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Identification and characterization of an enhancer in the coding region of the genome of human immunodeficiency virus type 1

(retrovirus gene expression/phorbol 12-myristate 13-acetate/transcription factor AP-1/transcription factor NF-κB)

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ABSTRACT **Transcription of human immunodeficiency virus type 1 (HIV-1) is regulated by cis-acting DNA elements located in the viral long terminal repeats, by viral transregulatory proteins, and by cellular transcription factors acting in concert to modulate the degree of viral expression. We demonstrate that a DNA fragment corresponding to the central portion of the HIV-1 genome exhibits enhancer activity when cloned upstream of the thymidine kinase promoter of herpes simplex virus. This enhancer is inducible by phorbol 12-myristate 13-acetate in HeLa cells and is independent of its position and orientation with respect to the promoter. We have mapped the activity of the enhancer to two independent domains encompassing nucleotides 4079–4342 (end of the *pol* gene) and nucleotides 4781–6026 (*vif* gene and first coding exon of *tat*). This intragenic enhancer and its subdomains demonstrate cellular specificity because they are only active in specific cell lines. The presence of similar intragenic enhancer elements in other retroviruses suggests that they might be a conserved feature of this family of viruses.**

The expression of human immunodeficiency virus type 1 (HIV-1), the etiologic agent of AIDS (1, 2), is regulated at both the transcriptional and postranscriptional levels (3). Transcription control elements of HIV, as for other retroviruses (4), are located in the viral long terminal repeats (LTRs) (5, 6). Functional studies have defined several domains in the U3-R region of the 5′ LTR of HIV (5), and binding sites for several transcription factors have been identified (for review, see ref. 7). The combined action of these factors, in concert with the viral transregulatory proteins, TAT and possibly NEF (3), determines the degree of viral expression at the transcriptional level. The potential significance of transcriptional control elements in retroviral pathogenesis is illustrated by the murine leukemia virus system where elements located in the U3 region of the viral LTR have been shown to be determinants of tissue tropism, disease specificity, and leukemogenesis (8–10). In addition, retroviral sequences located outside of the LTR may also play a role in the regulation of retrovirus gene expression. Several DNA-binding sites for the glucocorticoid receptor have been identified by footprinting analysis, both in the LTR and in the transcribed region of the mouse mammary tumor virus genome (11). In addition, DNA sequences derived from the *gag* gene of avian retroviruses were found to exhibit enhancer activity (12). An intragenic enhancer element has also been identified in the transcribed ε region of the transposon Ty*1* from *Saccharomyces cerevisiae* (13). These results suggest that intragenic enhancers may be a conserved feature of retroviruses and related elements and prompted us to test whether the genome of HIV might also contain intragenic regulatory elements.

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This report describes the identification and characterization of a fragment from the coding region of HIV presenting the properties of a eukaryotic transcriptional enhancer.

MATERIAL AND METHODS

Cells and Plasmids. HeLa, CV1, HEC1-B, and LS174T cells were obtained from the American Type Culture Collection. NIH 3T3 cells were a gift from Jean Rommelaere (Université Libre de Bruxelles, Brussels). All cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum, 2 mM glutamine, and kanamycin at 50 μg/ml except for the NIH 3T3 cell line, for which 10% bovine calf serum was used instead of fetal calf serum. All cells were grown at 37°C in a 5% CO_2/95% air atmosphere. The cloned provirus HXB-2 (14) was a gift from R. C. Gallo (National Cancer Institute). Plasmids pBLCAT2 and pBLCAT3 were a gift from Gunther Schütz (Heidelberg) (15).

