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Differential Regulation of the Human Immunodeficiency Virus Type 2 Enhancer in Monocytes at Various Stages of Differentiation

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We have demonstrated that stimulation of the human immunodeficiency virus type 2 (HIV-2) enhancer in T cells is dependent upon at least four *cis*-acting elements, including two purine-rich binding sites, PuB1 and PuB2, which are capable of binding members of the *ets* family of proto-oncogenes, the pets (peri-*ets*) site, which lies just upstream of the PuB2 site, and a single κB site (D. M. Markovitz, M. Smith, J. M. Hilfinger, M. C. Hannibal, B. Petryniak, and G. J. Nabel, J. Virol. 66:5479–5484, 1992). In this study, we examined the regulation of the HIV-2 enhancer in cells of monocytic lineage. We found that in immature monocytic cell lines, the HIV-2 enhancer is markedly induced by phorbol esters and that all four *cis*-acting elements are required for activation. In mature monocytic cells, constitutive activity is high, with only modest stimulation following phorbol ester treatment. Mutation of any of the four *cis*-acting elements resulted in greatly reduced basal expression in mature monocytes. This is in contrast to HIV-1, in which developmentally controlled expression of the enhancer in monocytes is mediated largely through the κB sites alone [G. E. Griffin, K. Leung, T. M. Folks, S. Kunkel, and G. J. Nabel, Nature (London) 339:70–73, 1989]. Further, we demonstrated that although both Elf-1, an *ets* family member with significant similarity to the drosophila developmental regulatory protein E74, and Pu.1, a monocyte- and B-cell-specific member of the *ets* family, bind the purine-rich enhancer region, Elf-1 is the protein which binds predominantly in vivo. A nuclear factor(s) which binds the pets site, an element which has been described only in HIV-2, was detected in extracts of all of the monocytic cells tested. These findings indicate that the mechanism by which cellular factors regulate HIV-2 enhancer function in monocytic cells differs significantly from that of HIV-1 and may offer a partial explanation for the differences in the biological and clinical characteristics of the two viruses.

Human immunodeficiency virus type 2 (HIV-2) was first identified as a causative agent of AIDS in West Africa (8–10) and has since been described in many other parts of the world (1, 10, 11, 38, 49). HIV-2 shows only approximately 42% nucleic acid sequence similarity to HIV-1 and is most closely related to two simian immunodeficiency viruses, simian immunodeficiency virus from macaque and simian immunodeficiency virus from sooty mangabey (6, 18). While HIV-2 and HIV-1 both cause AIDS, the two viruses differ considerably in clinical and biological behavior. HIV-2-infected individuals appear to have a significantly longer asymptomatic period than do individuals infected with HIV-1 (2, 31, 38). In addition, HIV-2 displays inefficient perinatal transmission relative to HIV-1 (3, 30, 32).

We have recently demonstrated that the enhancer-promoter region of HIV-2, located in the long terminal repeat, functions differently from the HIV-1 enhancer in T cells (37, 39, 42). HIV-1 enhancer activation is mediated by NF-κB, an inducible nuclear factor which binds to two adjacent *cis*-acting κB sites in the enhancer (40, 44). In contrast, HIV-2, in addition to a single functional κB site, contains at least three other inducible *cis*-acting elements: two purine-rich binding sites, PuB1 and PuB2, which can bind members of the *ets* family of proto-oncogenes, and the pets (peri-*ets*) site, located between the two purine-rich binding sites (37, 39, 42). These sites, which are not present in HIV-1, are conserved across a wide range of HIV-2 isolates (43). We have shown that these sites are important in the 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 (26, 39, 42).

