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The Peri-κB Site Mediates Human Immunodeficiency Virus Type 2 Enhancer Activation in Monocytes but Not in T Cells

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Human immunodeficiency virus type 2 (HIV-2), like HIV-1, causes AIDS and is associated with AIDS cases primarily in West Africa. HIV-1 and HIV-2 display significant differences in nucleic acid sequence and in the natural history of clinical disease. Consistent with these differences, we have previously demonstrated that the enhancer/promoter region of HIV-2 functions quite differently from that of HIV-1. Whereas activation of the HIV-1 enhancer following T-cell stimulation is mediated largely through binding of the transcription factor NF-κB to two adjacent κB sites in the HIV-1 long terminal repeat, activation of the HIV-2 enhancer in monocytes and T cells is dependent on four *cis*-acting elements: a single κB site, two purine-rich binding sites, PuB1 and PuB2, and a pets site. We have now identified a novel *cis*-acting element within the HIV-2 enhancer, immediately upstream of the κB site, designated peri-κB. This site is conserved among isolates of HIV-2 and the closely related simian immunodeficiency virus, and transfection assays show this site to mediate HIV-2 enhancer activation following stimulation of monocytic but not T-cell lines. This is the first description of an HIV-2 enhancer element which displays such monocyte specificity, and no comparable enhancer element has been clearly defined for HIV-1. While a nuclear factor(s) from both peripheral blood monocytes and T cells binds the peri-κB site, electrophoretic mobility shift assays suggest that either a different protein binds to this site in monocytes versus T cells or that the protein recognizing this enhancer element undergoes differential modification in monocytes and T cells, thus supporting the transfection data. Further, while specific constitutive binding to the peri-κB site is seen in monocytes, stimulation with phorbol esters induces additional, specific binding. Understanding the monocyte-specific function of the peri-κB factor may ultimately provide insight into the different roles monocytes and T cells play in HIV pathogenesis.

Human immunodeficiency virus type 2 (HIV-2), like HIV-1, causes AIDS (1, 9, 11, 63). While HIV-2 infection has been associated with AIDS cases in West Africa (1, 9–11, 13, 63), it is also becoming increasingly recognized in other areas of the world (1, 11–13, 48, 63). The genomes of HIV-1 and HIV-2 encode similar viral proteins, but HIV-2 shares only approximately 42% nucleic acid sequence similarity with HIV-1 (9, 10) and is instead more closely related to simian immunodeficiency virus (SIV), with which HIV-2 shares 75% or more sequence similarity, depending on the strain (6, 23).

HIV-1 and HIV-2 not only display significant nucleic acid sequence differences but also show striking biological and clinical differences. HIV-2 infection progresses to AIDS much more slowly than HIV-1 infection does and demonstrates less efficient perinatal and heterosexual transmission (2, 3, 13, 37–39, 48, 52). Furthermore, in tissue culture experiments, monoclonal antibodies to the T-cell receptor component CD3 stimulate production of HIV-2 but not HIV-1 from latently infected T-cell lines, and HIV-2 is less responsive to stimulation by tumor necrosis factor alpha than HIV-1 (33, 49).

The clinical differences between HIV-1 and HIV-2 infection remain largely unexplained, and it is unknown, for example, why HIV-2 infection displays a much more prolonged period of clinical latency than HIV-1 infection does. A correlation between clinical latency and latency of HIV on a cellular level has been suspected. Following infection of cells in vitro, HIV expression is suppressed for a variable period of time until cellular conditions are altered such that they permit replication of the virus (20, 44, 49, 56). In asymptomatic HIV-infected individuals, the number of infected cells in the peripheral blood is low and it is often difficult to demonstrate viral expression in these infected cells (18). Disease progression is associated with marked increases in viral expression in peripheral blood cells (36). Recently it has been noted that there may be a preferential localization of HIV-infected cells actively producing virus in the lymphoid tissue early in HIV-1 infection, despite very low levels of virus production in peripheral blood mononuclear cells (16, 59). However, even in the lymph nodes, cellular latency is an important phenomenon, as it has been demonstrated that only a small percentage of infected lymphoid cells are actively making virus at any given time (16).

Stimulation of T cells with cytokines such as tumor necrosis factor alpha or antibodies to the T-cell receptor increases transcription of HIV via cellular intermediaries (21, 33, 41, 49, 56). Similarly, exposure of HIV-infected macrophages and monocytes to activated, uninfected T cells or to cytokines can induce HIV replication (21, 25, 26, 61, 65). Furthermore, recent data suggest that the level of HIV mRNA expression in peripheral blood mononuclear cells in asymptomatic HIV-infected persons correlates with clinical disease progression over the subsequent 2 to 5 years (64). Since progression of clinical disease is associated with and likely accelerated by an increase in viral replication, we have been interested in the cellular events involved in effecting the transition from HIV latency to replication in monocytes and T cells. It is possible that differences in transcriptional activation of HIV-1 and HIV-2 may be responsible for the observed differences in the clinical latency period following infection with these viruses.