Plasmid Construction. Recombinant DNA techniques were performed according to Maniatis *et al.* (16) and Ausubel *et al.* (17). The complete genome of the cloned provirus HXB-2 was digested with *Hin*dIII, and the six fragments from the provirus resulting from this digestion were cloned in both orientations in the unique *Hin*dIII site of pBLCAT2, upstream of the thymidine kinase (TK) promoter. By definition, a fragment is cloned in the sense orientation (SO) in the vector when it is cloned in the natural orientation (5′ → 3′) with respect to the TK–chloramphenicol acetyltransferase (CAT) transcriptional unit and in the antisense orientation (ASO) when cloned in the inverse orientation (3′ → 5′). Plasmids containing the HIV fragments cloned in the ASO were called pULB5050 [containing nucleotides (nt) 1–531 of provirus HXB-2, where nt + 1 is the start of U3 in 5′ LTR]; pULB5052 containing nt 531–1084; pULB5054 containing nt 1084–1711; pULB5056 containing nt 1711–6026; pULB5067 containing nt 6026–8140; pULB5073 containing nt 8140–9615 (see Fig. 1).

A second group of constructions was generated by cutting the purified *Hin*dIII–*Hin*dIII fragment of pULB5056 (nt 1711–6026 of HXB-2) with a variety of enzymes generating blunt ends (*Pvu* II, *Dpn* I, and *Dra* I), adding *Hin*dIII linkers, and cloning the resulting fragments in both orientations in the unique *Hin*dIII site of pBLCAT2. The precise coordinates of these fragments with respect to the HXB-2 provirus are as follows: pULB5077(ASO), nt 1711–3298; pULB5074(ASO) and pULB5073(SO), nt 3298–4342; pULB5079(ASO) and pULB5078(SO), nt 4342–6026; pULB5101 (ASO), nt 3298–4079; pULB5103(ASO) and pULB5102(SO), nt 4079–4342;

Abbreviations: CAT, chloramphenicol acetyltransferase; HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; nt, nucleotide(s); 4αPDD, 4α-phorbol 12,13-didecanoate; SO, sense orientation; ASO, antisense orientation; TK, thymidine kinase; PMA, phorbol 12-myristate 13-acetate.

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pULB5107(ASO), nt 4342–4781; pULB5105(ASO) and pULB5104(SO), nt 4781–6026 (see Fig. 3). In addition, the *Hin*dIII–*Hin*dIII fragment from pULB5103 (nt 4079–4342) was cloned downstream of the CAT gene and poly(A) site in both orientations in the unique *Sma* I site of pBLCAT2 after filling in the recessed 3′ termini with the Klenow fragment of *Escherichia coli* DNA polymerase I. These plasmids are called pULB5167(ASO) and pULB5166(SO). A similar construction was performed with the *Hin*dIII–*Hin*dIII fragment from pULB5105 and called pULB5241(ASO) and pULB5240(SO). The *Hin*dIII–*Hin*dIII fragment containing nt 1711–6026 was also cloned in both orientations in the promoterless plasmid pBLCAT3 (15) [pULB5237(ASO) and pULB5238(SO)]. Plasmid pRSVCAT was constructed by cloning a 624-base-pair (bp) fragment containing the LTR from Rous sarcoma virus (nt 8907–9531) (18) in the *Sma* I site of pUC18, excising a *Xho* I–*Xba* I fragment by using sites from the polylinker of pUC18 and entering this fragment upstream of the CAT gene in the *Xho* I and *Xba* I sites of pBLCAT3. All plasmids used for transfection were purified twice by cesium chloride density-gradient centrifugation (16).

Transfection of DNA into Cells. Twenty hours before transfection, cells were seeded at a density of 6.4×10^3 cells per cm^2. The DNA to be tested (5 μg of pULB5056 or an equimolar amount of all other plasmids) was coprecipitated with 10 μg of pBluescript (Stratagene) (19), added to the cell culture medium, and incubated with the cells for 8–10 hr. The medium was replaced with fresh medium containing only 0.5% fetal calf serum and 20 hr later phorbol 12-myristate 13-acetate (PMA) (10 nM, except where indicated) in dimethyl sulfoxide (0.1% final concentration) or dimethyl sulfoxide alone (0.1%) as control was added to the medium. Cells were harvested 12–14 hr later, and cell extracts were prepared by freeze–thawing (3×) in 0.25 M Tris·HCl, pH 7.8, followed by heating at 65°C for 7 min and centrifugation at 14,000 × *g* for 10 min. The protein concentration of the extracts was measured by using a kit (Sopachem, Sopar-Biochem, Brussels) based on the formation of a pyrogallol red–molybdate complex (20). The CAT activity of the extracts was measured as described (19) by using 5 or 10 μg of protein for a 30-min reaction. Plasmids were tested in three independent transfections and, in most cases, with different plasmid preparations. Variation for a given plasmid between different experiments was <20% in most cases.

