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# Differential regulation of human immunodeficiency viruses (HIVs): A specific regulatory element in HIV-2 responds to stimulation of the T-cell antigen receptor

(HIV-1/viral gene expression/cofactors/CD3)

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ABSTRACT **The human immunodeficiency viruses (HIVs) types 1 and 2 have similar genetic organization but differ significantly in nucleic acid sequence. Although infection by either agent leads to symptoms of immunodeficiency, recent studies suggest potential differences in the time course and severity of these diseases. In this report, the transcriptional regulation and induction of these retroviruses were analyzed. We report that the regulation of HIV-2 differs from that of HIV-1: a distinct T-cell activation pathway, triggering of the CD3 component of the T-cell receptor complex, stimulates HIV-2 but not HIV-1 gene expression. The response to T-cell receptor stimulation in HIV-2 is mediated partly by an upstream regulatory element, termed CD3R, which is recognized by a sequence-specific DNA binding protein, NF-CD3R. Jurkat T leukemia cell lines containing HIV-2 provirus also showed increased viral replication after stimulation of the T-cell receptor complex, in contrast to HIV-1. These findings suggest that transcriptional regulation and induction of HIV-2 differ from HIV-1 and raise the possibility that different cofactors contribute to the activation of HIV-1- and HIV-2-associated AIDS.**

Although it is a distinct retrovirus, the human immunodeficiency virus type 2 (HIV-2) shares nucleic acid and protein similarity with HIV-1 (1–4). First described in western Africa, HIV-2 is also a causative agent of the acquired immunodeficiency syndrome (AIDS) and has begun to appear throughout the world (5–12). While HIV-1 and HIV-2 both cause AIDS, the length of the asymptomatic period following infection may differ for the two viruses (5, 13). Because increased viral replication is associated with progression of HIV-related disease (14), the rate of disease progression may be influenced by regulatory proteins synthesized by host cells, which activate replication of virus. Such proteins could be regulated by distinct cofactors that selectively stimulate cellular activation pathways. These T-cell activation pathways regulate specific transcription factors, which may contribute to the regulation of the latent phase of HIV infection.

Despite their sequence divergence, the organization of the HIV-2 enhancer is similar to that of HIV-1. Both viruses encode a transactivating protein, tat, which acts on a responsive element located downstream of the transcriptional initiation site (15). Like HIV-1, HIV-2 appears to have three Sp1 transcriptional regulatory sites upstream of the TATA box (4). We have previously shown that stimulation of the HIV-1 enhancer by phorbol 12-myristate 13-acetate (PMA) is mediated by κB, which is a cis-acting, twice repeated 11-base-pair (bp) regulatory element found in the long terminal repeat (16). κB-mediated stimulation may act synergistically with tat-I to enhance HIV-1 transcription in activated T cells (16). In contrast, the HIV-2 enhancer contains a single κB site, located immediately upstream of a related site, designated K, which differs at 4 bp (Fig. 1). Because NF-κB and other factors bind to a variety of related κB-like sites (17–22), it was unknown whether this variant was functional and contributed to HIV-2 transcriptional activation.

In this study, the cis-acting sequences in HIV-2 that respond to T-cell activation have been analyzed. Because T cells can be stimulated through multiple activation pathways, we have analyzed whether HIV regulatory elements respond differentially to such stimuli and whether they affect induction of provirus. We have found that activation of the HIV-2 enhancer is partly mediated by an upstream site not found in HIV-1, termed CD3R, since it is responsive to stimulation of the CD3 component of the T-cell receptor complex. This site also contributes to HIV-2 activation by PMA and phytohemagglutinin (PHA). In contrast, HIV-1 is minimally responsive to stimulation of the T-cell receptor complex, and stimulation by PMA and PHA is mediated solely by κB, the major regulatory element of its enhancer (16). Similarly, production of HIV-2, but not HIV-1, is stimulated with anti-CD3 antibody in latently infected lines.

## MATERIALS AND METHODS

**Preparation of Site-Specific Mutants.** Sequences upstream of −256 were removed by digestion with the *Kpn* I restriction enzyme. A deletion to about −107 was prepared by using *Kpn* I and the *Bgl* II site introduced at the mutant HIV-2 κB site (see Fig. 1). Site-specific mutants of HIV-2 chloramphenicol acetyltransferase (CAT) were prepared as described (16). HIV-1-CAT (pU3R-III) and its κB mutant have been described elsewhere (16, 23).

