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Activation of the Human Immunodeficiency Virus Long Terminal Repeat by Varicella-zoster Virus IE4 Protein Requires Nuclear Factor-κB and Involves Both the Amino-terminal and the Carboxyl-terminal Cysteine-rich Region

Defechereux-Thibaut de Maisieres P[1], Baudoux-Tebache L[1], Merville M-P[2], Rentier B[1], Bours V[2] Piette J[1]

[1]*Laboratory of Fundamental Virology and Immunology, Institute of Pathology, University of Liege, B-4000 Liege, Belgium*
[2] *Laboratory of Medical Chemistry and Medical Oncology, Institute of Pathology, University of Liege, B-4000 Liege, Belgium*

Abstract : Varicella-zoster virus open reading frame 4-encoded protein (IE4) possesses transactivating properties for varicella-zoster virus genes as well as for those of heterologous viruses such as the human immunodeficiency virus type 1 (HIV-1). Mechanisms of HIV-1 LTR (long terminal repeat) transactivation were investigated in HeLa cells transiently transfected with an IE4 expression plasmid and a CAT reporter gene under the control of the HIV-1 LTR. These results demonstrated that IE4-mediated transactivation of the HIV-1 LTR in HeLa cells required transcription factor *κB* (NF-κB). Using the gel retardation assay, it was shown that transfection of the IE4 expression vector in HeLa cells was not associated with induction of NF-κB under the p50-p65 het-erodimeric form and that no direct binding of IE4 to the κB sites could be detected. Both Western blot and immunofluorescence analyses suggested that the ability of IE4 to activate transcription through *κB* motives was not connected with its capacity to override the inhibitory activities of *IκB-a* or p105. Finally, *in vitro* protein-protein interactions involving IE4 and basal transcription factors such as TATA-binding protein and transcription factor IIB were carried out. A direct interaction between IE4 and TATA-binding protein or transcription factor IIB components of the basal complex of transcription was evidenced, as well as binding to the p50 and p65 NF-κB subunits. Mutagenesis analysis of IE4 indicated that the COOH-terminal cysteine-rich and ar-ginine-rich regions (residues 82-182) were critical for transactivation, whereas the first 81 amino acids appeared dispensable. Moreover, the arginine-rich region is required for the *in vitro* binding activity, whereas the COOH-terminal end did not appear essential.

Varicella-zoster virus (VZV)[1] is an α-herpesvirus, which causes two distinct diseases in man: chicken pox and shingles. Shortly after entry into the infected cells, VZV genes are expressed in a temporal cascade. The immediate-early genes are expressed first; these stimulate early gene expression, providing most of the proteins necessary for viral DNA replication. After DNA synthesis has occurred, genes of the late class, which mainly encode structural proteins, are expressed. This orderly pattern of expression has been proposed mainly by comparison with herpes simplex virus type 1 (HSV-1) (1), another α-herpesvirus that has been intensively studied. The use of transient transfection assays has clearly shown that several VZV proteins, *i.e.* those encoded by ORFs (open reading frames) 4, 10, 29, 61, 62, and 63, possess regulatory properties (2–8). Three of these polypeptides, encoded by ORFs 4, 62, and 63, are expressed during the immediate-early phase of lytic infection (9–11), and are thus referred to as IE4, IE62, and IE63. Hence, VZV immediate-early proteins contribute to the control of the viral cycle progression as in other herpesviruses.
The IE4 protein is a transactivator of gene expression whose regulatory properties are not yet fully understood (2, 4, 11–14). IE4 stimulates VZV gene expression regardless of the cell type envisaged, *i.e.* monkey fibroblasts or human T lymphocytes (2, 4, 11, 12). It also appears that IE4 is capable of heterologous transactivation (11–13). The available data suggest that IE4 could exert its functions through transcriptional and post-transcriptional mechanisms (11–13). Evidence for transcriptionally mediated activation was provided by the characterization of critical *cis*-elements necessary for the IE4 inducibility of heterologous viral promoters (13). The presence of a TATA box alone is apparently not sufficient to convey transactivation by IE4, because transactivation of a minimal promoter containing only a TATA box is not possible (13). Two different factor-binding sites, the Sp1 and nuclear factor **κ**B (NF-κB)-respon-sive elements, have been proposed as critical for IE4-mediated transactivation (13).

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Protein-encoding genes in eukaryotes are transcribed by RNA polymerase II associated with general transcription factors. Control of their expression requires specific regulatory

[1] The abbreviations used are: VZV, varicella-zoster virus; LTR, long terminal repeat; HIV-1, human immunodeficiency virus type 1; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; HSV, herpes simplex virus; ORF, open reading frame; IE, immediate-early; NF-κB, nuclear factor-κB; USA, upstream factor stimulatory activity; USF, upstream stimulatory factor; CMV, cytomegalovirus; EBV, Epstein-Barr virus; WT, wild-type; CAT, chloramphenicol acetyltransferase; TBP, TATA-binding protein; TFIIB, transcription factor IIB; PAGE, polyacrylamide gel electrophoresis; HTLV, human T cell leukemia virus.