RESULTS

A DNA Fragment from the Coding Region of the HIV Genome Increases TK-Directed Gene Expression in a PMA-Dependent Fashion. To determine whether the genome of HIV contains an intragenic regulatory element, we first tested the activity of six nonoverlapping *Hin*dIII restriction fragments from the molecular clone of HIV-1, HXB-2 (14), which were cloned in the unique *Hin*dIII site of the vector pBLCAT2 (15). This vector contains the TK promoter, derived from herpes simplex virus, driving the expression of the bacterial CAT gene. The vector also contains the small tumor antigen intron and polyadenylylation signals, which are derived from simian virus 40 (15). Presence of the weakly active TK promoter allows analysis of the effects of putative regulatory elements on a heterologous promoter and has been used for this purpose (21, 22). Because of the demonstrated dependence of HIV expression on the degree of activation of its host cell (23–26), the potential enhancer activity of these fragments was examined with and without PMA after transfection in HeLa cells. Without PMA, no plasmids tested presented any significant activity when compared with pBLCAT2 (Fig. 1). However, with PMA, transfection of pULB5056 markedly increased CAT activity from a conver-

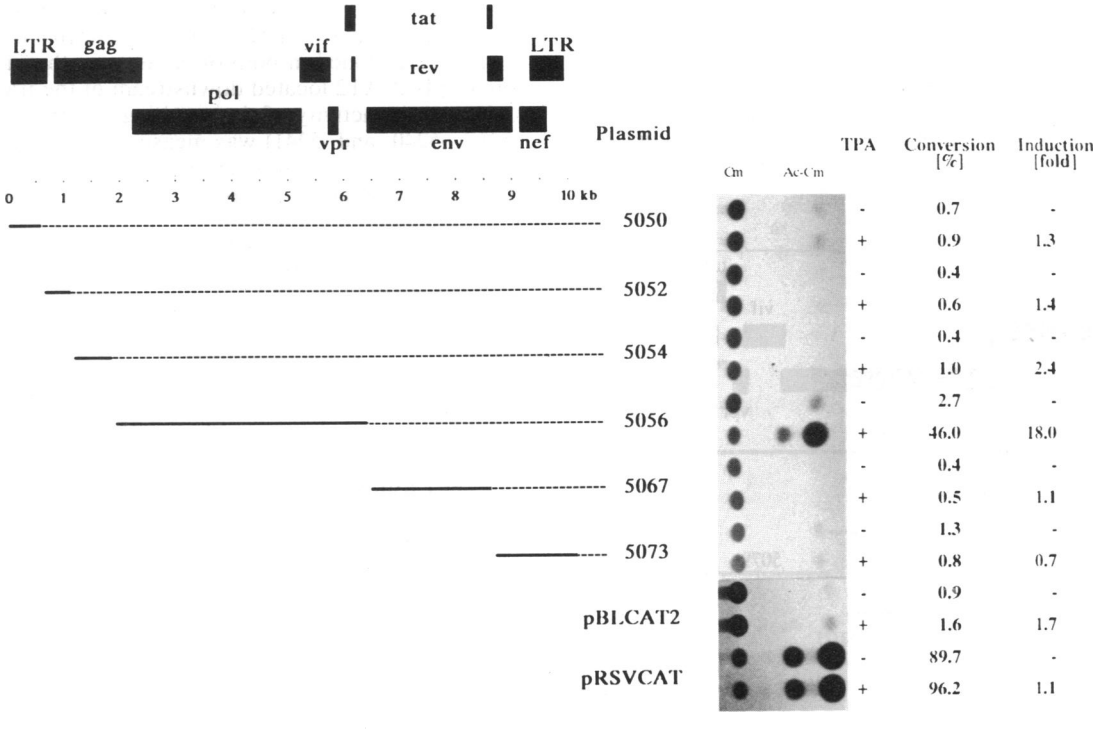

FIG. 1. Identification of an intragenic enhancer in the HIV genome. Enhancer activity of six fragments representing the complete HIV genome was examined after cloning the individual fragments upstream of the TK promoter in pBLCAT2 and transfection in HeLa cells with or without PMA (TPA). The coordinates of each DNA fragment are indicated on the graph by position relative to the HIV-1 genome. A representative result from TLC, separating [^{14}C]chloramphenicol (Cm) from its acetylated products (Ac-Cm), is shown next to the average conversion of [^{14}C]chloramphenicol to acetylated products from three independent transfections. Conversion is defined as the ratio [Ac-Cm/(Cm + Ac-Cm)] × 100, and induction is the ratio between conversion with and without PMA.

sion of 2.7% to 46% (Fig. 1), a 28-fold increase in CAT activity when compared with the pBLCAT2 vector.

Next, the enhancer activity of the fragment cloned in pULB5056 was examined in response to different concentration of PMA and of 4α-phorbol 12,13-didecanoate (4αPDD), an inactive analog of PMA, as control (Calbiochem). Stimulation of enhancer activity occurred at doses as low as 1 nM of PMA, peaked at 10 nM to decrease with higher doses (data not shown). No or minimal enhancement of CAT activity was seen after treatment with 4αPDD, as expected (data not shown).

