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A Discrete cis Element in the Human Immunodeficiency Virus Long Terminal Repeat Mediates Synergistic trans Activation by Cytomegalovirus Immediate-Early Proteins†

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The major immediate-early (IE) promoter of human cytomegalovirus directs the expression of several differentially spliced and polyadenylated mRNAs that encode isomeric proteins with apparent molecular masses of 55, 72, and 86 kDa. All of these proteins are potent transcriptional regulatory proteins. We are interested in the collateral interactions between human cytomegalovirus and human immunodeficiency virus (HIV) in the context of dual infection of a cell. The roles of the specific IE protein isoforms and their respective response elements involved in trans activation of the HIV long terminal repeat (LTR) are not known. Here we present evidence that major HIV proteins IE86, IE72, and IE55 are capable of trans-activating the HIV LTR in a T-cell line, HUT-78. The IE55 isoform noncooperatively stimulates the HIV LTR in the presence of either isoform IE72 or IE86. Interactions between isoforms IE72 and IE86, however, result in strong synergistic activation of the LTR. Our results suggest that a specific 155-amino-acid protein domain that is unique for the IE86 protein participates in this synergistic interaction. Point-mutational analysis of the LTR identified a distinct cis-acting target site, located between nucleotide positions −174 and −163, that mediates exclusively synergistic trans activation by the IE72 and IE86 proteins. Finally, this study underscores the role of a cellular intermediate(s) for communicating the synergistic interactions between two IE trans activators.

AIDS is clinically characterized by severe immunosuppression followed by subsequent unretarded growth of opportunistic infections. Manifestation of AIDS may not occur until years after initial infection by human immunodeficiency virus (HIV). The precise pathogenic mechanism which determines the development of the disease state in HIV-infected people is unknown. The diversity of responses to HIV infection suggests that a number of factors contribute to the pathogenesis of the disease. Human cytomegalovirus (HCMV) is a critical cofactor in AIDS associated with more rapid progression to disease (14, 32, 51). The possible interactions between these two viruses may exist at a number of distinct levels (reviewed in reference 32). At the molecular level, the transcriptional regulatory proteins of one virus may be able to influence the other. The observation that the same cell can be infected by HIV and HCMV in vivo (5, 34) indicates the importance of the direct interactions that may occur.

The major immediate-early (IE) gene of HCMV (11, 23, 30, 45, 49, 50, 52) expresses multiple nuclear phosphoproteins which act as either activators or repressors of transcription, depending on the cell type and target promoter (1–4, 8–10, 12, 13, 22, 26, 28, 29, 31, 33, 35–39, 42–44, 48). The major IE gene of HCMV has been assigned two main regions, 1 (IE-1/UL123) and 2 (IE-2/UL122) (7, 45–47). Formally, the IE gene contains at least seven exons which, because of differentially spliced and polyadenylated mRNAs, translate into three variant isoforms with apparent molecular masses of 55, 72, and 86 kDa (42). In addition, region 2 contains an internal late promoter that directs the synthesis of an unspliced mRNA encoding an in-frame polypeptide with part of exon 7 coding sequences and has an apparent molecular mass of 40 kDa (42). The 72-kDa protein (IE72) is the product from IE region 1 mRNA assembled from exons 1, 2, 3, and 4, inclusive. In contrast, the 86 (IE86)- and 55 (IE55)-kDa variant phosphoproteins are the products of mRNAs assembled from IE region 1 exons 1, 2, and 3 spliced with IE region 2 sequences encompassing exons 5 and 6 for the IE55 protein mRNA and exon 7 for the IE86 protein-encoding mRNA, respectively (Fig. 1). Previously, we have isolated cDNA clones of the major IE gene products encoding isoforms IE72, IE86, and IE55 (43). The availability of cDNA clones corresponding to the various IE proteins has greatly facilitated the identification of protein products responsible for mediating the specific activity that had previously been ascribed to IE regions 1 and 2. The IE72 and IE86 proteins were identified as positive activators of early cytomegalovirus gene expression and demonstrated strong synergistic activity (28, 43). The IE86 protein was characterized as the repressor for autoregulation of the major IE promoter (MIEP), while IE72 was shown to be an activator of the MIEP (43). Recently, the IE55 protein has also been shown to be a potent transcriptional activator of the MIEP (2).