Purine-rich sites PuB1 and PuB2 contain the conserved AGGAA pentanucleotide motif characteristic of binding sites for the *ets* family of proto-oncogenes (4, 5, 52, 53, 55). The *ets* proteins are involved in the life cycle of several retroviruses, including human T-cell leukemia virus type I (21), Friend murine leukemia virus (4), and Moloney murine sarcoma virus (24). These proteins contain highly conserved basic and putative α-helical domains responsible for DNA binding (35, 55). Elf-1 (E74-like factor), an *ets* family member which is very similar to drosophila development factor E74, and *ets-1*, which binds to eukaryotic enhancers (29, 56), can both bind the PuB2 site of the HIV-2 enhancer in vitro (42). Elf-1 is also capable of binding to the PuB1 site (55). While Elf-1 is the predominant protein that binds to both of these sites in T cells in vivo (37), the identity of the factor that binds these sites in monocytic cells has not been identified. Recently, an *ets*-like factor, Pu.1, was shown to be a transcriptional activator that is expressed in macrophages and B cells (33). Altered expression of Pu.1 leads to a block in the cellular commitment to terminally differentiate, as in the case of Friend erythroleukemia (46).

Monocytic cells play a crucial role in the pathogenesis of HIV (16). These cells likely provide a reservoir for the virus in vivo and are involved in HIV-related encephalopathy (19, 20, 45). Griffin et al. have shown that HIV-1 enhancer activation during monocyte differentiation is regulated by

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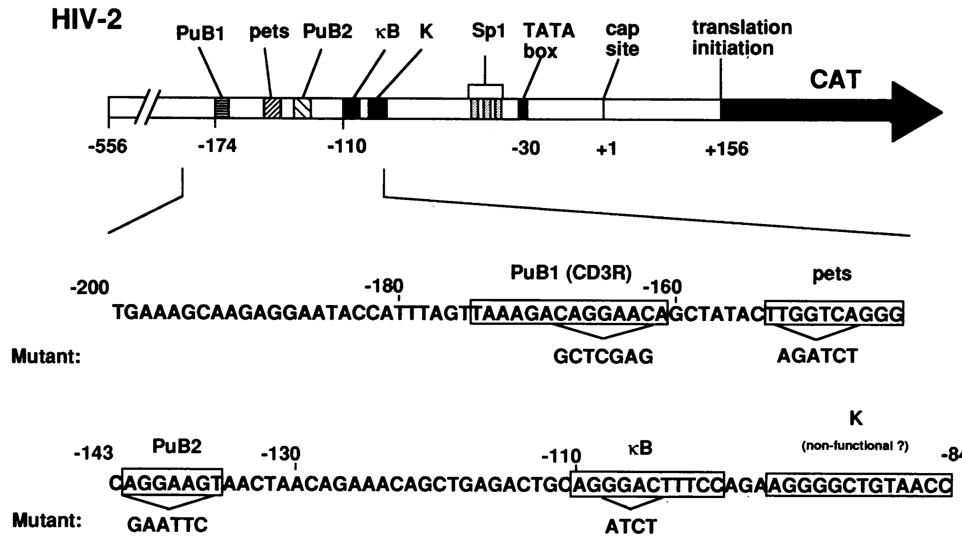

FIG. 1. Enhancer region of HIV-2_{rod}. The *cis*-acting elements within the enhancer region are identified. Altered sequences in the mutant plasmids, introduced by the gap-heteroduplex method of site-directed mutagenesis (15, 37, 39, 42), are identified below the wild-type sequence. The sequence of HIV-2_{rod} has been published previously (25).

NF-κB binding to the two κB sites (23). In immature monocytic cell lines, activation of the HIV-1 enhancer by PMA is mirrored by the induction of NF-κB. In mature monocytic cell lines, NF-κB is constitutively expressed and basal expression of the HIV-1 enhancer is very high and not inducible by phorbol myristate acetate (PMA). Because of the importance of monocytes to HIV pathology and the differences between the HIV-2 and HIV-1 enhancers, we examined the function of the HIV-2 enhancer in cells representing different stages of monocytic differentiation and in peripheral blood monocytes.