In studying the transcriptional regulation of HIV-2, we and

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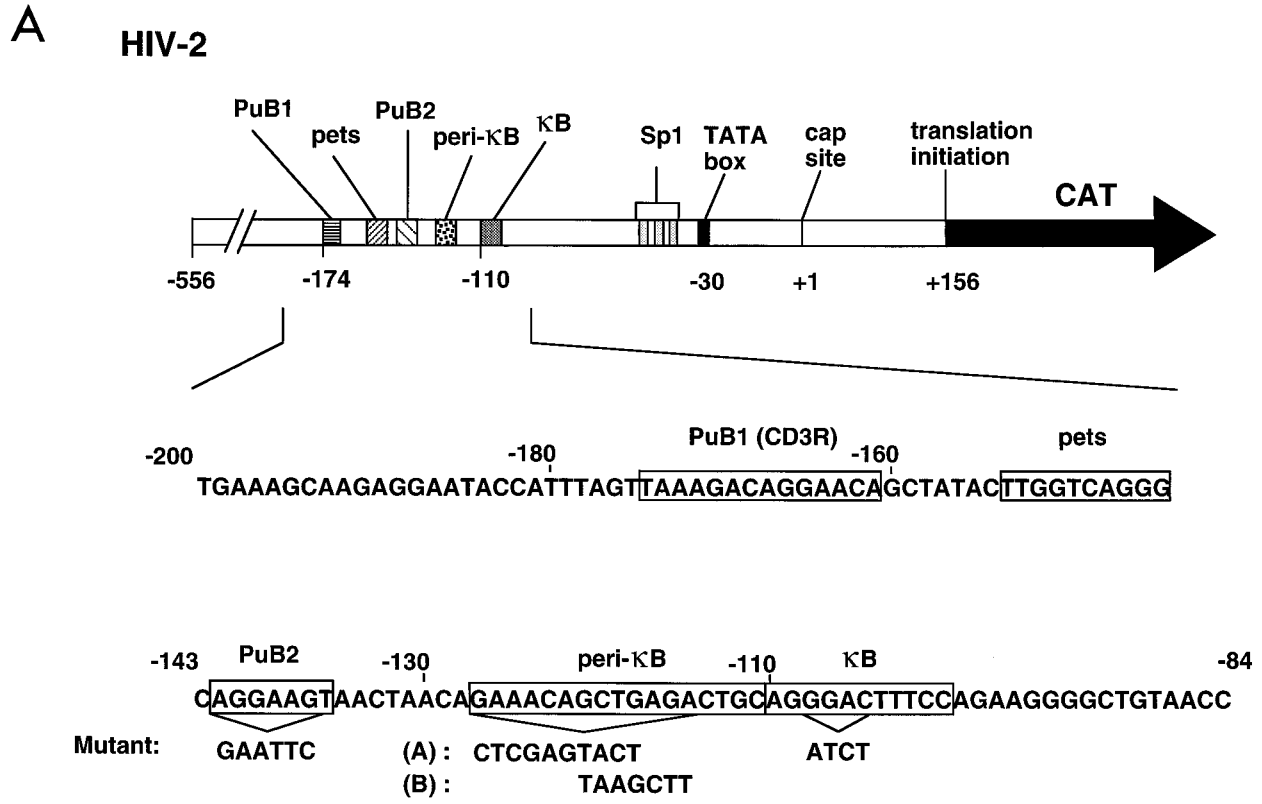

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CONSENSUS	aaac?aGctgagac?GC
HIV-2 ROD	GAAACAGCTGAGACTGC
HIV-2 NIHZ	.GAACAGCTGAGACTGC
HIV-2 ISY	.AAACAGCTGAGACTGC
HIV-2 ST	.AAACAGCTGAGACTGC
HIV-2 BEN	.AAACAGCTGAGGCTGC
HIV-2 CAM2	.AAACAGCTGAGACTGC
HIV-2 D194	.AA.CAGCTGAGACTGC
HIV-2 GH1	GCTGAGGCTGCAGCTGC
HIV-2 D205	AAACTAGCAGACACTGC
SIV MM251	AAACTCGCTGAGATAGC
SIV MM1A11	AAACTAGATGAGACAGC
SIV MM32H	AAACTCGCTGAGACAGC
SIV MM142	AAACTAGCTGACACAGC
SIV MM239	AAACTCGCTGAAACAGC
SIV MNE	AAACTAGCTGAGACAGC
SIV SMMH4	AAACAAGCTGAGACAGC
SIV SMMPBJ	AAACAAGCTAAGACAGC
SIV STAK1	AAACAAGCTGAGACGGC
SIV STM	AAACAAGCTGAGACAGC

FIG. 1. (A) Enhancer region of HIV-2. Relevant sites within the LTR are identified. Altered bases within the mutant plasmids used are shown below the wild-type sequence; (A) denotes the mutation contained in Δperi-κB(A), and (B) denotes the Δperi-κB(B) mutation. The Δperi-κB(A)+ΔκB mutant plasmid contains both the Δperi-κB(A) and the ΔκB mutations. The PuB1, PuB2, pets, and κB sites have been described previously (49, 51), and the sequence of HIV-2_{rod} has been published elsewhere (32). Site-directed mutations were introduced by the gap-heteroduplex method as previously described (56). CAT, chloramphenicol acetyltransferase. (B) Conserved nucleotides in the peri-κB region of the LTRs of HIV-2 and SIV isolates, modified from Myers et al. (55). Uppercase letters in the consensus sequence indicate 100% conservation of nucleotide bases in a given position of the alignment, and lowercase letters represent bases conserved in at least 50% of the sequences examined (55). Dots are introduced where necessary to conserve alignment.

others have found that the enhancer/promoter region of HIV-2, located in the viral long terminal repeat (LTR), functions quite differently from that of HIV-1 in T cells and monocytes (33, 35, 43, 49, 51, 68). Stimulation of the HIV-1 enhancer/promoter in activated T cells is mediated predominantly by binding of the cellular protein NF-κB to two adjacent κB sites in the HIV-1 enhancer (50, 56). The HIV-2 enhancer, unlike the HIV-1 enhancer, contains only a single functional κB element and also contains at least three other inducible *cis*-acting elements which we have previously described: two purine-rich binding sites, designated PuB1 and PuB2, and a pets (peri-ets) site, located between the PuB sites (see Fig. 1A) (43, 49, 51). The purine-rich PuB1 and PuB2 sites contain the conserved GGA core binding motif which is recognized by members of the Ets family of proto-oncogenes (43, 67, 69). The predominant protein binding the PuB sites in vivo appears to be Elf-1, an Ets family member which is related to the *Drosophila* development protein E74 (35, 43). The PuB and pets sites of HIV-2 are not found in HIV-1 but are conserved among HIV-2 isolates and are also found in another human pathogenic retrovirus, human T-cell leukemia virus type 1 (8). These sites are important for activation of the HIV-2 enhancer in T cells stimulated with phorbol esters, phytohemagglutinin, antibody to the CD3 component of the T-cell receptor complex, and antigen (33, 34, 49, 51).

Differences in transcriptional regulation of HIV-1 and HIV-2 have also been noted in monocytic cells, which play an important role in HIV infection. It is believed that monocytes act as a reservoir for HIV in vivo and are involved in the pathogenesis of HIV-related encephalopathy (24, 25, 35, 58,

71). Griffin et al. have shown that activation of the HIV-1 enhancer in immature monocytic cell lines by phorbol myristate acetate (PMA) is dependent on the induction of NF-κB and its binding to the κB sites in the HIV-1 enhancer (30). In mature monocytes, NF-κB is constitutively expressed, leading to a high level of basal expression of HIV-1 and lack of induction by PMA. We have shown that HIV-2 expression in monocytes following PMA stimulation requires intact PuB and pets enhancer sites in addition to the κB site (35). The different clinical and biological characteristics of infection with HIV-1 compared with HIV-2 may thus in part be a result of the different patterns of transcriptional control of the two viruses.