Several studies have examined the role of the major IE gene products in trans activation of the HIV long terminal repeat (LTR) with expression vectors based on genomic fragments from regions 1 and 2 (1, 3, 6, 10, 31, 32, 37, 38). However, little is known about which precise isoform is
responsible for the \textit{trans} activation event. For example, Davis et al. (10) demonstrated that region 2, but not region 1, products were responsible for \textit{trans} activation of the HIV LTR. However, increased LTR activity was observed when both region 1 and 2 products were present in the cell. To date, HIV LTR sequences encompassing the cap site (nucleotides -6 and +20) have been shown to mediate responsiveness to the cytomegalovirus IE proteins (3). In addition, region 1 products have been proposed to induce NF-\kappa B in autoregulating the MIEP (39). Since NF-\kappa B elements exist in the HIV LTR, those sites may be potential targets of the IE proteins. Therefore, we have sought to delineate the role of the major IE gene products in activating the HIV LTR and determine whether they act via specific target sequences.

\section*{MATERIALS AND METHODS}

\textbf{Recombinant plasmids.} Recombinant plasmids pSVH, pSVVm17, pSVCC3, pIE55, pIE72, and pIE86 have already been described (43). The cDNAs for the 55-, 72-, and 86-kDa IE proteins were cloned into a Rous sarcoma virus (RSV) expression vector (pBlueRSV). The structures of the major IE gene and transcript products are illustrated in Fig. 1. To construct pBlueRSV, the 592-bp NdeI-HindIII fragment (containing the entire RSV LTR) from pNM52 was isolated, mung bean nuclease treated, and subcloned into the blunted EcoRI site of pBluescriptIIISK (Stratagene). For construction of the 72-kDa effector plasmid (pRSV72), the 2.2-kb EcoRI-HindIII fragment containing the entire coding sequence of pIE72 was purified, and the EcoRI end was blunted and ligated into the EcoRV and HindIII sites of pBlueRSV. The cDNAs of the 55- and 72-kDa proteins have exons in common with the 86-kDa cDNA (42). This was exploited in the construction of effector plasmids pRSV55 and pRSV86. The pIE55 and pIE86 plasmids were digested with \textit{Hpai} and \textit{HindIII}. The purified 1.8- and 2.5-kb fragments of pIE55 and pIE86, respectively, were substituted for the 1.7-kb \textit{Hpai}-HindIII fragment of pRPSV72. The resulting constructs, pRSV55 and pRSV86, contain the complete coding sequences of the IE55 and IE86 proteins, respectively. HIV LTR chloramphenicol acetyltransferase (CAT) reporter plasmids and the construction of oligonucleotide-directed mutations in the LTR have been previously described (16-18, 20, 21, 53). These mutant constructs were previously assayed in both DNase I footprinting assays and transient expression assays. The mutations eliminate the binding of their cognate cellular DNA-binding protein. For a summary in schematic form of the HIV LTR constructs used in this study, see Fig. 5.

\textbf{Transient transfections and CAT assays.} Suspension cultures of HUT-78 cells were maintained in RPMI medium with 7% fetal calf serum; 1% each penicillin, streptomycin, amphotericin B; and 1.2% glutamine. Approximately 2 \times 10^8 cells per 500-\mu l aliquot were incubated on ice for 20 min with effector and reporter DNAs, as specified in the figure legends. Electroporations were performed with a Bio-Rad Gene Pulser at a capacitance of 960 \textmu F and 250 m\textsuperscript{V} in a 0.4-cm electrode gap sterile gene pulser cuvette (Bio-Rad). Samples were further incubated on ice for an additional 15 min and subsequently plated on 60-mm-diameter tissue culture dishes with 3 ml of RPMI medium. After 24 h, the transfected cells were harvested and lysates were prepared by the freeze-thaw method. CAT enzymatic assays were performed as previously described (19) and quantitated by scintillation counting.

\section*{RESULTS}

\textbf{trans activation of the HIV LTR by specific IE isoforms.} To determine whether IE isoforms IE86, IE72, and IE55 can potentially activate the HIV LTR, cDNA expression vectors encoding the respective isoforms under the control of the RSV LTR were cotransfected in a dose-response manner with the HIV LTR (-453 to +80) (WT\textsubscript{453}) linked to the CAT reporter gene. Increasing amounts of pRSV without the IE cDNAs did not influence the level of expression of the HIV LTR (data not shown). In contrast, increasing amounts of RSV6, RSV72, and RSV55 cotransfected with plasmid pLTR\text{CAT}-453 (WT\textsubscript{453}) resulted in increasing levels of expression from the HIV LTR (Fig. 2A). The levels of stimulation observed were approximately 22-, 36-, and 5-fold for pRSV86, pRSV72, and pRSV55, respectively (Fig. 2B). These results suggest that all three isoforms examined are capable of \textit{trans} activating the HIV LTR in the HUT-78 T-cell line. Significantly, the IE86 and IE72 isoforms represent strong activators of the HIV LTR while IE55 stimulates expression to a lesser degree.