To examine the regulation of the HIV-2 enhancer in monocytic cells, site-directed HIV-2 enhancer–chloramphenicol acetyltransferase (CAT) mutant constructs (39, 42; Fig. 1) were first transfected by the DEAE-dextran method (48, 50) into two cell lines, HL-60 and KG-1, which represent an immature state of monocytic differentiation. At 20 h after transfection, PMA (16 nM) was added, and after an additional 20 h, cultures were assayed for CAT activity by standard procedures (22). As shown in Fig. 2A and B, basal activity of the enhancer was low and enhancer activity was markedly induced by PMA. Mutation of any one of the *cis*-acting elements, PuB1, pets, PuB2, or κB, was sufficient to completely eliminate enhancer induction. This is in contrast to the results seen upon transfection of the HIV-2 enhancer–CAT constructs into the mature monocytic cell line THP-1 (Fig. 2C). Basal activity in the THP-1 cells was high (note the change of scale on the *y* axis), while little PMA-mediated activation was seen. In this case, mutation of any one of the *cis*-acting elements reduced basal activity up to 10-fold (Fig. 2C) and mutation of two sites (PuB1 and either PuB2 or κB) greatly reduced basal expression. Similar results were obtained with the mature mouse monocytic cell lines P338D_1 and PU5-1.8 (data not shown). Therefore, both PuB sites, the pets site, and the κB site were required for inducible activation in immature monocytic cells and for basal expression in mature cells. In U937 cells (Fig. 2D), which represent an intermediate stage of development (Table 1), the HIV-2 enhancer was induced approximately 42-fold. While mutation of any one of the *cis*-acting elements

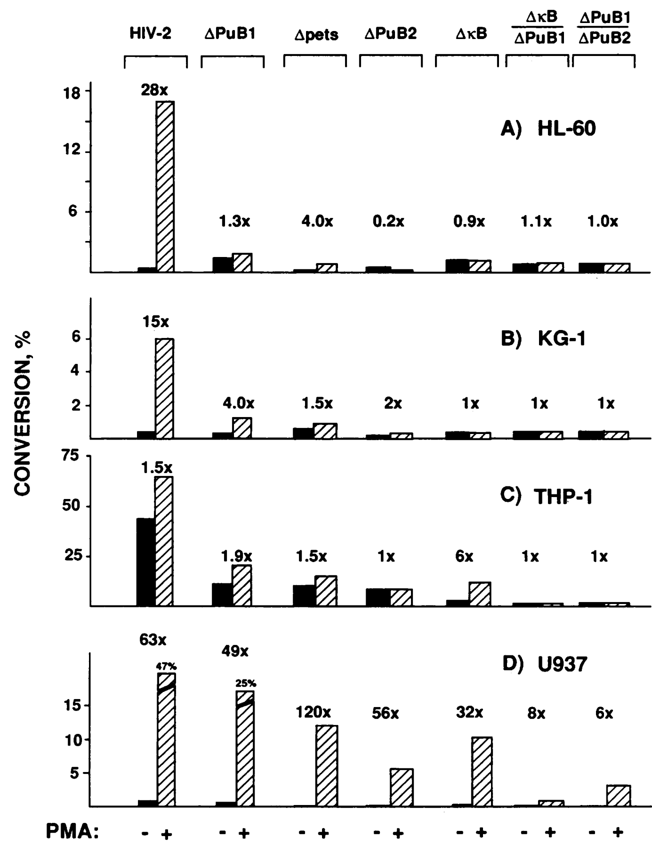

FIG. 2. Activation of the HIV-2 enhancer in transfected HL-60, KG-1, THP-1, and U937 monocytic cells. HIV-2 enhancer–CAT constructs containing mutations in the indicated sites were transfected into HL-60, KG-1, THP-1, and U937 cells. The bars represent percent conversion of chloramphenicol to its acetylated form measured in extracts of cells that had been incubated in the absence (black bars) or presence (hatched bars) of PMA. Fold activation is given above each pair of datum points. The data presented are representative of at least four separate experiments.

TABLE 1. Properties of monocytic cell lines[a]

Cell line	Morphology	Fc receptors	C3b receptors	Adherence	Phagocytosis (%)
HL-60	Myeloblast, promyelocyte	Few	Few	Little	1–2
KG-1	Myeloblast, promyelocyte	Few	Not done	Little	~3
U937	Monoblast	Few	Few	Little	1–2
THP-1	Monocyte	99%	69%	Little	13

[a] The data shown are from Koeffler et al. (34), Harris and Ralph (27), and Tsuchiya et al. (54).

resulted in only a slight decline in PMA-stimulated CAT activity, mutation of any of these elements reduced basal expression up to 10-fold (Fig. 2D). Mutation of two sites within the same construct (PuB1 and PuB2 or PuB1 and κB; Fig. 2D) greatly reduced inducible enhancer function. Therefore, regulation of the HIV-2 enhancer in U937 cells, which are moderately differentiated, demonstrates characteristics seen in both immature and mature monocytic cells.