To demonstrate that the enhancing activity detected in pULB5056 is not secondary to the presence of an undetected promoter, we cloned this fragment upstream of a promoterless CAT gene (pBLCAT3) (15) in both orientations (pULB5237, ASO and pULB5238, SO). The vector pBLCAT3 is identical to pBLCAT2, except for the absence of the TK promoter upstream of the CAT gene. Very low CAT activity (<1%) was measured after transfection of the resulting plasmids in HeLa cells with and without PMA (data not shown), indicating that no detectable promoter activity is present in the fragment cloned in pULB5056.

Surprisingly, no enhancer activity was demonstrated for the fragments cloned in pULB5050 and pULB5073, which contain the previously identified enhancer located in the 5′ and 3′ LTRs, respectively (5). However, because the HIV promoter is also present in these constructs, it is likely that the HIV enhancer acts on its own promoter rather than on the more distant TK promoter. Indeed, experiments examining the activity of a single enhancer on two distinct promoters located on the same plasmid have shown that the enhancer only activates the more proximal promoter (27, 28), which in these constructs is the HIV promoter. Because the HIV LTR is cloned in the ASO with respect to the CAT transcription unit, its activity is thus not measured in these experiments.

Mapping of Enhancer Activity of the Intragenic Fragment Defines Two Functionally Independent Domains. To map more precisely the DNA fragment responsible for the enhancer activity, we have used a series of restriction sites to divide the *Hin*dIII–*Hin*dIII (nt 1711–6026) fragment into smaller fragments (Fig. 2; the exact coordinates of these fragments are given under *Materials and Methods*). These experiments indicated that most activity of the fragment cloned in pULB5056 was located in pULB5079 (30.5% conversion after PMA treatment) (Table 1). However, a small activity (4.3% conversion) was reproducibly associated with the central fragment cloned in pULB5074 (Table 1). The active domain in each of these two fragments (5074 and 5079) was further defined. These experiments indicated that two fragments, located respectively at the carboxyl-terminal portion of the *pol* gene (pULB5103) and in a region overlapping the end of the *pol* gene, the *vif* gene, the *vpr* gene, and the first coding exon of *tat* and *rev* (pULB5105) present PMA-dependent enhancer activity (Fig. 2, Table 1). Note that the activity of the fragment cloned in pULB5103 is several-fold higher than the activity of the fragment from which it was derived in pULB5074 (32.9% conversion vs. 4.3% conversion) (Table 1). The portion deleted from pULB5074 to pULB5103 may contain a transcriptional silencer. In addition, a decrease in activity from 30.5% conversion to 17.4% was noted between pULB5079 and pULB5105 (Fig. 2, Table 1), indicating that additional regulatory elements may be located in pULB5107.