\textbf{The IE72 isoform is an activator of HIV LTR activity.} Numerous investigators have shown that genomic expression vectors of IE region 2 of the major IE gene that code for proteins IE55 and IE86 can potently activate the HIV LTR (1, 10, 32, 38). Thus, it was not unexpected when IE86 and IE55 were found to \textit{trans} activate the HIV LTR. What was surprising, however, was that IE72 was also found to \textit{trans} activate this promoter. Therefore, the role of IE72 in activating the HIV LTR was investigated further. Since previous investigators have used exclusively genomic expression constructs for the MIEPs, the ability of the genomic region 1 expression vector (pSVCC3) (Fig. 3A), as well as the IE72 cDNA under control of the MIEP (pIE72) (Fig. 3A) and the RSV LTR (pRSV72) (Fig. 3A), to \textit{trans} activate the HIV LTR was examined. Figure 3B shows that the genomic and cDNA expression vectors \textit{trans} activated pLTR\text{CAT}-453 to the same extent. To confirm that the observed \textit{trans} activation resulted from IE72 activity in the context of the complete genomic region of the major IE gene, an IE region 1 mutant containing a four-amino-acid insertion at residue position 325 (Fig. 4) was tested. This mutant, pSVVm17, has been previously shown to eliminate IE72 activity completely (43). In comparison with that caused by wild-type expression
vector pSVH, the level of stimulation of the HIV LTR by pSVHm17 was severely diminished (Fig. 4). Reduced stimulatory activity was also observed for mutations in the IE region 2 coding sequences (data not shown). Importantly, the four-amino-acid insertion mutant (pSVHm17) that resides exclusively in the IE72 coding sequences abrogates trans activation. Thus, we conclude that the IE72 protein is important for activation of the HIV LTR by IE region 1 sequences.

Synergistic and noncooperative interactions between IE isoforms. To assess whether the various isoforms are capable of interacting in either a cooperative or a noncooperative manner, dual-cotransfection experiments were performed. Dual-cotransfection experiments with pRSV72 and pRSV55, together with the reporter pLTR3CAT-453, resulted in an approximately 23-fold additive stimulation effect (Fig. 2). We interpret this effect as being additive, since the combination of IE72 and IE55 resulted in activation of the HIV LTR that approximated the sum of the activation potentials of the individual proteins. A similar additive activation response (approximately 10-fold) was also observed in dual-cotransfection experiments involving pRSV86 and pRSV55 (Fig. 2). However, examination of the response produced by cotransfection of the pRSV72 and pRSV86 effectors with pLTR3CAT-453 demonstrated strong (approximately 100-fold) stimulation of the HIV LTR that significantly exceeded the sum of the individual activation potentials (Fig. 2). We take these results to mean that the IE72 and IE86 activators work synergistically (cooperatively). Thus, IE55 noncooperatively activates the HIV promoter in conjunction with IE72 and IE86. In contrast, the IE86 and IE72 proteins together synergistically stimulate the HIV LTR.

Mutational analysis of the HIV LTR and trans activation by IE proteins. To determine whether a specific cis-responsive element mediates trans activation by the IE72 and IE86 proteins, point mutations throughout the LTR were introduced and examined for the ability to be trans activated. These mutations were directed to sites of known protein-DNA interactions defined by DNase I footprinting studies and have previously been shown to abolish the binding of their respective factors (16–18, 20, 21, 53). Schematics of the deletion and point mutations analyzed in this study for responsiveness and synergistic stimulation by the IE72 and IE86 trans activators are shown in Fig. 5. The mutant HIV LTR templates (listed in Fig. 5) were analyzed for the ability to be trans activated by IE72 and IE86 independently (Fig. 6). Cotransfection of either pRSV72 or pRSV86 with the mutant LTR-reporter constructs demonstrated that all were responsive to trans activation by the individual proteins (Fig. 6). In some cases, such as the TATA box mutation (NTATA) and those mutations in the NF-κB binding site (NENHB), LTR mutants were weakly activated relative to the wild type (Fig. 7). However, since the mutations have a dramatic effect on the basal level of expression, in particular the mutant NTATA, the possibility that these mutant constructs are still fully responsive to the IE proteins cannot be ruled out. It is not possible to distinguish between the mutations that affect the ability of the promoter to be trans activated and those effects that are manifested simply because the promoter has been severely compromised. For this reason, the responsivenesses of the NTATA and NENHB mutations were scored as ± and +, respectively (Fig. 5). We infer from these results that the known sites of protein-DNA interaction are not responsible for mediating the independent mode of trans activation by IE72 and IE86. Alternatively, multiple targets may be involved by which the mutation of one is compensated for by a different target site.