The pets site is required for HIV-2 enhancer function in both immature and mature monocytic cells. To examine whether monocytic nuclear factors interact with the pets site, we employed electrophoretic mobility shift assays as previously described (37, 39, 42), except that the reaction mixture was 200 mM KCl (Fig. 3). Nuclear factor binding was seen in all monocytic extracts (Fig. 3, lanes 1, 4, 7, and 10). The binding was specific, since incubation with an excess of the unlabeled probe greatly reduced the observed band (Fig. 3, lanes 2, 5, 8, and 11), while a 500-fold excess of an unrelated oligomer, Sp1, failed to reduce factor binding (Fig. 3, lanes 3, 6, 9, and 12). Similar behavior was seen with other, unrelated competitors (data not shown). A similar pattern of pets binding was seen with nuclear extracts made from peripheral blood monocytes (Fig. 3, lanes 13 to 15). pets-specific binding was seen regardless of whether cells were preincubated with PMA.

We have shown above that the PuB1 and PuB2 sites are important to HIV-2 enhancer function in monocytic cells. Two *ets* family members appeared to be likely candidates for binding to these sites: Elf-1, which has been shown to bind to PuB1 and PuB2 in vitro and in vivo (37, 42), and Pu.1, the macrophage- and B-cell-specific *ets*-related transcription factor involved in erythroblast differentiation (33, 46, 47). Both Elf-1 and Pu.1 were detected by Northern RNA hybridization analysis in all of the monocytic cell lines tested, as well as in peripheral blood monocytes (data not shown). This expression was independent of PMA treatment.

Having previously shown that Elf-1 and *ets-1* bind to PuB2 in vitro (37, 42), we next wished to determine whether Pu.1 is capable of binding PuB2 in vitro. Full-length Pu.1, synthesized in vitro with the TNT T7 coupled reticulocyte lysate system (Promega), was incubated with the PuB2 probe (Fig. 4) in the presence of oligonucleotide competitors (lanes 3 to 6), Pu.1-specific antiserum (lane 7), or preimmune serum (lane 8) and used in electrophoretic mobility shift assays (37, 39, 42) and supershift assays (37, 42). The Pu.1-specific band (solid arrow) was eliminated upon incubation with Pu.1-

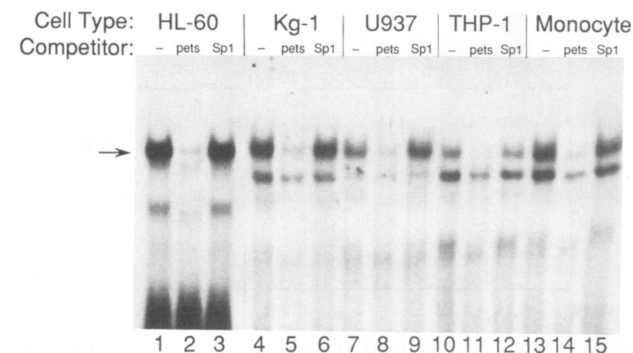

FIG. 3. pets site binding in HL-60, KG-1, U937, and THP-1 cells and peripheral blood monocytes. The pets site probe (−131 to −162, containing a mutated PuB2 site; Fig. 1) was incubated with 10 μg of nuclear extract, prepared by a modification of the method of Dignam et al. (13, 39–42) in the presence of 50 ng (500-fold molar excess) of a competitor, which in this case was either an unlabeled pets site oligonucleotide or the unrelated oligonucleotide Sp-1. The arrow marks the mobility of the specific band.