In the course of performing DNase protection analysis of the HIV-2 LTR, a footprint was noted immediately upstream of the κB site (Fig. 1A). Analysis of this DNA sequence revealed that the motif, designated the peri-κB site, is conserved across isolates of HIV-2 and SIV (Fig. 1B) (55) and appears to be unique. Given these findings and the proximity of the site to κB, we were interested in the ability of the peri-κB site to confer, along with the other *cis*-acting elements, inducible activation of the HIV-2 enhancer in T cells and/or monocytes.

In the experiments described below, we show that the peri-κB site is involved in regulation of HIV-2 enhancer function in monocytes but unlike the PuB1, PuB2, pets, and κB sites, not in T cells. Mutation of this site leads to markedly diminished transcriptional activation following stimulation in monocytic cells but not in T cells. Furthermore, the peri-κB site is specifically recognized by either a different cellular protein in monocytes than in T cells or by a single protein which is differentially modified in the two cell types. A monocyte-specific transcriptional activator has not previously been described for HIV-2 or clearly delineated for HIV-1, and such a finding suggests one possible cellular regulatory mechanism which might contribute to the different roles monocytes and T cells play in HIV pathogenesis.

MATERIALS AND METHODS

Plasmids. Construction of HIV-2/CAT and HIV-2 ΔκB/CAT has been described elsewhere (17, 49). HIV-2 Δperi-κB(A)/CAT, HIV-2 Δperi-κB(B)/CAT, HIV-2 peri-κB $DEL_{(-111/-127)}$/CAT, and HIV-2 Δperi-κB(A)+ΔκB/CAT (Fig. 1A) were constructed by using the gap-heteroduplex site-specific mutagenesis protocol as previously described (56). The HIV-2 mutants were sequenced by the dideoxy method (kit from U.S. Biochemical).

Cell transfections and chloramphenicol acetyltransferase assays. The Jurkat T leukemia cell line, CEM T leukemia cell line, and the U937 monocytic cell line were maintained in RPMI with 10% fetal bovine serum (FBS) prior to transfection and RPMI with 5% FBS after transfection. The HL-60 cell line was maintained in RPMI with 20% FBS before and after transfection. The THP-1 monocytic cell line was maintained in RPMI with 10% FBS and 50 μM β-mercaptoethanol before and after transfection. Cells were grown under standard conditions. Cells (10^7) were transfected with 5 μg of the indicated plasmid by the DEAE-dextran method (62). Twenty hours after transfection, certain cell groups were incubated for an additional 20 h with 16 nM PMA. Cell extracts were prepared, and chloramphenicol acetyltransferase activity was determined by standard methods (29). Transfection efficiencies were normalized by protein concentration.

DNase protection assays. Nuclear extracts were prepared by a modification of the method of Dignam et al. (14). The Jurkat extract was additionally purified through a KCl step gradient, with the fraction eluting at 0.4 M being used. For the assays using HeLa cell extracts, a radiolabeled antisense strand probe extending from −51 to −231 was used. For the experiments using U937 nuclear extracts, both radiolabeled sense and antisense strand probes extending from −96 to −147 were employed, and the probes contained mutations in both the PuB2 and κB sites (the sequences of these mutations are shown in Fig. 1A). For the experiments using Jurkat nuclear extracts, a radiolabeled sense strand probe extending from −110 to −190 was employed. The probes were generated and DNase protection assays were performed as previously described (15, 22, 49, 51).

EMSAs. Jurkat, U937, and HL-60 nuclear extracts were prepared as previously described (14) from cells which were either unstimulated or treated for 5 to 8 h with 16 nM PMA. Human peripheral blood mononuclear cells were isolated as follows: buffy coats prepared by the American Red Cross from samples of whole blood from healthy donors were diluted 1:2 with phosphate-buffered saline and layered onto Histopaque-1077 (Sigma). The cells were centrifuged at 400 × *g* for 30 min at 25°C, and the mononuclear cell layer was aspirated and washed three times with RPMI containing 5% FBS. The cells were then layered on a continuous Percoll (Pharmacia) gradient and centrifuged at 1,000 × *g* for 20 min for separation of monocytic cells from lymphocytes (28). The monocytes and lymphocytes were aspirated separately, and each was washed three times with RPMI containing 5% FBS. Unstimulated nuclear extracts were prepared from half of the monocytes (14), and the rest were incubated with 16 nM PMA in RPMI with 5% FBS at 37°C for 6 to 8 h. Nuclear extracts were subsequently prepared from these cells as well. The lymphocytes were layered onto a discontinuous Percoll gradient and centrifuged at 1,750 × *g* for 10 min to separate B and T lymphocytes (31). The T cells were aspirated and washed three times with RPMI containing 5% FBS and nuclear extracts were prepared (14). The sense and antisense strands for the oligonucleotide probe used in the electrophoretic mobility shift assays (EMSAs) were synthesized with an Applied Biosystems 300B synthesizer. The sequence corresponded to positions −111 to −137 of the HIV-2 enhancer (Fig. 1A). The single-stranded oligonucleotides were purified on a 15% polyacrylamide gel, and each strand was labeled with polynucleotide kinase in the presence of [γ-^{32}P]ATP. Equimolar amounts of each strand were annealed by incubation at 80°C followed by slow cooling in 500 mM NaCl. The double-stranded probe was then purified with a Sephadex G-50 column. The peri-κB(C) oligonucleotide used as a competitor consisted of the sequence corresponding to positions −111 to −135, the κB competitor oligonucleotide consisted of the sequence corresponding to positions −93 to −114, the ΔκB competitor oligonucleotide corresponded to positions −93 to −114 with positions −105 to −108 mutated (Fig. 1A), the PuB1 competitor sequence corresponded to positions −151 to −182, and the pets competitor sequence corresponded to positions −131 to −162 with the PuB2 site mutated at positions −137 to −142 (Fig. 1A). These oligonucleotides were synthesized with an Applied Biosystems 300B synthesizer and were annealed in an identical manner to the peri-κB probe described above. The binding reaction for the EMSAs (8, 30, 49, 66) contained 10 μg of nuclear extract, 500 ng of poly(dI-dC), and 15,000 cpm of radiolabeled oligonucleotide probe in 10 mM Tris (pH 7.5), 0.1 M NaCl, and 4% glycerol in a final reaction volume of 20 μl. Following incubation at room temperature for 15 min, DNA-protein complexes were run on a 4% polyacrylamide gel at room temperature in TGE buffer (0.05 M Tris, 0.5 M glycine, 0.5 mM EDTA) at 100 V for 2.5 h.