To determine whether a specific target site communicates the synergistic mode of trans activation of the LTR, the promoter mutants were analyzed by dual-cotransfection experiments. Promoter mutant NREP, which was found to be responsive to trans activation by IE72 and IE86 independently, abolished the ability of the HIV LTR to be synergistically trans activated by those IE proteins (Fig. 5 to 7). In contrast, synergistic trans activation was observed for mutant LTR templates NABS, NENHB, 748, NENH02, SP1ALL, NTARP, NTATA, NPOLT, NCAP, NSENSE, and NUBP (Fig. 6 and 7). The observation that the NREP mutant was unable to be synergistically trans activated by IE72 and IE86 without affecting the degree to which those individual proteins activate the LTR suggests that the two modes of trans activation, the synergistic and independent modes, are distinct. Promoter mutant NREP introduces
The results of experiments that used cDNA expression constructs to examine the roles of the IE proteins in activating the HIV LTR independently showed that IE72 can trans activate the LTR in addition to IE86 and IE55. Previous studies have indicated that the region 1 IE72 protein is not sufficient in activating the HIV LTR (10). This was based on the results of cotransfection experiments in HeLa cells that used genomic expression vectors of the HCMV major IE gene. These seemingly contradictory results may be due to the use of different expression vectors. However, constructs were not a factor in this case, as both genomic fragments containing only IE72 coding sequences and IE72 cDNA expression vectors under the control of different promoters stimulated the HIV LTR to the same extent in dose-response experiments. Furthermore, a four-amino-acid insertion mutant (exclusive for the IE72 coding sequences) in the context of the complete IE region 1 and 2 genomic fragment abrogated the level of trans activation. From these data, it is apparent that the IE72 protein has the ability to activate the HIV LTR independently of proteins IE55 and IE86. In agreement, recent studies by a number of different investi-

**FIG. 3.** IE72 trans activation of the HIV LTR. (A) Schematic representation of the expression vectors based on the genomic fragment for IE72 (pSVCC3) and the cDNA expression vectors for the IE72 isoform under the control of the MIEP, pIE72, and the RSV LTR, pRSV72, respectively. (B) Autoradiogram of CAT assays from dose-response cotransfection experiments with 5 μg of WT<sub>943</sub> and increasing amounts of pSVCC3, pIE72, and pRSV72. Lanes: 1, no effector plasmids; 2, 3, and 4, 5, 10, and 20 μg of pSVCC3, respectively; 5, 6, and 7, 5, 10, and 20 μg of pRSV72, respectively.

**DISCUSSION**

In this study, we demonstrated that multiple IE proteins (IE86, IE72, and IE55), encoded by both regions 1 and 2 of the major IE gene of HCMV, cooperatively and noncooperatively trans activate the HIV LTR in T-cell line HUT-78. Importantly, this study illustrates the possible role of a cellular factor(s) in mediating synergistic trans activation of the LTR by proteins IE72 and IE86.

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**FIG. 4.** trans activation of the HIV LTR by insertion mutant pSVHm17. (A) Cotransfection of WT<sub>943</sub> with 10 μg of pSVH and mutant pSVHm17, respectively. (B) Four-amino-acid insertion (in brackets) at amino acid residue 325 of the IE72 reading frame in mutant pSVHm17.

![Image](image_url)
FIG. 5. Schematic representation of the HIV LTR point mutants examined for the ability to be trans activated by isoforms IE72 and IE86. The full-length LTR is shown on the top (WT943), and the locations of the enhancer, TATA box, and TAR domain are shown by white boxes. The arrow marks the point of transcription initiation (+1). Signed numbers refer to nucleotide positions relative to the transcription initiation site. Black boxes indicate the positions of the point mutations, and the site-specific changes are shown above the wild-type sequence. A summary of the responsiveness and synergistic trans activation by IE72 and IE86 is shown to the right of the respective clones.

The mutational analysis of the HIV LTR performed in this study did not reveal target sites for the independent mode of trans activation by the IE proteins. A number of investigators have attempted to define the target site for trans activation of the HIV LTR by HCMV IE proteins (1, 3, 6, 29, 31, 38). Consistent with our findings, many of these studies also failed to detect a specific response element (1, 6, 29, 31, 38). However, Barry et al. (3) have shown that a region between -6 and +20 is essential for trans activation by an IE region 1 and 2 genomic expression vector. Although point mutations throughout this region were examined, we did not analyze the exact mutations used in those studies. Furthermore, the suggestion that NF-kB is required for trans activation of the MIEP by IE72 (39) implicates the NF-kB

FIG. 6. CAT assays of cotransfection and dual-cotransfection experiments with no trans activator (C) and with effector plasmids RSV72 (72) and pRSV86 (86) with the indicated mutant HIV LTR reporters.