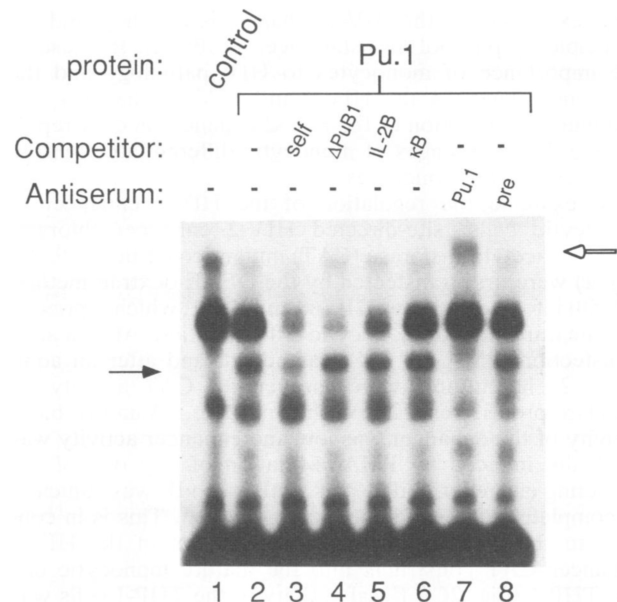

FIG. 4. Pu.1 binds to the PuB2 site in vitro. In vitro-translated Pu.1 (3 μl out of a total reaction volume of 25 μl) was mixed with water (lane 2); the unlabeled, double-stranded oligonucleotide competitor PuB2 (lane 3), ΔPuB1 (containing the sequences from −155 to −182 [Fig. 1], including the indicated mutated sequence [lane 4]), IL-2B (lane 5), or κB (lane 6); Pu.1-specific antiserum (lane 7); or preimmune serum (lane 8) prior to the binding reaction with the PuB2 probe (containing sequences from −155 to −182 [Fig. 1]) and separation on a 0.25× Tris-borate-EDTA gel. The solid arrow marks the Pu.1-PuB2 complex. The unfilled arrow marks the shifted Pu.1–PuB2–Pu.1-specific antibody complex. The control (lane 1) was reticulocyte lysate with no exogenously added DNA. Oligonucleotide competitors were used at a concentration of 50 ng/20 μl of the binding reaction mixture.