**Cell Transfections and CAT Assays.** Cells ($10^7$) were transfected with 5 μg of the indicated plasmid by using DEAE-dextran (24). Twenty hours after transfection, cells were incubated with either 16 nM PMA, PHA (2 μg/ml), or anti-CD3 antibody (1:10,000 dilution of murine monoclonal antibody; ≈100 ng/ml), for an additional 20 hr. Experiments involving cotransfection with the immediate-early transactivator of human cytomegalovirus (CMV-IE) used 2 μg of the CMV-IE-expressing plasmid pHD101SV1, a gift from Michelle Davis (25), and 5 μg of the indicated HIV-CAT plasmid. Cell extracts were prepared and CAT activity was determined according to standard methods (26), and transfection efficiencies were normalized by using protein con-

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Abbreviations: HIV, human immunodeficiency virus; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; CAT, chloramphenicol acetyltransferase.

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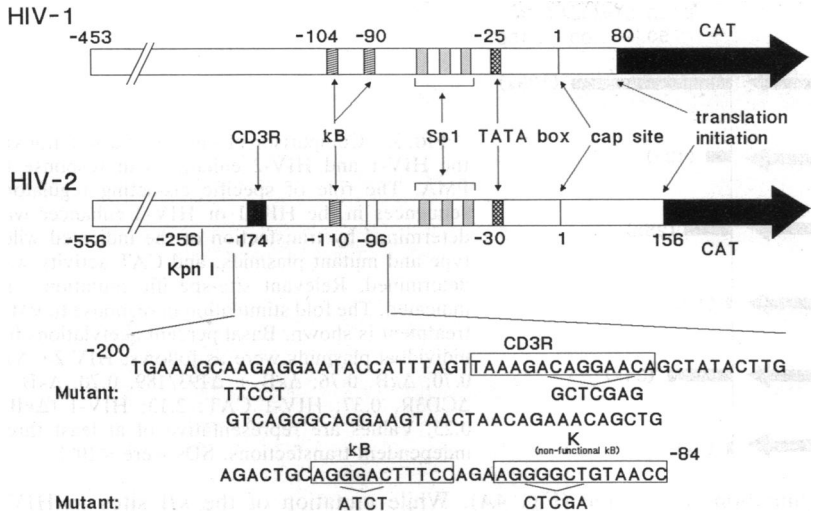


FIG. 1. Comparison of the enhancer regions of HIV-2 and HIV-1; relevant sites within each long terminal repeat are identified. Altered bases within the mutant plasmids used in this study are shown below the wild-type sequence. Identification of the CD3R site is described in the text. The sequence of HIV-$2_{rod}$ has been published (4). HIV-2-CAT was kindly provided by M. Emerman (15). Site-directed mutations were introduced by a previously described method (16).

centration or transfection with an independent reporter plasmid as described (16, 27).

**Analysis of DNA Binding Proteins.** Nuclear extracts were prepared by a modification of the method of Dignam *et al.* (28). Activated Jurkat cells were incubated in RPMI 1640 medium containing 10% fetal calf serum for 2 hr before preparation of nuclear extract. The procedure for the electrophoretic mobility-shift assay has been described (29); the gel running buffer was 50 mM Tris base/380 mM glycine/2 mM EDTA, pH 8.5. Ten micrograms of nuclear protein was used in DNA binding reactions. Oligonucleotides were synthesized with an Applied Biosystems 300B synthesizer. The CD3R probe extended from position −183 to −155 in the sequence (see Fig. 1). Equimolar amounts of each strand were combined by incubating at 80°C and then slowly cooling in 500 mM NaCl. Radiolabeled probe for DNase protection experiments was prepared by incubation of the ΔκB+Δ193/189 mutant plasmid with *Bgl* II and calf intestinal phosphatase, labeling with T4 polynucleotide kinase in the presence of [$\gamma$-$^{32}$P]ATP. This fragment was subsequently digested with *Hin*dIII and purified by PAGE.

**Virus Infection and Detection.** Jurkat cells ($1 \times 10^5$) containing either HIV-1 or HIV-2 provirus were incubated with 10 μg of anti-CD3 antibody per ml (Ortho Diagnostics) bound to goat anti-mouse immunoglobulin (Jackson Laboratory), which was used to coat a 96-well microtiter plate. Supernatants were collected after 24 or 48 hr and p24 antigen levels were determined (Coulter).