FIG. 7. Summary of fold trans activation of the mutant HIV LTR templates from cotransfection experiments with pRSV72 (72) and pRSV86 (86) and dual-cotransfection experiments with pRSV72 and pRSV86 (72/86).
binding sites of the HIV LTR as a potential target. Point mutations that prevent binding of NF-kB to the HIV LTR (18) were still responsive to trans activation by IE72 and IE86. The level of stimulation was reduced; however, since these mutations may affect basal promoter functions, the possibility that these mutant constructs are still fully responsive to the IE proteins cannot be ruled out. A similar explanation may be given for the TATA box mutant, although it is likely that the ultimate effects of the IE proteins are manifested at this region. In light of the promoter and cell type dependence of IE protein function, the resolution of the target sites for the independent mode of activation of the LTR must remain an open question.

An important issue raised in this study is whether the different IE isoforms interact in the regulation of promoters. Here we show that isoform IE55 noncooperatively interacts with the IE72 and IE86 proteins in stimulating the HIV LTR. In contrast, the IE72 and IE86 proteins synergistically interact in activation of the LTR. While this work was being submitted for publication, a report on the activation of the HIV LTR by genomic (IE region 1 and 2) expression vectors in fibroblast cells, in agreement with our findings, implicated possible synergism between products derived from IE regions 1 and 2 (6). However, since those investigators used genomic expression vectors, it was not clear which protein products were responsible for the observed augmented activity of the HIV LTR.

The IE55 protein is an isoform variant of the IE86 protein that differs by a 155-amino-acid deletion between amino acid residues 365 and 519 of IE86. This deletion arises from an in-frame splicing event in IE region 2 (Fig. 1) (42, 43). The finding that IE55 does not functionally synergize with IE72 implies that the specific domain unique to IE86 (i.e., residues 365 to 519) is involved in the synergistic response. Thus, it is likely that the IE72-IE86 synergism involves protein-protein interactions that are mediated through this domain. This protein domain contains a putative zinc finger adjacent to a leucine-rich region (33, 43) and may mediate protein-protein interactions by either coordination with Zn$^{2+}$ (15) or hydrophobic contacts via the leucine-rich region (25).

Mutational analysis showed that a discrete sequence element located between −174 and −163 in the HIV LTR is required for IE86-IE72 synergistic activation. This sequence element is a target site for the interaction of a host-encoded nuclear protein (17). Point mutations that abolish the ability of this factor to interact with the HIV LTR ablate the synergistic trans activation by IE86 and IE72 without affecting the ability of the promoter to be trans activated by the proteins individually. Thus, these experiments provide evidence that the independent and synergistic modes of trans activation are distinct. UV cross-linking experiments have identified a 50-kDa polypeptide that interacts with this sequence domain (40) and suggested that it is transcription factor USF-MLTF (27). Although the precise identity of this cellular factor is not known, the data presented suggest that a cellular intermediate, such as USF, communicates the synergistic action of IE72 and IE86. Direct biochemical and genetic evidence for a physical interaction between this factor and the IE isoforms has yet to be provided. However, the data certainly do not preclude a direct involvement for functional synergism.

Clearly, the ability of the USF-binding site to mediate IE86-IE72 synergism may play an important role in the HCMV life cycle. For example, early promoters of HCMV appear to be highly dependent on synergistic activation by IE72 and IE86 (12, 28, 43). At least one cytomegalovirus early promoter, the 2.7-kb early RNA promoter, has been shown to contain a USF-binding site that is critical for regulated expression (24, 41). In this case, we predict that the USF-binding site in the early promoter would be responsible for mediating synergistic activation by IE72 and IE86. Thus, it is unlikely that the HIV LTR has had selective pressure to maintain a USF-binding site simply for the benefit of cross-trans activation by HCMV IE proteins. The role of the USF site in the HIV life cycle has yet to be established.

In conclusion, we have demonstrated that major IE isoforms IE72, IE86, and IE55 can independently trans activate the HIV LTR. IE55 noncooperatively stimulates the HIV LTR in the presence of isoforms IE72 and IE86. In contrast, the IE72 and IE86 proteins interact in a manner that synergistically stimulates the LTR. The synergic interactions between IE72 and IE86 are mediated by a sequence element (at nucleotide positions −174 to −163) that interacts with a host-encoded DNA-binding protein. The precise identity of this third target protein and the question of whether or not it complexes with the IE isoforms remain to be explored.

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