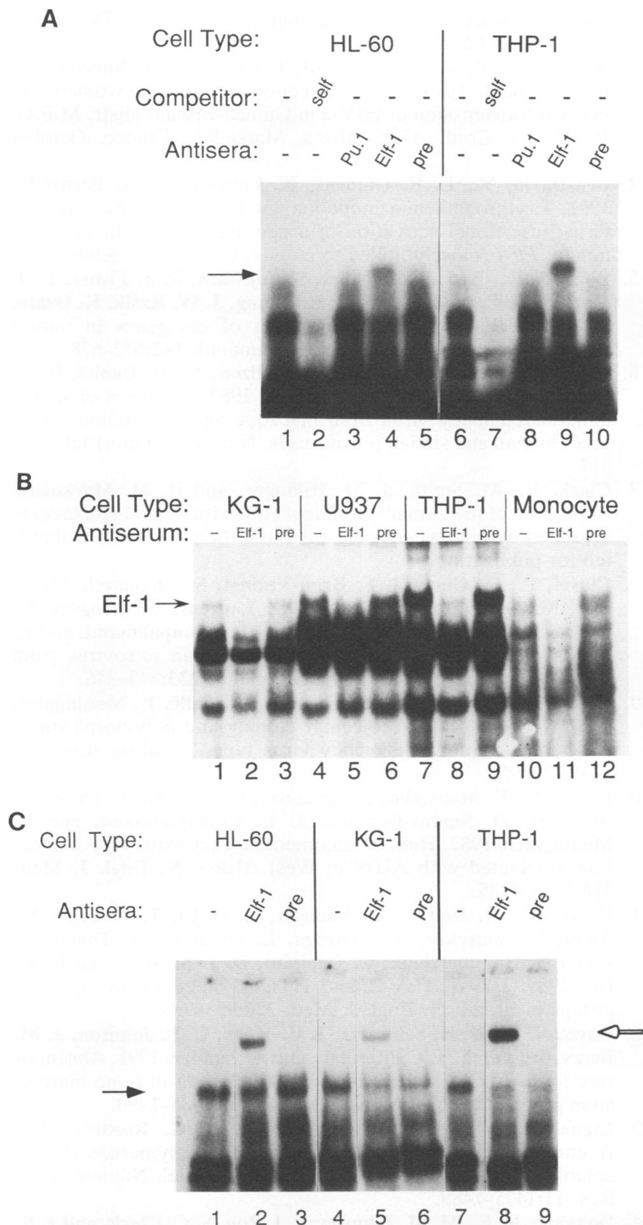

FIG. 5. Detection of Elf-1 binding to the PuB2 (A and B) and PuB1 (C) sites in nuclear extracts from monocytic cell lines and peripheral blood monocytes. (A) Nuclear extracts from HL-60 (lanes 1 to 5) or THP-1 (lanes 6 to 10) cells were mixed with water (lanes 1 and 6), 50 ng of unlabeled PuB2 oligonucleotide (lanes 2 and 7), Pu.1-specific antiserum (lanes 3 and 8), Elf-1-specific antiserum (lanes 4 and 9), or preimmune serum (lanes 5 and 10) prior to the binding reaction with the PuB2 probe and separation on a 0.25× Tris-borate-EDTA gel. The arrow marks the Elf-1–PuB2–Elf-1-specific antibody complex. (B) Nuclear extracts from each of the cell lines were incubated with either Elf-1 antiserum of preimmune serum prior to the binding reaction with the PuB2 probe and separation on a 1× Tris-glycine-EDTA gel. The arrow marks the Elf-1–PuB2 complex. (C) Nuclear extracts from HL-60 cells (lanes 1 to 3), KG-1 cells (lanes 4 to 6), or THP-1 cells (lanes 7 to 9) were mixed with water (lanes 1, 4, and 7), Elf-1-specific antiserum (lanes 2, 5, and 8), or preimmune serum (lanes 3, 6, and 9) prior to the binding reaction with the PuB1 probe and separation on a 0.25× Tris-borate-EDTA–polyacrylamide gel. The solid arrow marks the Elf-1–PuB1 complex. The unfilled arrow marks the shifted Elf-1–PuB1–Elf-1-specific antibody complex.

specific antiserum (lane 7) and replaced by the supershifted complex (open arrow). These findings show that the Pu.1 transcription factor can bind the PuB2 site.

To determine which *ets* family member binds to the PuB2 site in monocytic cells in vivo, electrophoretic mobility shift assays and supershift analysis were performed with nuclear extracts from monocytic cells and antisera directed against Elf-1 or Pu.1 (Fig. 5A). Specific binding to the PuB2 site was seen in the extracts of immature (HL-60) and mature (THP-1) monocytic cells (lanes 1, 2, 6, and 7). A supershifted protein-DNA complex was seen upon addition of Elf-1-specific antiserum (lanes 4 and 9) but not upon addition of Pu.1-specific (lanes 3 and 8) or preimmune sera (lanes 5 and 10). Further, as seen in Fig. 5B, supershift assays with nuclear extracts from KG-1, U937, and peripheral blood monocytes also showed Elf-1-specific binding. In this experiment, note that addition of antibody to Elf-1 resulted in disappearance rather than supershifting of the specific band (lanes 2, 5, 8, and 11). This was our experience when supershift assays were performed with Tris-glycine-EDTA buffer in place of 0.25× Tris-borate-EDTA buffer (Fig. 5A) during electrophoresis. As with anti-Pu.1 antibody, antiserum directed against *ets-1* failed to block or shift the specific band (data not shown). Therefore, Elf-1 binds to the PuB2 site in extracts of monocytic cells and is most likely the predominant factor that binds to this site in vivo. Elf-1 was also the predominant protein that bound to the PuB1 site (Fig. 5C). Elf-1-specific binding was seen in both immature monocytic cells, HL-60 (lanes 1 to 3) and KG-1 (lanes 4 to 6), and mature monocytic cells, THP-1 (lanes 7 to 9). No Pu.1-specific binding to PuB1 was detected in these cell lines (data not shown). Our data indicate that Elf-1 is likely the predominant protein factor that binds to the PuB1 and PuB2 sites in vivo and strongly suggest that, in monocytic cells, the Elf-1 transcription factor acts along with other cellular factors, such as NF-κB and the pets factor, to regulate the HIV-2 enhancer.

