to be functionally significant in several cell types.

Transactivation by viral genes also leads to HIV enhancer activation. In the case of the tax_I gene of human T-cell lymphotropic virus type I (HTLV-I), deletion of upstream enhancer sequences has been suggested to increase the minimal stimulation of the HIV-1 enhancer (28), possibly due to a negative regulatory element between −278 and −117 which impairs the tax_I response. To investigate whether the PuB or NF_P site acts as a negative regulatory element for tax_I, Jurkat cells were cotransfected with a tax_I expression plasmid and HIV-1-CAT or mutant plasmids. Cotransfection with tax_I resulted in a ca. threefold stimulation of HIV-1-CAT, which did not differ significantly from the result after cotransfection of tax_I with the PuB or NF_P

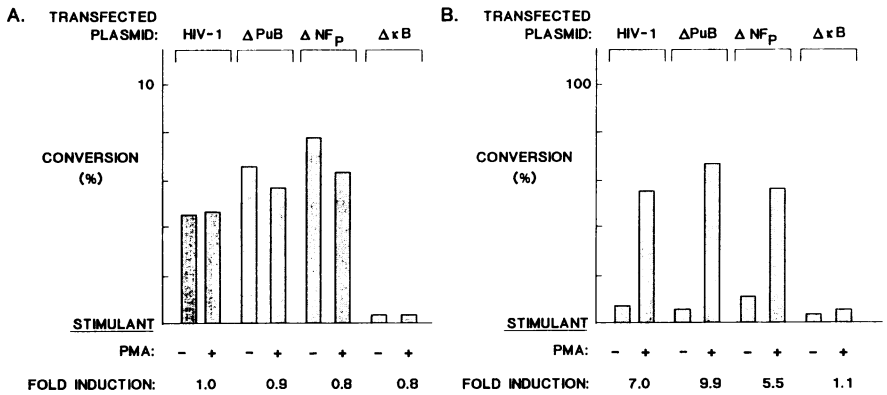

FIG. 2. Effect of site-specific HIV-1 enhancer mutations on expression in (A) the THP-1 macrophage leukemia line and (B) the clone 13 Epstein-Barr virus-transformed B-cell line. The mature monocyte line THP-1 was transfected with the indicated plasmid as described by Griffin et al. (11). Clone 13, an Epstein-Barr virus-transformed B-cell line (14), was transfected with HIV-1-CAT or mutant plasmids as described above. CAT assay results represent the average of two independent transfections.

mutant plasmid (Fig. 3). Similarly, no increase in CAT activity was detected in unstimulated or mitogen-activated Jurkat cells (Fig. 1B, C, and D), suggesting that neither site functioned as a negative regulatory element.

Because functional activation of the HIV-1 enhancer in Jurkat cells by PMA, PHA, or immobilized antibodies to the T-cell receptor was mediated by the two κB sites in each case, we examined whether these stimulants induced NF-κB binding activity in electrophoretic mobility shift assays. Both PMA and PHA induced binding activity which was demonstrated in competition experiments to be specific for NF-κB (not shown). As reported previously (26), cyclosporin A reduced induction of the κB complex by PHA but not PMA (Fig. 4A). This effect was specific, because cyclosporin A had no effect on binding to an unrelated octamer probe (Fig. 4B). Although a nonspecific band was noted with cross-linking, no increase in NF-κB binding was detected when cells were treated with cross-linked anti-CD3 for 2 h (Fig. 4A) or for 0.5, 1, 4, 24, 48, or 72 h (not shown). The inability to detect an increase in NF-κB binding with this method is consistent with the very weak induction of the HIV-1 enhancer seen with treatment by anti-CD3.