## RESULTS

**Definition of HIV-2 cis-Acting Regulatory Elements.** To determine which cis-acting sites modulate HIV-2 gene expression, Jurkat cells were transfected with a plasmid containing the HIV-2 enhancer linked to the CAT gene. When transfected cells were incubated with PMA, CAT activity increased 116-fold. Although mutation of the κB site diminished this response by 19-fold, it remained PMA responsive, as did a plasmid containing mutations in both the κB and K sites, which was stimulated 7-fold by PMA (Fig. 2), similar to the single κB mutant. To localize the additional cis-acting element(s) responsive to PMA, several deletion mutant plasmids were analyzed. Deletion of a sequence in the enhancer region to position −256 did not affect the response to PMA; however, deletion of a sequence between −256 and −107 abolished stimulation when the κB site was mutated (Fig. 2). The second PMA-responsive element was thus localized to this 149-bp region of the HIV-2 enhancer.

To define the cis-acting regulatory element(s) within this region, we compared the sequences of HIV-2 and the simian immunodeficiency viruses (SIV), which share ≈75% nucleic acid similarity (30). Two sequences downstream of the *nef* open reading frame, between −197 and −187 as well as −174 and −161 (subsequently termed CD3R), were highly conserved among various HIV-2 and SIV isolates within this 149-bp region (Fig. 1; see also ref. 31). Mutations were introduced into each of these sites in HIV-2-CAT plasmids containing a mutant κB site. Although mutation of one site

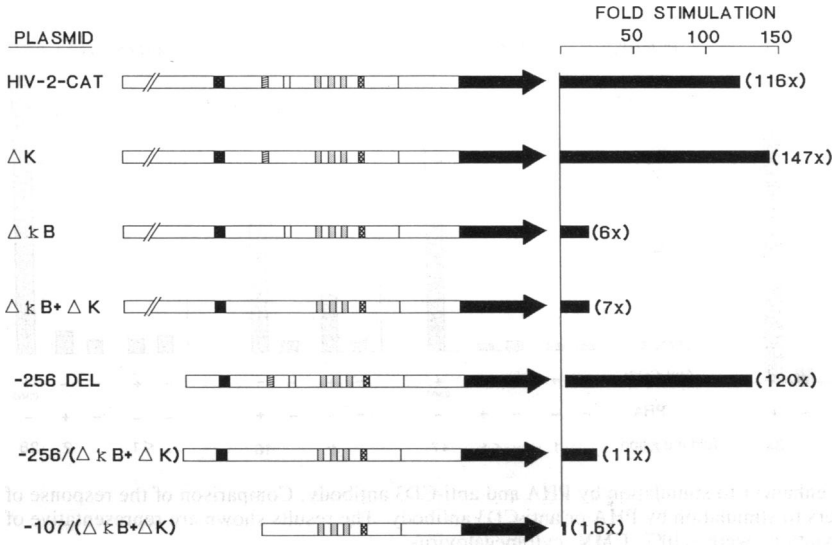


FIG. 2. Mutational analysis of PMA-responsive elements in the HIV-2 enhancer. Cis-acting regulatory sequences in HIV-2 were determined by transfection of deletion mutant and wild-type HIV-2-CAT plasmids. Deletions and site-specific mutations are indicated. The fold stimulation in response to PMA treatment is shown. Basal percent acetylations for individual plasmids were as follows: HIV-2-CAT, 0.16; ΔK, 0.12; ΔκB, 0.14; ΔκB + ΔK, 0.11; −256 DEL, 0.18; −256/(ΔκB + ΔK), 0.16; −107/(ΔκB + ΔK), 0.68. Values are representative of at least three independent transfections. SDs were <10%.