RESULTS

The peri-κB site is recognized by a nuclear factor in T cells and monocytes. As noted above, the sequence immediately upstream of the κB site in the HIV-2 enhancer, the peri-κB site, is conserved among isolates of HIV-2 and SIV (Fig. 1B). DNase footprint analysis was performed, initially with unstimulated HeLa cells, and revealed two footprints: one which included the κB site as well as 5 bp upstream and 4 bp downstream; and a second footprint farther upstream of the κB site, located between positions −123 and −126 (Fig. 2A). DNase footprint analysis was also performed with PMA-stimulated U937 nuclear extracts and both sense-labeled (Fig. 2B) and antisense-labeled (Fig. 2C) probes. As with the HeLa cell nuclear extracts, experiments employing U937 nuclear extracts showed a footprint upstream of the peri-κB site; with the sense-labeled probe, a footprint between positions −117 and −124 was seen, and with the antisense-labeled probe, a protected region was noted between positions −119 and −126. DNase protection assays were also performed with unstimulated Jurkat T-cell nuclear extracts, and these experiments revealed a larger protected sequence which encompassed a region between positions −127, 17 bp upstream of the κB site, and −110, which is the endpoint of the probe and the start of the κB site (data not shown). This large footprint was used to define the boundaries of the peri-κB site. From these data, then, we conclude that the peri-κB site is not only a conserved sequence, but it is bound by one or more nuclear factors found in monocytic cells, unstimulated T cells, and HeLa cells.

Mutation of the peri-κB site disrupts HIV-2 enhancer function in monocytes but not in T cells. To determine the ability of the peri-κB site to mediate HIV-2 enhancer activation, we introduced site-specific mutations into the peri-κB site and the κB site of an HIV-2/CAT construct (Fig. 1A). The sites were mutated either singly or in combination. The plasmid desig-

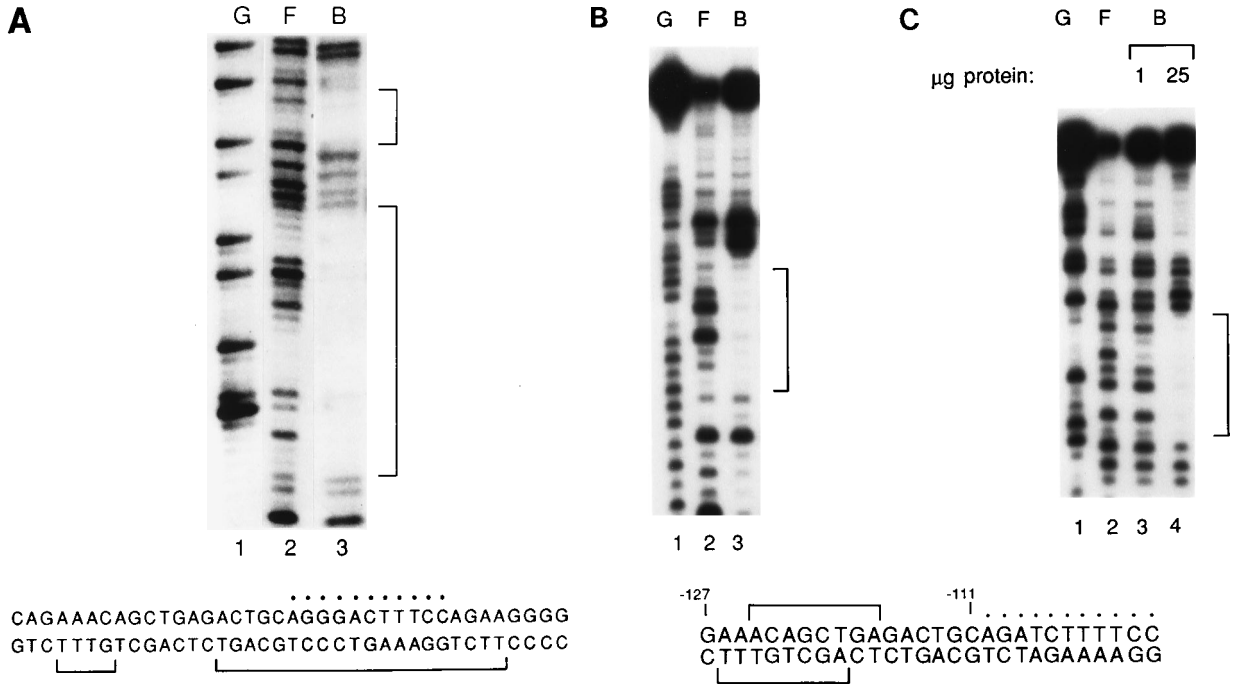

FIG. 2. DNase protection assays demonstrate a footprint immediately upstream of the κB site in the HIV-2 enhancer. DNase protection was performed as described previously (15, 22, 49). The DNase protection assays were performed with 150 μg of unstimulated HeLa cell nuclear extract and an antisense strand probe (A), 25 μg of U937 nuclear extract and a sense strand probe (B), and 1 or 25 μg of U937 nuclear extract and an antisense strand probe (C). The sequences of the peri-κB and κB elements are shown below the gels, with dots indicating the bases which constitute the κB site and brackets indicating the protected bases. The probe used for Fig. 2B and C contained mutated κB and PuB2 sites. G, G ladder; F, free probe; B, bound probe.

nated Δperi-κB(A) contains a site-directed mutation at the 5′ end of the peri-κB site, and the plasmid designated Δperi-κB(B) contains a mutation near the 3′ end of the peri-κB site. The peri-κB $DEL_{(-111/-127)}$ contains a site-directed deletion of the 17 bp constituting the peri-κB site. The plasmid Δperi-κB(A)+ΔκB contains both the peri-κB(A) and the κB mutations shown in Fig. 1A. These plasmids were transfected into the Jurkat and CEM T-cell lines and several monocytic cell lines, including the HL-60 immature cell line, the U937 promonocytic cell line, and the THP-1 mature monocytic cell line. The data derived from these experiments are shown in Fig. 3.