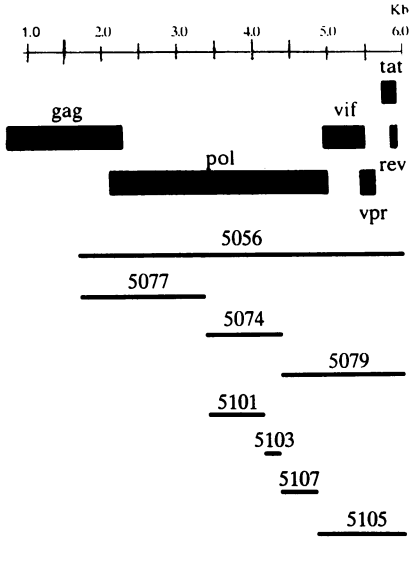

FIG. 2. Mapping of the intragenic enhancer of HIV-1. Subfragments generated by digestion of the fragment in pULB5056 were cloned in pBLCAT2. The location of the individual fragments is shown above the plasmid derived from them relative to the HIV genome.

The Enhancing Activity of the Fragments Is Independent of Their Orientation and Position with Respect to the TK Promoter. Eukaryotic transcriptional enhancers can function independently of their orientation and position with respect to the promoter on which they act (29). To determine whether HIV fragments identified above as enhancers present this property, fragments with enhancing activity in the ASO (pULB5056, -5074, -5079, -5103, and -5105; see Fig. 2) were tested in the SO as well (Table 1). All fragments with enhancer activity in the ASO also showed enhancer activity in the SO (Table 1). The larger fragments carried in plasmids pULB5056, -5055, -5074, -5073, -5079, and -5078 showed higher activity in the ASO than in the SO (Table 1). This orientation preference was lost when smaller fragments were examined (compare pULB5103 with -5102, and pULB5105 with -5104, Table 1). A similar orientation preference has been noted for other enhancer sequences (30–32).

To examine the position independence of the enhancer, fragments from pULB5103 and pULB5105 (Fig. 2) were individually cloned in both orientation in the unique *Sma* I site of pBLCAT2 located downstream of the transcriptional unit, and the activity of the resulting plasmids (pULB5166, -5167, -5240, and -5241) was measured in HeLa cells. Both fragments increased transcription when cloned in the SO downstream of the transcriptional unit, although with a lower

Table 1. Mapping, orientation, and position independence of the intragenic enhancer of HIV-1

Plasmid	Conversion, % PMA −	Conversion, % PMA +	Induction, -fold
pULB5056(ASO)	2.7	46.0	17.0
pULB5055(SO)	2.6	38.2	14.7
pULB5077(ASO)	0.5	0.6	1.2
pULB5074(ASO)	0.7	4.3	6.2
pULB5073(SO)	0.7	2.7	3.6
pULB5079(ASO)	2.4	30.5	15.3
pULB5078(SO)	2.1	9.3	4.4
pULB5101(ASO)	0.5	0.8	1.5
pULB5103(ASO)	2.0	32.9	16.4
pULB5102(SO)	2.0	31.5	15.8
pULB5107(ASO)	1.0	0.9	0.9
pULB5105(ASO)	2.3	17.4	7.6
pULB5104(SO)	1.3	19.8	15.2
pULB5167(ASO)	1.2	12.1	10.1
pULB5166(SO)	1.2	6.4	5.3
pULB5241(ASO)	0.8	1.7	2.1
pULB5240(SO)	0.7	6.0	8.6
pBLCAT2	0.9	1.6	1.8

efficiency than when placed upstream of the TK promoter (compare pULB5166 with -5102 and pULB5240 with -5104, Table 1). In the ASO, the fragment from pULB5103 was also functional when placed downstream of the CAT gene (compare pULB5167 and -5103, Table 1); however, only marginal enhancement was noted with the fragment from pULB5105 (compare pULB5241 and -5105, Table 1).