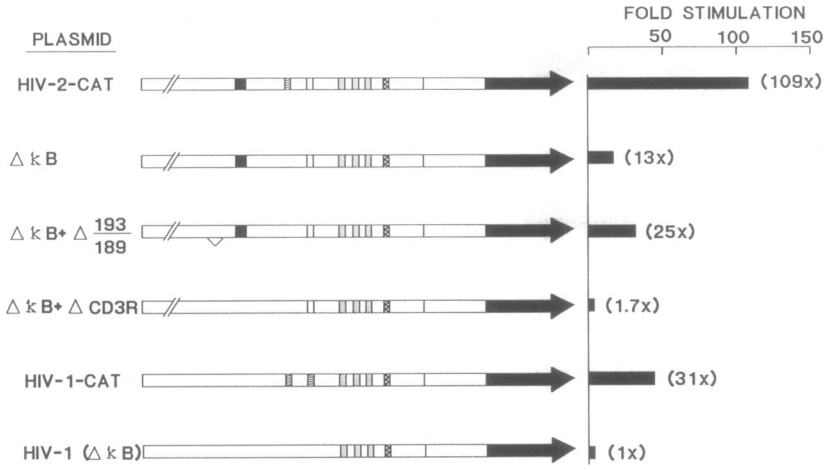


FIG. 3. Comparison of site-specific mutations in the HIV-1 and HIV-2 enhancers in response to PMA. The role of specific cis-acting regulatory sequences in the HIV-1 or HIV-2 enhancer was determined by transfection of the indicated wild-type and mutant plasmids, and CAT activity was determined. Relevant site-specific mutations are indicated. The fold stimulation in response to PMA treatment is shown. Basal percent acetylations for individual plasmids were as follows: HIV-2-CAT, 0.70; ΔκB, 0.76; ΔκB + Δ193/189, 0.70; ΔκB + ΔCD3R, 0.37; HIV-1 CAT, 2.13; HIV-1 (ΔκB), 0.25. Values are representative of at least three independent transfections. SDs were <10%.

(Δ-193/189) did not alter PMA responsiveness, mutation of the second site reduced the response to background levels (Fig. 3). The response of these site-directed mutants of the HIV-2 enhancer to PMA was compared to the response of the HIV-1 enhancer. As shown previously, PMA induction of the HIV-1 enhancer is mediated entirely by the two κB sites (Fig. 3; see also ref. 16). Thus, the cis-acting regulatory elements that respond to PMA in HIV-2 differ from those in HIV-1.

**Response of HIV-2 Enhancer Plasmids to Different Stimulants.** In addition to PMA, stimulants such as anti-CD3 antibody or PHA induce the expression of T-cell activation genes (32–36). To determine whether the HIV-2 enhancer is stimulated through these pathways, Jurkat cells were transfected with HIV-1-CAT or HIV-2-CAT plasmids and incubated with anti-CD3 antibody or PHA. When stimulated with anti-CD3 antibody, the HIV-2 enhancer displayed a markedly different response than HIV-1. Incubation with anti-CD3 antibody did not stimulate HIV-1-CAT activity, whereas CAT activity of the HIV-2 enhancer increased 7-fold (Fig. 4*A*). This effect was mediated both by κB (≈2-fold) (Fig. 4*A Left* vs. Fig. 4*B Center*, anti-CD3 stimulation), and by the CD3R site (≈4-fold) (Fig. 4*B Right*, anti-CD3 stimulation), identical to the second PMA-responsive site identified above. This reduction in CAT activity was specific, since the κB + CD3R mutant was stimulated 28-fold by the human cytomegalovirus immediate-early gene transactivator (Fig. 4*B*), a nonspecific activator. Although the enhancer activity of both HIV-1 and HIV-2 increased in the presence of PHA, the HIV-2 enhancer responded with consistently higher induction (Fig. 4*A*). While mutation of the κB sites in HIV-1 eliminated this modest PHA response (Fig. 4*B*), mutations in both κB and the CD3R site were required to abolish the PHA response in the HIV-2 enhancer (Fig. 4*B*). Finally, the effect of mutation of the CD3R site alone (between −168 and −162) was assessed. This mutation reduced the response to PMA, PHA, and anti-CD3 antibody 9- to 14-fold in the HIV-2 enhancer (Fig. 5).

**Definition of CD3R Binding Protein.** To determine whether a sequence-specific DNA binding protein could recognize the CD3R site, an electrophoretic mobility-shift assay was performed with nuclear extracts from Jurkat cells that were either unstimulated or incubated with PMA and PHA. Using a radiolabeled CD3R site probe, we observed a specific complex in extracts from both unstimulated and induced cells (Fig. 6*A*, lanes 1 and 2). This complex was observed in the presence of a mutant CD3R site or sequences that bind other transcription factors found in nuclear extracts of activated T cells (16, 20, 37, 38), such as NF-κB, interleukin octamer, or NF-AT1 but was not seen in the presence of an excess of unlabeled CD3R site (Fig. 6*A*, lanes 3 and 4 vs. 5–12). No such complex was seen when the mutant CD3R site sequence was used as a probe (Fig. 6*B*, lane 1). Further evidence that a sequence-specific DNA binding protein recognizes this region was obtained by DNase footprinting where protection was observed between about −168 and −177 (Fig. 6*C*).