In Jurkat cell transfections, mutation of the κB site produced a marked reduction in the response of the HIV-2 enhancer to PMA. However, neither deletion of the peri-κB site nor mutations within the site had any effect on the responsiveness of the enhancer, as stimulation with PMA consistently led to a >20-fold activation of the mutant enhancer constructs, similar to levels seen with the wild-type enhancer (Fig. 3A). Furthermore, mutation of the peri-κB site in combination with the κB site produced no significant effect beyond that seen following mutation of κB alone. Similar results were seen in transfection experiments using CEM cells, another human leukemic T-cell line. As with Jurkat cells, mutation of the κB site of the HIV-2/CAT construct in CEM cells resulted in a dramatic decrease in the enhancer response to PMA, whereas mutation or deletion of the peri-κB site did not significantly affect inducible enhancer function (data not shown).

In HL-60 cells, the wild-type HIV-2/CAT construct was markedly activated (43-fold) by the addition of PMA (Fig. 3B). However, in contrast to the results seen in the Jurkat and CEM transfections, experiments employing either the Δperi-κB(A) or Δperi-κB(B) mutant construct demonstrated significantly reduced inducible enhancer function in the HL-60 cells, an effect which was further enhanced by mutation of κB in combination with the peri-κB(A) mutation (threefold activation of the Δperi-κB(A) mutant compared with less than onefold activation using the double mutant). Deletion of the peri-κB site also resulted in significantly reduced enhancer function. Results similar to these were seen in transfection experiments using the mature monocytic cell line THP-1, with mutations in the peri-κB site leading to a marked reduction in the level of PMA-induced enhancer function (data not shown). In the U937 monocytic cell line, transfections using mutant Δperi-κB(A) or Δperi-κB(B) alone had no effect on enhancer function (Fig. 3C), but when the plasmid containing both Δperi-κB(A) and a mutated κB site was employed, activation declined from 56-fold with the wild-type enhancer to only 2.5-fold. This decline represents a pronounced reduction in the ability of PMA to activate the HIV-2 enhancer beyond that seen with the κB site mutated alone: the HIV-2 enhancer was activated 11.5-fold by PMA (4.8% acetylation) in experiments using the single κB site mutant compared with an activation of only 2.5-fold (2.4% acetylation) with both κB and peri-κB sites mutated. These studies indicate that the peri-κB site is important for the function of the HIV-2 enhancer in monocytes but not in T cells.

The peri-κB site binds a specific nuclear factor(s) which is different in monocytes and T cells. In order to further characterize protein binding to the peri-κB site, EMSAs were performed with a radiolabeled peri-κB site probe and nuclear extracts from U937 and HL-60 cells, unstimulated or incubated with PMA (Fig. 4). Binding characteristics were then compared with those found in EMSAs which employed Jurkat T-cell extracts. A specific complex was observed in extracts from both unstimulated and PMA-induced U937 cells (Fig.