Like that of HIV-1, regulation of the HIV-2 enhancer-promoter varies significantly in monocytic cell lines representing different states of monocytic differentiation. In contrast to HIV-1 (23), in which the κB sites are the dominant regulatory elements, the PuB1, PuB2, and pets sites, along with the single κB site, respond to these differences in cellular maturation. In the immature cell lines HL-60 and KG-1, PMA-mediated activation is dependent upon all four *cis*-acting elements and a mutation in any one site is sufficient to inhibit activation completely. However, in the mature monocytic cell lines, the HIV-2 enhancer shows high levels of basal activity, with little PMA-mediated activation. A mutation in any one of the *cis*-acting elements markedly reduces basal activity. Enhancer activity in the U937 cell line, which represents an intermediate stage of monocyte development, shows characteristics of both immature and mature cell lines. This is in contrast to HIV-1, in which the enhancer functions in U937 cells just as it does in HL-60 cells, with low basal activity and the κB sites alone mediating enhancer induction (23). These differences between HIV-2 and HIV-1 most likely reflect the greater complexity of the HIV-2 enhancer. Similar to our previous findings with T cells (39, 42), regulation of the HIV-2 enhancer in monocytes is dependent upon at least four *cis*-acting elements which work in concert to control enhancer activity. However, in contrast to T cells, in which even in mature cells enhancer-driven expression is low unless induced, basal enhancer function is low in immature monocytic cells but high in mature cell lines. As hematopoietic stem cells can be

infected by HIV (14, 17), the mechanism by which monocytes might serve as a renewable reservoir of infection could involve infection of cells of monocytic lineage in the marrow with increased HIV production as the cells mature. This hypothesis is, however, controversial (12, 51).

Previous studies have shown that in vitro-synthesized Elf-1 and *ets-1* are both capable of binding the PuB2 site (37, 42). Our findings demonstrate that in nuclear extracts from monocytic cells or peripheral blood monocytes, Elf-1 is the predominant cellular protein which binds to PuB2 (Fig. 5A and B). While in vitro-synthesized Pu.1 was capable of binding the PuB2 site (Fig. 4), supershift analysis failed to show any evidence of *ets-1* or Pu.1 binding to the PuB2 site in vivo. In addition, we demonstrated that Elf-1 is also the predominant protein factor that binds to PuB1 (Fig. 5C). In view of the functional importance of the purine-rich elements and these binding studies, it is likely that Elf-1 plays an important role in HIV-2 enhancer regulation in monocytes.

Recent evidence indicates that *ets* transcription factors often recruit a second factor for transcriptional activation (46, 52, 53). We detected a specific nuclear factor that binds to the pets site, which is located proximal to the PuB2 site (Fig. 1), in both immature and mature monocytic cells. Both the PuB2 and pets sites are functionally significant, and therefore it is possible that the pets-specific factor and Elf-1 work in tandem to regulate HIV-2 enhancer function. The identity of the pets factor, which contributes to regulation of the HIV-2 enhancer in T cells and monocytes and to regulation of the human T-cell leukemia virus type I enhancer (7), is not known. While the pets site bears some resemblance to an AP-1 site and AP-1 and Elf-1 often cooperate (36), supershift assays indicate that *jun* family members are not part of the pets-binding complex (28). Binding of the pets factor, like that of Elf-1 (39, 42), is constitutive, and therefore posttranslation modification of these DNA-binding proteins or involvement of other inducible nuclear factors is likely required for transcriptional regulation. In this regard, our preliminary data suggest that different, or modified, factors can bind to the pets site, depending upon the monocytic cell type and the length of PMA treatment (28). It is also plausible that NF-κB, which is induced with PMA treatment in immature monocytic cells and is constitutively expressed in mature monocytic cells (23), interacts with the Elf-1 and pets-specific nuclear factors to bring about transcriptional activation of the HIV-2 enhancer. Regulation of the HIV-2 and HIV-1 enhancers by different combinations of cellular factors may offer a partial explanation for the dissimilar clinical courses seen in HIV-2- and HIV-1-infected individuals.

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