**Differential Activation of HIV-1 and HIV-2 by Anti-CD3 Antibody.** Having established differences in enhancer function of HIV-1 and HIV-2, we extended this analysis to intact

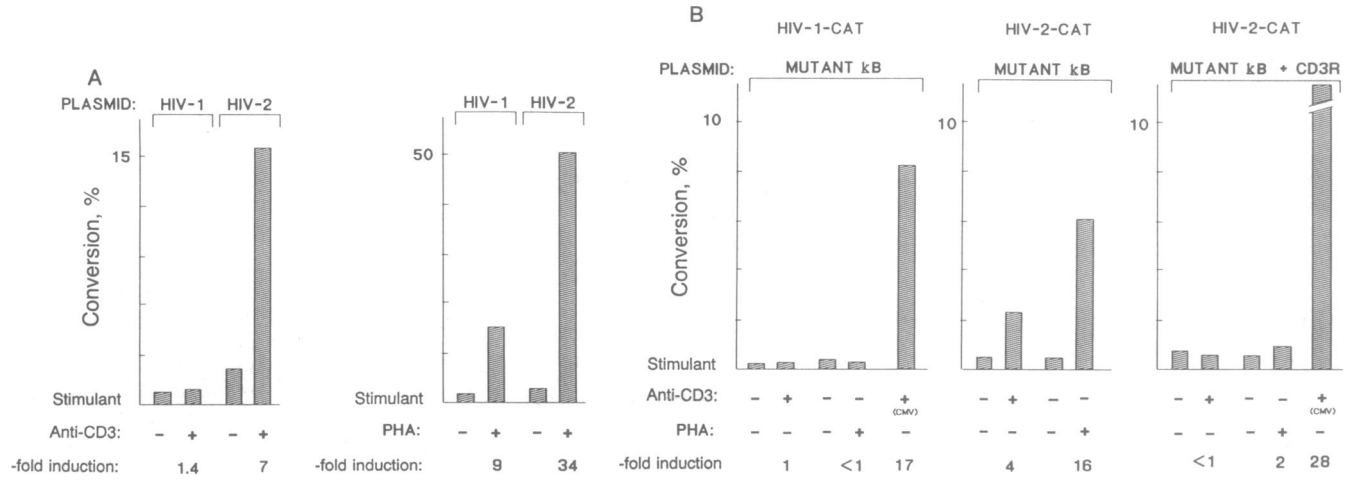


FIG. 4. Differential response of HIV-1 and HIV-2 enhancer to stimulation by PHA and anti-CD3 antibody. Comparison of the response of wild-type (*A*) or mutant (*B*) HIV-1 and HIV-2 enhancers to stimulation by PHA or anti-CD3 antibody. The results shown are representative of at least three independent transfections. Standard deviations were <10%. CMV, cytomegalovirus.

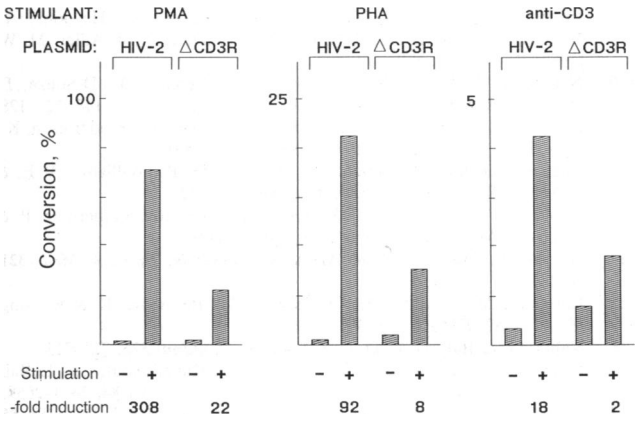


FIG. 5. Functional analysis of the HIV-2 CD3R site in Jurkat cells. The effect of mutation of CD3R alone was assessed by transfection with HIV-2-CAT and mutant plasmids. Transfected plasmids and added stimulants are indicated. Results are means of two independent transfections.