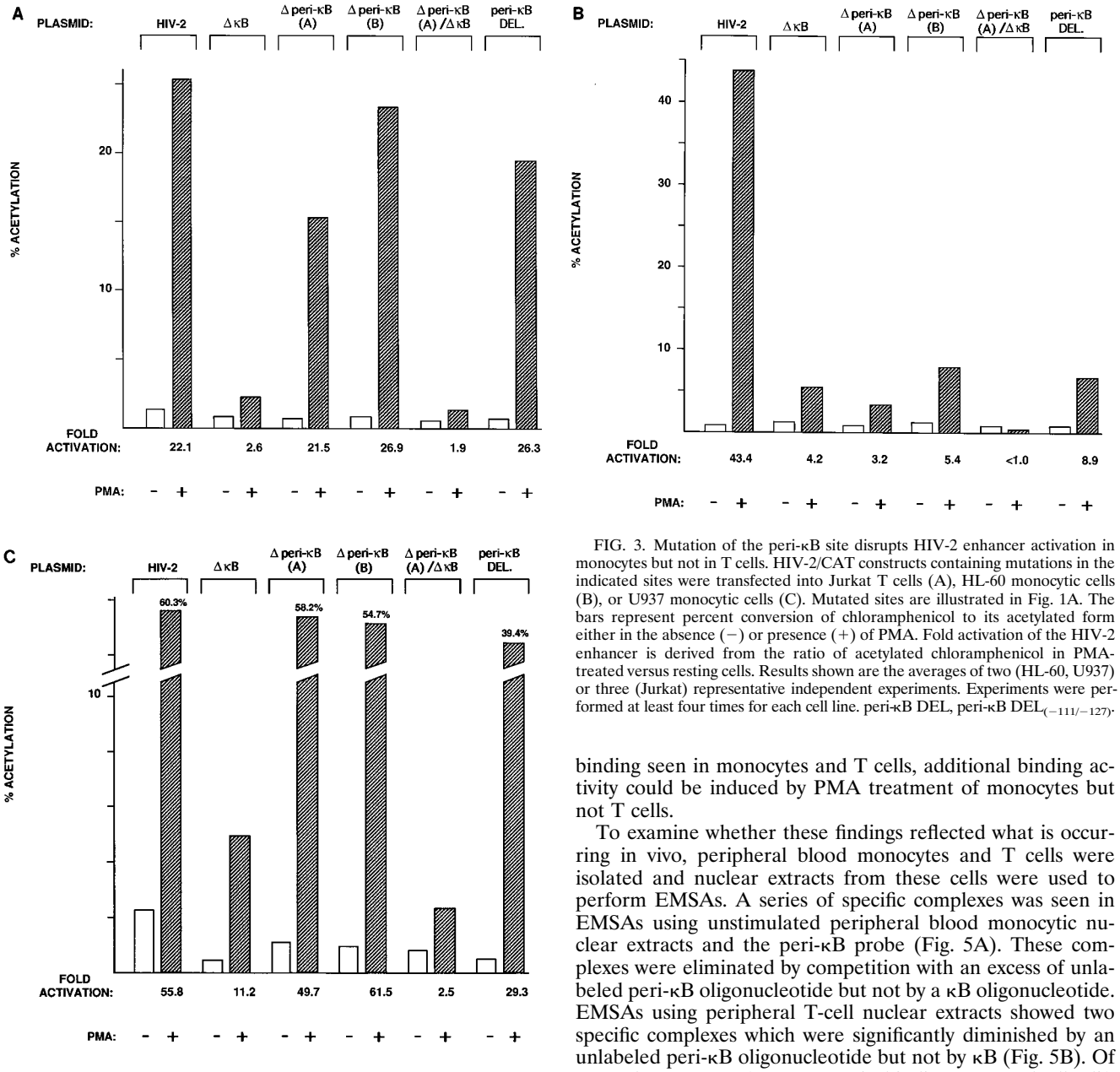

FIG. 3. Mutation of the peri-κB site disrupts HIV-2 enhancer activation in monocytes but not in T cells. HIV-2/CAT constructs containing mutations in the indicated sites were transfected into Jurkat T cells (A), HL-60 monocytic cells (B), or U937 monocytic cells (C). Mutated sites are illustrated in Fig. 1A. The bars represent percent conversion of chloramphenicol to its acetylated form either in the absence (−) or presence (+) of PMA. Fold activation of the HIV-2 enhancer is derived from the ratio of acetylated chloramphenicol in PMA-treated versus resting cells. Results shown are the averages of two (HL-60, U937) or three (Jurkat) representative independent experiments. Experiments were performed at least four times for each cell line. peri-κB DEL, peri-κB $DEL_{(-111/-127)}$.

4A, lanes 1 and 2). This complex was significantly diminished by competition with an excess of unlabeled peri-κB oligonucleotide (lane 3) and to a lesser degree by the slightly shorter peri-κB(C) oligonucleotide (lane 4) but not by the κB site, a mutant κB site, or the PuB1 site of the HIV-2 enhancer (lanes 5 to 7). With HL-60 nuclear extracts, there was again a specific complex seen which was almost completely eliminated by competition with an unlabeled peri-κB site oligonucleotide (Fig. 4B, lanes 1 to 3). This complex was not significantly diminished by unrelated competitor oligonucleotides (lanes 5 to 7). Experiments employing Jurkat nuclear extracts showed a specific nuclear factor–peri-κB complex as well (Fig. 4C). In addition, in many (although not all) of the EMSA experiments using U937 or HL-60 nuclear extracts but not in any experiments using Jurkat extracts, the complex appeared much more prominent following PMA treatment of the extracts (Fig. 4A and B, lanes 2). This result suggests that in addition to the constitutive binding seen in monocytes and T cells, additional binding activity could be induced by PMA treatment of monocytes but not T cells.

To examine whether these findings reflected what is occurring in vivo, peripheral blood monocytes and T cells were isolated and nuclear extracts from these cells were used to perform EMSAs. A series of specific complexes was seen in EMSAs using unstimulated peripheral blood monocytic nuclear extracts and the peri-κB probe (Fig. 5A). These complexes were eliminated by competition with an excess of unlabeled peri-κB oligonucleotide but not by a κB oligonucleotide. EMSAs using peripheral T-cell nuclear extracts showed two specific complexes which were significantly diminished by an unlabeled peri-κB oligonucleotide but not by κB (Fig. 5B). Of note, the pattern of DNA-protein binding was markedly different between the two cell types, suggesting the possibility of binding by different cell-specific proteins or by a protein(s) which is differentially modified in the two cell types, thus altering the binding pattern. In addition, when peripheral blood monocytes were activated with PMA and nuclear extracts from these cells used in EMSAs, the pattern of DNA-protein binding was dramatically different from that seen in extracts from resting monocytes (Fig. 5C), consistent with the EMSA data from monocytic cell lines, suggesting that both constitutive and inducible peri-κB binding activity is found in monocytes.

DISCUSSION

We have previously demonstrated that regulation of the HIV-2 enhancer in both T cells and monocytes is dependent upon conserved sequences in the LTR including κB, PuB1, PuB2, and the pets site (35, 49, 51). We have now shown that