The Enhancer Is Functional in a Variety of Cell Types. Enhancer elements can confer tissue- and cell-specific transcription to their cognate promoter (33, 34). This specificity is, in part, secondary to the ability of sequence-specific DNA-binding proteins to interact with defined elements located in enhancers (33, 34). To determine whether the enhancer identified above was functional in different cell types, we have tested its activity after transfection in a variety of cell lines. These included the CV1 cell line (African green monkey kidney), NIH 3T3 (mouse embryo), HEC1-B (human endometrial adenocarcinoma), and LS174T (human colon adenocarcinoma). The fragment identified as an enhancer in HeLa cells (pULB5056) was also able to activate the TK promoter after transfection and PMA treatment in CV1 and HEC1-B cells (Fig. 3, lanes A). No or little CAT activity was detected in NIH 3T3 and LS174T cells (Fig. 3, lanes A). The low level of CAT activity in these cells was not the consequence of a lower efficiency of transfection because the two plasmids pRSVCAT and pSV2CAT (19, 35) induced a high level of CAT synthesis in all cell lines tested (Fig. 3, lanes E and F). Remarkably, the activity of the two plasmids containing the subfragments pULB5103 (lane B) and -5105 (lane C) differed between cell lines; both pULB5103 and -5105 were active in HeLa and CV1 cells, whereas only pULB5103 was active in the HEC1-B cell line (Fig. 3, lanes B and C). These results indicate that the enhancer located in the coding region of HIV can function in different cell lines and that distinct domains are active, depending on the cell examined.

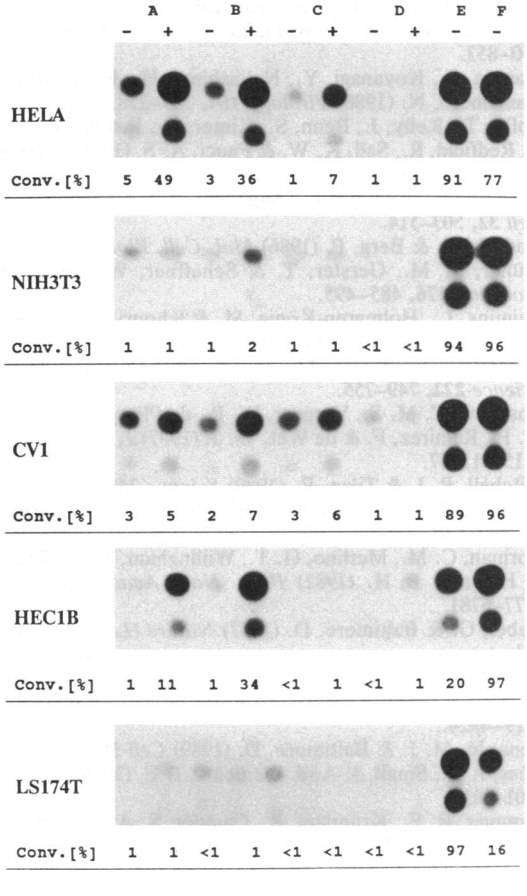

FIG. 3. The intragenic enhancer of HIV-1 is active in a variety of cell types. Six different plasmids, including pULB5056 (lane A), pULB5103 (lane B), pULB5105 (lane C), pBLCAT2 (lane D), pRSVCAT (lane E), and pSV2CAT (lane F), were transfected in several cell lines, and CAT activity was measured in extracts from cells treated or not treated with PMA. The two acetylated forms of [^{14}C]chloramphenicol (1- and 3-acetylchloramphenicol) and the conversion obtained with these plasmids in each cell line are shown. This experiment was repeated once with similar results.