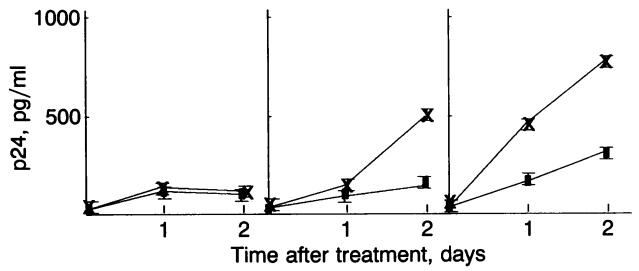


FIG. 7. Effect of anti-CD3 antibody on HIV-1 and HIV-2 expression in Jurkat cells. Independent Jurkat T leukemia lines were chronically infected with HIV-1 (*Left*, clone J1.1) or HIV-2 (*Center* and *Right*, clones J2.1 and J2.2, respectively). The indicated cell lines were incubated with (×) or without (■) monoclonal anti-CD3 antibody, and p24 antigen levels were measured at 24 and 48 hr. Parallel cultures stimulated for 48 hr with 1000 units of recombinant tumor necrosis factor α (Genzyme) per ml yielded 1810 (*Left*), 551 (*Center*), and 777 (*Right*) pg of p24 antigen per ml, respectively. Samples were measured in duplicate or triplicate and varied by <10%.

virus. Several independent Jurkat T leukemia lines containing proviral HIV-1 or HIV-2 were examined. Although it was induced 14-fold by tumor necrosis factor α or by PMA (data not shown), the HIV-1-containing clone was not induced by treatment with anti-CD3 antibody (Fig. 7). In contrast, at least two independent clones containing HIV-2 displayed increased p24 production in the presence of anti-CD3 antibodies (Fig. 7). Differential regulation of HIV-2 is therefore observed not only when the enhancer region of this virus is examined but applies also to induction of provirus, since replication of HIV-2, but not HIV-1, is stimulated by treatment of Jurkat cells with anti-CD3 antibodies.

## DISCUSSION

Although both the HIV-1 and HIV-2 enhancers respond to mitogenic stimulation in Jurkat T leukemia cells (Fig. 2; refs. 16, 41–43), we have shown that the cis-acting regulatory sequences that mediate these effects differ. When these cells are activated by specific agents, such as stimulation of the T-cell antigen receptor complex by monoclonal antibodies, regulation of these retroviral enhancers also differs. In the case of HIV-2, stimulation by anti-CD3 antibody is mediated partly through an upstream regulatory element, CD3R, which is also responsive to PMA and PHA. This site is recognized by a factor present in both unstimulated and activated Jurkat cells. In contrast to NF-κB and NF-AT1, whose binding activity correlates with cellular activation, the binding of nuclear factor of the CD3R site (NF-CD3R) is independent of cellular activation. Similar to other DNA binding proteins that display no change in binding activity after activation (44–48), NF-CD3R could undergo posttranslational modification to increase its ability to transactivate. While the mechanism of NF-CD3R activation is unclear, agents that stimulate either protein kinase C (PMA) or increased intracellular free calcium (anti-CD3 antibody) mediated this effect, raising the possibility of a common intermediate in these pathways.

Our findings suggest that transcriptional regulation of HIV-2 differs from that of HIV-1 and is dependent on the mode of T-cell activation. HIV-1 gene expression can be