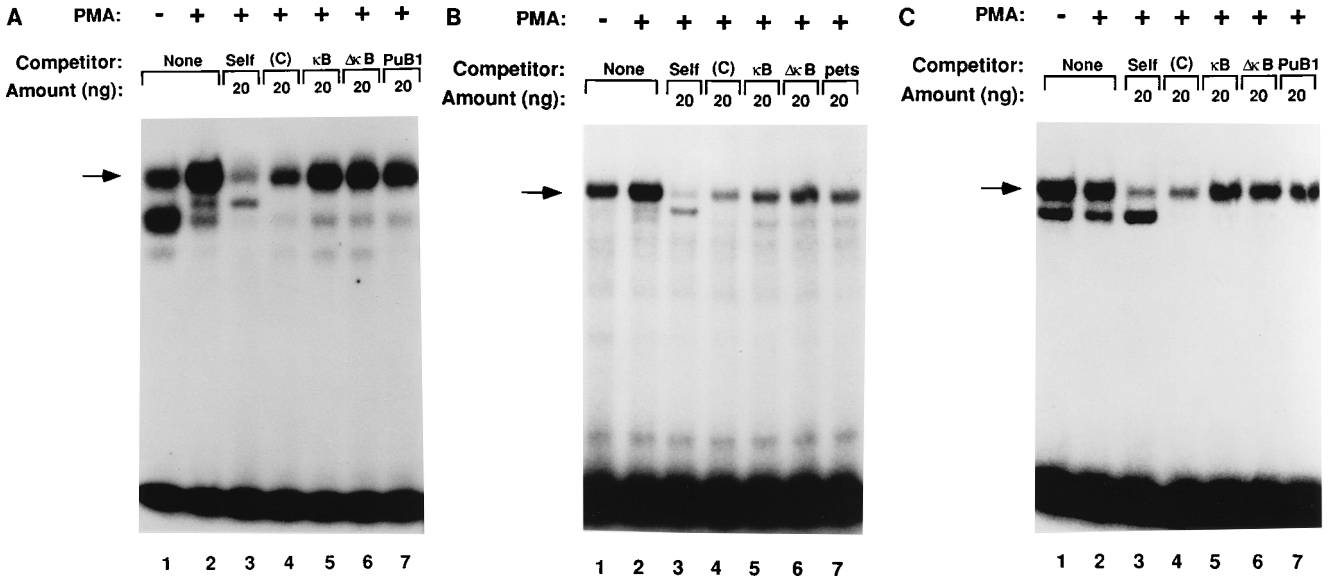

FIG. 4. A nuclear factor(s) binds to the isolated peri-κB site in cell lines. EMSAs were performed with a peri-κB oligonucleotide probe (−111 to −137) and nuclear extracts from either U937 (A), HL-60 (B), or Jurkat (C) cells prepared as described previously (14), which were unstimulated (−) or activated with PMA (+). Extracts were incubated with the peri-κB probe alone (lanes 1 and 2) or in the presence of 20 ng of unlabeled competitor oligonucleotide (lanes 3 to 7). The competitor oligonucleotides are sequences from the HIV-2 enhancer and include the unlabeled peri-κB site, a truncated peri-κB site (−111 to −135) indicated as (C) in the figure, a κB oligonucleotide (−93 to −114), a mutated κB sequence (ΔκB) (Fig. 1A), and either a PuB1 site (−151 to −182) in panels A and C or a sequence containing a pets site and a mutated PuB2 site (Fig. 1A) (−131 to −162) in panel B. The arrow in each figure indicates the specific DNA-peri-κB factor complex.

in addition to these *cis*-acting elements there is another functional site, the peri-κB site, which mediates HIV-2 enhancer activation in monocytic cells but not in T cells. Mutation of the peri-κB site alone results in a marked reduction in inducible enhancer function in HL-60 and THP-1 monocytic cells, and mutation of the site in combination with a mutated κB site causes significant disruption of enhancer activity in U937 monocytic cells. The observation that a single mutation in the peri-κB site does not significantly reduce HIV-2 enhancer induction in U937 cells is consistent with previous findings; we have shown that disabling HIV-2 enhancer function in U937 cells appears to require mutation of more than one enhancer element (35).

Through DNase protection analysis and EMSAs, we have further demonstrated that the peri-κB site is bound specifically by one or more nuclear factors (the peri-κB factor) found in monocytes and T cells as well as HeLa cells. EMSAs using peripheral blood monocyte nuclear extracts show several specific complexes. Whether these complexes represent binding of multimeric forms or degradation products of a single protein or binding of distinct proteins to the peri-κB site is unknown and will require further investigation. Compatible with the functional data, the binding pattern seen with T-cell nuclear extracts is different from that seen with monocytic nuclear extracts, and while binding activity is seen in resting monocytic cells, greater activity as well as a change in the binding pattern is seen following stimulation of peripheral blood monocytes with PMA.

Additional studies will be needed to determine the identity of the peri-κB factor. With the exception of a short motif in the antisense strand (CTTTG), the peri-κB site bears little similarity to the region upstream of κB in the HIV-1 enhancer which is recognized by hLEF/TCF-1α (5, 70). Furthermore, hLEF/TCF-1α appears to be specific to lymphoid cells, and the factor recognizing the peri-κB site in the HIV-2 enhancer is present in monocytes and HeLa cells in addition to T cells. A search of the Transcription Factor Database (27) for similarities between the sequence of the peri-κB site and sequences of known transcription factor binding sites revealed some similarity to two known binding sites. One of these sites is located in the simian virus 40 enhancer and is known as the GT-IIB motif (ACAGCTG; 72), and the other is present in a number of cellular enhancers and binds helix-loop-helix proteins, and is known as the E2-box sequence (RCAGNTG; 54). It is not yet known whether the factors which bind these elements are similar to the peri-κB factor.

A potential interaction of the peri-κB factor with NF-κB in mediating transcriptional activation is suggested by several observations. The two enhancer sites lie in close proximity to one another in the HIV-2 LTR, and DNase footprint analysis in HeLa cells shows a footprint overlapping both the κB and peri-κB sites. In addition, the synergistic decline in inducible enhancer function seen when both the κB and peri-κB sites are mutated in U937 cells gives weight to the notion that the peri-κB factor and NF-κB may interact functionally. Of note, subunits of NF-κB do not bind directly to the peri-κB site in EMSAs (7).

We have demonstrated in EMSAs that DNA binding of the peri-κB factor is partially inducible; there is binding in unstimulated monocytes and T cells, but enhanced binding is usually seen in EMSAs using PMA-treated HL-60 and U937 nuclear extracts. Enhanced binding and an alteration in the binding pattern are consistently seen in EMSAs using nuclear extracts from PMA-treated peripheral blood monocytes. These results suggest that cellular activation in monocytes causes a posttranslational modification of the peri-κB factor and/or an interaction of the peri-κB factor with additional cellular proteins leading to enhanced binding and activation of transcription. These findings would not be incompatible with a posttranslational modification involving, for example, phosphorylation of the peri-κB factor. Alternatively or additionally, an interaction between the peri-κB factor and another cellular factor, such as NF-κB, might mediate transcriptional activation.