We have examined a region of the HIV-1 enhancer postulated to mediate induction in activated T cells through binding of an NFAT-like factor (18, 19, 27). This consideration was based primarily on sequence homology and binding of partially purified protein to DNA. The regions of IL-2 homology and sites protected by partially purified NFAT-1 differ. Because these sites, NF_P and NF'_P, lack homology to the NFAT-1 region of the IL-2 enhancer, this binding may be due to contaminants other than NFAT-1, further suggesting that NFAT-1 is not involved in HIV-1 enhancer function. Lu et al. (18, 19) have previously introduced a broad deletion (between −253 and −213) into an HIV-1 long terminal repeat (LTR)/CAT construct as well as an infectious proviral clone and tested these mutant plasmids in Jurkat T cells. This deletion had no effect on basal CAT activity and led to various modest effects on induction following treatment with

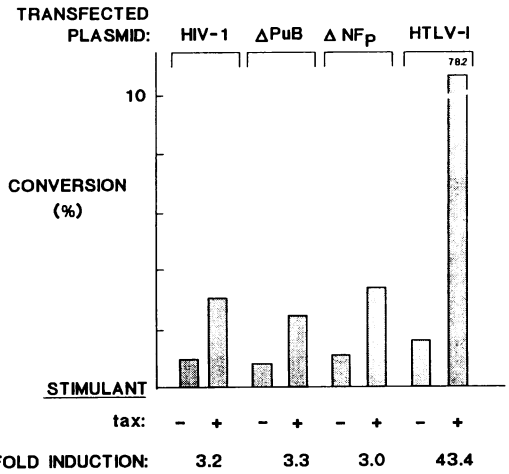

FIG. 3. HTLV-I *tax* stimulation of the HIV-1 enhancer and potential NFAT-1 site-specific mutants. Jurkat cells were transfected with 5 μg of HIV-1 or the indicated mutant enhancer plasmids and 1 μg of a plasmid expressing tax_I (+) or an inactive tax_I frameshift mutant (−) (17). CAT assay results are representative of three independent transfections.

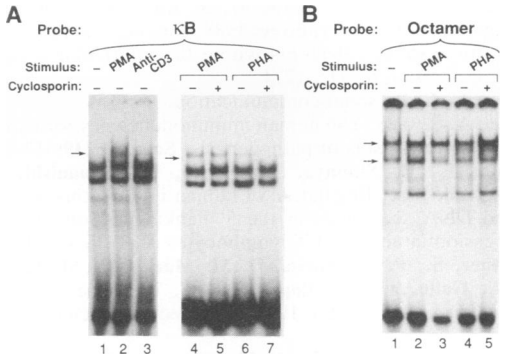

FIG. 4. Effect of PMA, PHA, and anti-CD3, with and without cyclosporin A, on NF-κB binding. Electrophoretic mobility shift assays were performed with radiolabeled oligonucleotide probes containing either the κB site (A) or two copies of the IL-2 octamer (B) as described before (17). Nuclear extracts (2) were prepared from unstimulated (−) Jurkat cells or Jurkat cells incubated for 2 h with the indicated combination of anti-CD3 (OKT3; see Fig. 1 legend), PMA (16 nM), or PHA (1 μg/ml) with cyclosporin A (1 μg/ml) as described previously (20, 21). Arrows denote specific complexes.

PMA and a ca. fivefold increase in viral replication. Although these authors suggested that these effects could be mediated by NFAT-1, this deletion eliminated other potential binding sites and disrupted the spatial organization of the LTR. In addition, because T-cell activation generally increases NFAT-1 binding activity and also stimulates HIV-1 replication, the data from these mutant viruses would ascribe a previously undetected activity to NFAT-1. Using site-specific mutagenesis, we now show that neither the region of IL-2 homology in the HIV-1 LTR nor the region which binds NFAT-1 mediates stimulation through the T-cell antigen receptor and that mutation of these sites does not significantly affect basal or induced levels of HIV-1 expression in several cell types. These observations are consistent with our previous data (20, 21) and with data from linker-scanning mutants (34), chimeric promoter-enhancer constructs (30), and deletion mutants (28). Taken together, it appears unlikely that NFAT-1 plays a direct role in induction of the HIV-1 enhancer or that the purine-rich region of IL-2 homology regulates HIV-1 expression in monocytes or lymphocytes. Although it remains possible that stimulation of the T-cell antigen receptor may indirectly activate T-cell gene products, for example, tumor necrosis factor alpha, which activate the HIV-1 enhancer through NF-κB, these data suggest that CD3-mediated activation plays no direct role in activating HIV-1 gene expression through a site recognized by the NFAT-1 transcription factor.

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