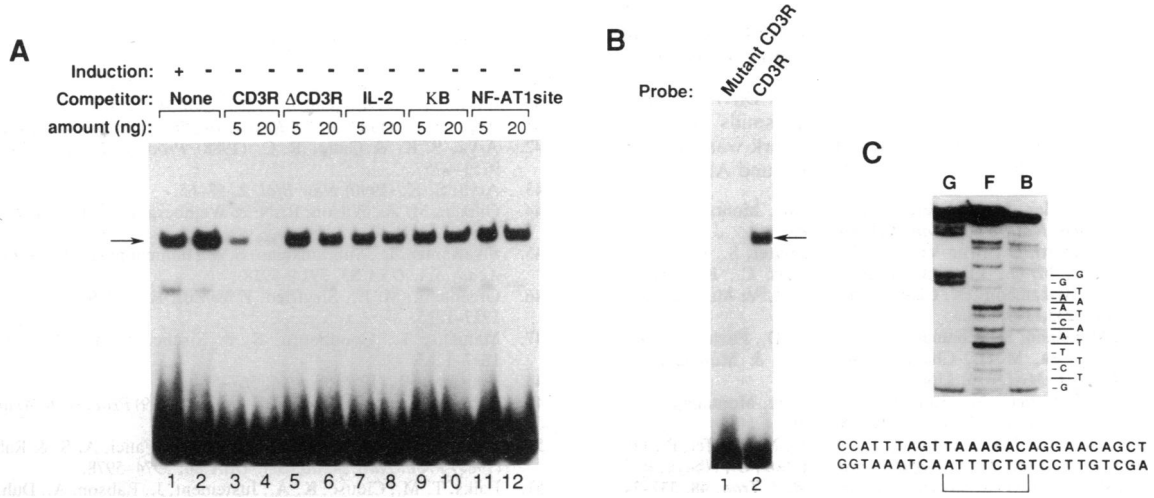


FIG. 6. Characterization of a nuclear factor that specifically binds the CD3R site. Analysis of the CD3R binding factor was determined by the electrophoretic mobility shift assay using CD3R site probe (*A*) mutant vs. wild-type CD3R site probe (*B*) or by DNase protection (*C*). Competition studies using the CD3R binding factor were performed with nuclear extracts from Jurkat cells, either activated with PMA and PHA (+) or unstimulated (−). (*A*) Extracts were incubated with a radiolabeled double-stranded oligonucleotide probe containing the CD3R site alone (lanes 1 and 2) or in the presence of the indicated amounts of the unlabeled CD3R (lanes 3 and 4), mutant CD3R site as indicated in Fig. 1 and described below (lanes 5 and 6), interleukin (IL-2) octamer (lanes 7 and 8), κB (lanes 9 and 10), or NF-AT1 site (lanes 11 and 12) competitors (16, 20, 37, 38). (*B*) Mutant CD3R site probe was compared to the wild-type CD3R element by using unstimulated Jurkat nuclear extracts (lane 1 vs. lane 2). Similar results were found by using stimulated extracts (data not shown). (*C*) DNase protection was performed as described (39, 40) using a G ladder (lane 1), no nuclear extract (lane 2), or 150 μg of nuclear extract from unstimulated Jurkat cells (lane 3).

stimulated through multiple independent cellular activators, including mitogens (16) or cytokines, such as tumor necrosis factor $\alpha$ (49–52). Although it can also be induced by such agents (Fig. 7), HIV-2 replication was stimulated in these studies by treatment of cells with anti-CD3 antibodies, which activated the T-cell antigen receptor complex and had no stimulatory effect on HIV-1 replication. The mechanism of virus induction has not been precisely determined, but the CD3R site of HIV-2 likely contributes to this effect. Although a site related to another element responsive to triggering of the T-cell antigen receptor complex, NF-AT1 (38), has been described in HIV-1, this site, which is not present in HIV-2, does not contribute to functional activation of the HIV-1 enhancer (Fig. 4*A*; see also ref. 53). A small degree of stimulation of the HIV-1 enhancer by anti-CD3 antibodies coupled to beads has been reported (54), but this induction was mediated by NF-$\kappa$B, suggesting that activation occurred through a protein kinase C-related pathway (33) and not through the effect on intracellular free calcium predominantly seen with stimulation of the antigen receptor. In the present study, incubation with native anti-CD3 antibody increased HIV-2 enhancer activity but had no effect on HIV-1 enhancer function or HIV-1 replication (Fig. 7). The enhancer stimulation is mediated partly by the CD3R site, which is not present in the HIV-1 enhancer, and suggests that the CD3R site may provide the basis for differential regulation of these human retroviruses. These results therefore suggest that activation of HIV-2 may be regulated by multiple activation pathways within T cells and that this virus can respond to stimulation of the T-cell receptor complex. Despite their common progression to immunodeficiency, the asymptomatic phase of HIV-2 infection does not appear to strictly parallel that of HIV-1 (5, 13). In particular, the asymptomatic phase of HIV-2 infection appears to be longer than that of HIV-1, and HIV-2 progresses to AIDS less frequently. It is possible that the differences in enhancer function reported here may contribute to the disparities in these diseases. For example, HIV-2 contains only a single $\kappa$B site, and the CD3R element appears to be a weaker regulatory element, which may not fully compensate for the absence of a $\kappa$B site. At the same time, the possibility remains that other genetic regions of HIV-2 contribute to these effects. Finally, because of the differences in retroviral gene regulation, this study raises the possibility that different cofactors may determine the asymptomatic phase of HIV-1- and HIV-2-associated disease.

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