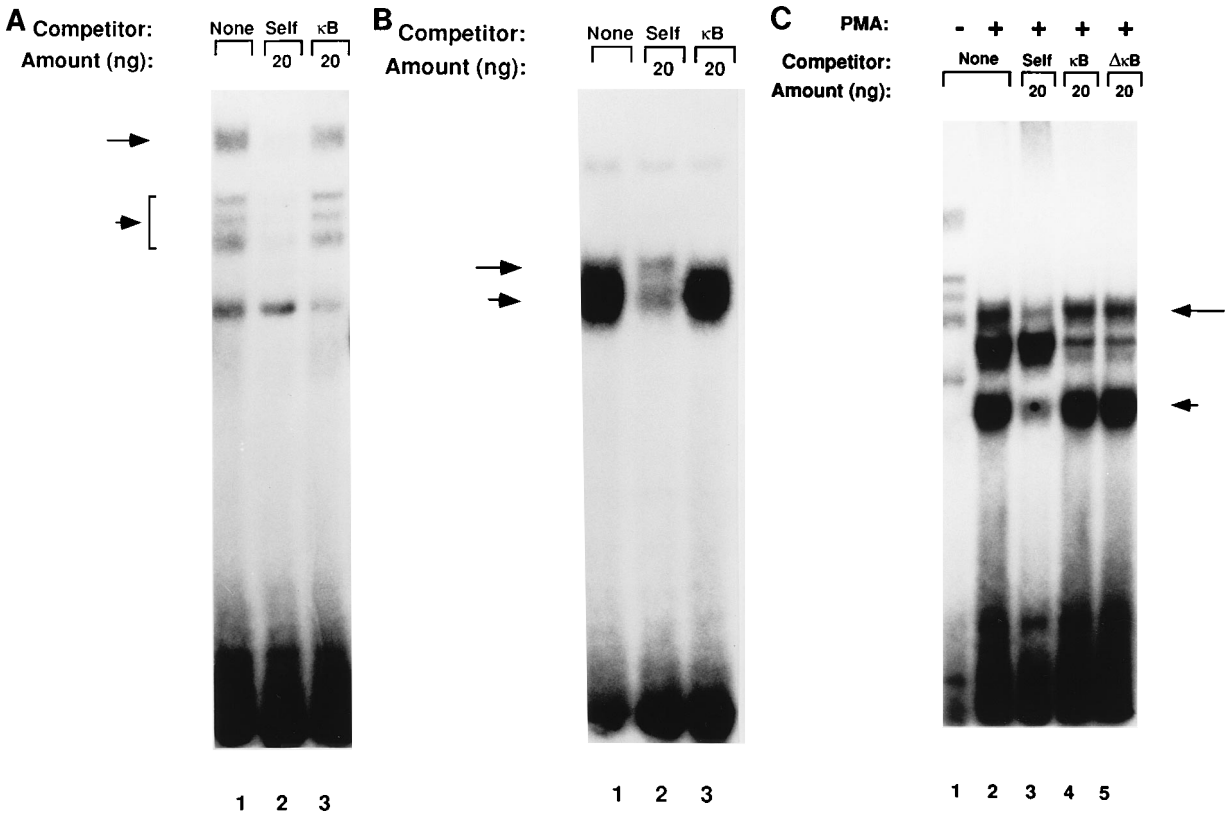

FIG. 5. Specific nuclear factors bind the peri-κB site in peripheral blood monocytes and T cells. EMSAs were performed as described in the legend to Fig. 4, using the same probe and nuclear extracts from unstimulated peripheral blood monocytes (A) or peripheral blood T cells (B). Extracts were incubated with the peri-κB probe alone (lanes 1 in panels A and B) or in the presence of 20 ng of unlabeled competitor oligonucleotide (lanes 2 and 3 in panels A and B). The competitor oligonucleotides were an unlabeled peri-κB site and a κB oligonucleotide. (C) Nuclear extracts from either unstimulated peripheral monocytes (lane 1) or monocytes stimulated with PMA (lanes 2 to 5) were incubated with the peri-κB probe alone (lanes 1 and 2) or in the presence of 20 ng of unlabeled peri-κB oligonucleotide (lane 3), κB oligonucleotide (lane 4), or a mutated κB oligonucleotide (lane 5). The arrows in each figure indicate the specific DNA–peri-κB factor complexes from uninduced (A and B) or induced (lanes 2 to 5 in panel C) cell extracts.

The monocyte-specific function of the peri-κB factor is a significant and unusual feature for an HIV transcriptional enhancer. In fact, such an enhancer element has not been described previously for HIV-2 nor clearly shown for HIV-1. Nakanishi et al. have described a *cis*-acting element in the HIV-1 LTR, known as URE (which spans the site recognized by hLEF/TCF-1α), which may have cell type-specific function (57). However, the deletion of this element has resulted in only a very modest decrease in transcription in MOLT-4 T cells and U937 cells (4.3- and 1.6-fold, respectively), and an increase in transcription in other T-cell lines (57). In addition, these studies employed large deletions of the HIV-1 LTR, a technique which modifies the spatial orientation of the enhancer and often removes multiple enhancer elements, making it difficult to interpret the contributions of individual elements. Several other studies have raised the possibility that another element of the HIV-1 enhancer, the USF binding site, also acts in a cell type-specific manner, but the data thus far appear contradictory (45, 46, 53, 73). Recently, a negative regulatory element of the HIV-2 LTR located upstream of the PuB and pets sites was described, with its effects apparently cell line specific (4). These experiments again used deletion rather than site-specific mutants, and deletion of this site resulted in increased basal but not inducible transcription of HIV-2/CAT constructs in CEM cells. These effects were not seen in U937 cells or HeLa cells, and they did not appear specific to T cells in general, as deletion of this site in Jurkat T cells had minimal effect on the basal level of transcription.

Given that the peri-κB factor is present at least in HeLa cells, T cells, and monocytes, its differential function among cell types cannot be explained on the basis of differential expression of a single DNA-binding protein. However, the means by which the peri-κB factor acts in a cell type-specific manner may be analogous to that described for the transcription factor Oct-1. The ubiquitous Oct-1 is a transcriptional activator which binds the octamer motif ATTTGCAT (for a review, see reference 40) and is involved in regulation of a histone H2B gene and the constitutive expression of the small nuclear RNA genes (19, 42). It has been demonstrated that the ability of Oct-1 to induce high-level, octamer-dependent transcription of immunoglobulin genes is dependent on the presence of a distinct B-cell-specific factor (47, 60), thus providing evidence that interactions of cell type-specific cofactors with ubiquitous transcription factors are a mechanism for tissue-specific function. The monocyte-specific activity of the peri-κB factor, then, could be explained by its interaction with a cofactor whose expression is restricted to monocytes.

The peri-κB site is an important element of the HIV-2 LTR. While it binds one or more specific nuclear factors from both T cells and monocytes, its functional activity is apparent in monocytic cells but not T cells. The monocyte-specific function of this element could be important for understanding the dif-

ferent roles monocytes and T cells play in HIV pathogenesis. Further studies, including purification and cloning of the peri-κB factor, are in progress and should allow a more definitive understanding of the function of the peri-κB factor in activating HIV-2 transcription, its interaction with other cellular factors, and perhaps its role in normal monocyte physiology.

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