DISCUSSION

We demonstrate in this report that a 4.3-kilobase DNA fragment from the central coding region of the genome of HIV functions as an enhancer on the heterologous TK promoter from herpes simplex virus. As predicted for an enhancer, activity of the fragment is independent of its orientation and position with respect to the TK promoter and depends on a functional heterologous promoter in the transcriptional unit. Activity of the fragment can be mapped to two domains that are differentially active in distinct cell lines.

To determine the physiological role of this enhancer in the life cycle of HIV, the activity of the enhancer has been studied in a variety of human CD4-positive and monocytic cell lines. The enhancer is functional in these cells but, contrary to what is seen in HeLa cells, appears to be constitutive (N.B. and E.V., unpublished work). Recently, the effect of mutations of transcription factor NF-κB-binding sites located in the LTR [shown to be crucial for enhancer and promoter activity (36)] on the replication of HIV has been examined. Unexpectedly, these studies showed that mutated proviruses with no functional NF-κB-binding sites are competent in terms of viral replication (ref. 37; M. Feinberg and D. Baltimore, personal communication), indicating that mutations in the NF-κB-binding sites can be rescued by other cis-acting elements located in the viral genome. Our results suggest that the intragenic enhancer described in this report could play such a role.

A search for the presence of binding sites for known transcription factors in the intragenic enhancer has identified several discrete DNA elements described in Table 2. The 263-base-pair fragment of pULB5103 contains two potential binding sites for transcription factor AP-1 (22) (Table 2). AP-1 binding sites have been identified in the genome of other viruses, including the closely related lentivirus visna, where they play a critical role in basal activity and transactivation of the viral LTR (39). In the sequence of the fragment from

Table 2. Potential binding sites for transcription factors in the intragenic enhancer of HIV-1

Gene	Sequence
AP-1-binding site	
Simian virus 40	ATTAGTCAGC
Collagenase	ATGAGTCAGA
Polyoma	GTTAGTCACT
HIV-1 (4096)	GTTAGTCAAT
HIV-1 (4198)	ATTAGTCAGT
NF-κB-binding site	
HIV-1 (-91)	GGGGACTTTCC
HIV-1 (-105)	AGGGACTTTCC
IL-Rα	GGGAATCTCC
β-Interferon	GGGAAATTCC
HIV-1 (+5287)	AGGGAGTCTCC

The sequence of two potential AP-1-binding sites in the intragenic enhancer is aligned with the sequence of described AP-1 sites in other genes (22). NF-κB-binding sites in the HIV LTR and in other genes (38) are compared with the potential site found in the intragenic enhancer of HIV-1; this site is most closely related to the site found in the interleukin 2 Rα (IL-Rα) promoter.

pULB5105, we have also noted a potential binding site for transcription factor NF-κB (38) (Table 2). The functional significance of these similarities can be determined experimentally by site-directed mutagenesis.

Why does HIV need two enhancers? Other genes have been shown to possess several enhancers the cooperative or possibly antagonistic activities of which modulate in concert the rate of transcription initiation (29, 40). The enhancer described in this report could either bring additional cellular specificity or increase the strength of the promoter/enhancer unit located in the LTR.

If this intragenic enhancer is important in the viral life cycle, it should be a conserved feature among closely related retroviruses. By using the approach outlined in this report, the genomes of different lentiviruses can be examined for the presence of an intragenic enhancer. Preliminary results show that the genome of simian immunodeficiency virus type mac also contains an intragenic enhancer, located in the same transcribed region (F.B. and A.B., unpublished results).

Identification of an enhancer in the transcribed region of the HIV genome brings an additional factor in an already complex network of regulators affecting the level of HIV transcription. Study of the interaction between the enhancers located in the LTR and in the transcribed region of HIV should contribute towards increased understanding of the regulation of HIV transcription.

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