factors with DNA binding and transactivation domains that interact with the general transcription factors. These interactions influence RNA polymerase II recruitment to the promoter and stabilize and/or modify polymerase II holoenzyme activity (15). The ubiquitous Sp1 factor is a transcription factor that participates in the basal activity of the transcription machinery by binding to specific sites that have been found in numerous core promoters. Sp1 possesses a zinc finger DNA-binding domain as well as a glutamine-rich transactivation domain, and its activity seems to be controlled by its phosphorylation status (16–18). NF-κB consists of a group of dimeric complexes composed of varied combinations of polypeptides of the Rel/NF-κB family (19). These factors are ubiquitous and have been involved in transcription regulation of cellular genes important in the inflammatory response and oxidative stress (19–21). The activation of NF-κB is dependent on the binding of these complexes to *cis*-sequences found in the promoter regions of target genes (20, 22). The prototypic form of NF-κB is a heterodimer containing the p50 and p65 proteins (19, 21). In most cells, NF-κB activity is kept under control through cytoplasmic sequestration by members of a family of inhibitory proteins, including IκB-α and p105, the latter being the cytoplasmic precursor of p50 (23). The majority of induction signals lead to the rapid phosphorylation of IκB-α at serine 32 and serine 36 (24), which targets it to the ubiquitin-proteasome pathway (25). Subsequent proteolytic degradation of IκB-α allows the nuclear translocation of the active NF-κB dimer. A large number of stimuli such as mitogens, cytokines, stress, and viruses have been shown to induce NF-κB (19, 21).

Because viruses are intracellular parasites, they appropriate the cellular machinery for their own benefit. NF-κB belongs to the host transcription factors used by a number of viruses to induce their own expression or that of specific host genes (19). Inducible human immunodeficiency virus type 1 (HIV-1) gene expression is generally mediated by the binding of NF-κB to the enhancer κB-binding sites in the long terminal repeat (22, 26, 27). Molecular interactions among herpesviruses and HIV-1 have been frequently investigated, as the most common opportunistic viral infections in individuals with AIDS are caused by herpesviruses (28–30). *Herpesviruses* could act as co-factors in enhancing HIV-1 replication. Direct effects of herpesvirus proteins on HIV-1 LTR activity have been detected in the case of HSV-1 (31–33), cytomegalovirus (CMV) (34, 35), and Epstein-Barr virus (EBV) (36). Some of these effects could be associated with NF-κB induction (27, 33, 36–38), although some controversy exists as to the identity of the responsible proteins in HSV-1 (32, 37, 39, 40) and CMV (34, 35,41). Such discrepancies might possibly be attributed to the different cell lines used in the various studies (31, 34, 35). The mechanisms of NF-κB induction by the latent membrane protein (LMP1) of EBV, for example, are post-translational and involve IκB-α degradation (38). Little is known about the mechanism of LTR induction by VZV, but it was shown that VZV infection of HeLa cells could stimulate HIV-1 LTR activity (42). It has also been demonstrated that a DNA fragment carrying ORFs 61, 62, and 63 could transactivate the HIV-1 LTR in transient transfection (32). We and others have shown that IE4 could also stimulate the LTR in CAT assays (12, 13, 43). This transactivation seems to occur transcriptionally (11, 13).

The purpose of this report was to clarify the molecular mechanisms of IE4-mediated transactivation of the HIV-1 LTR. Our results indicate that κB-binding sites are essential for LTR transactivation by IE4. However, under our experimental conditions, neither significant NF-κB induction nor degradation of IκB-α or p105 could be detected. *In vitro* protein interaction experiments showed that IE4 could interact with TBP, TFIIB, p50, and p65, which suggests that IE4 could enhance the efficiency of the NF-κB complexes bound to their sites in the LTR through interactions with the activators and the general transcription machinery. Mutational analysis was performed in an attempt to identify the critical domains of IE4 involved in these protein-protein interactions.

Materials and methods

Cells and Transfections—The HeLa human cervical epithelioid carcinoma cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were

performed using the LipofectAMINE reagent (Life Technologies, Inc.). The total amount of DNA was kept constant under the different conditions by addition of sonicated herring sperm DNA or pCMV expression vector without insert. Whole cell extracts were prepared by the freeze-thaw method, and CAT assays were performed as described previously (12). CAT assay data were collected from at least four independent transfec-tion experiments.

Plasmids

Plasmid pCMV-4, which carries VZV ORF4 under the control of the powerful CMV promoter-enhancer, was a gift from Dr. P. Kinchington (University of Pittsburgh, Pittsburgh, PA). In pGi4, IE4 expression is directed from the cognate ORF4 promoter (2). Plasmid pCMV-Tax directed *HUMAN t CELL LEUKEMIA VIRUS* (HTLV-1) Tax expression from the CMV promoter/enhancer (44). IκB-α was expressed from pRSV-IκB in which the IκB-α gene was under the control of the Rous sarcoma virus LTR (a gift from Dr. U. Siebenlist, National Institutes of Health, Bethesda, MD). Plasmids pHIV-CAT and pHIV*-CAT have been described previously (28). In these constructs, the wild-type LTR of HIV-1 and an LTR mutated in both **κ**B sites, respectively, are cloned upstream of the CAT gene. pκB-CAT contains the HIV-1 **κ**B enhancer element upstream of the *c-fos* TATA box (45, 46). The expression plasmids used for eukaryotic expression of p50 and p65 were those employed previously (45), and p105 was similarly cloned at the *EcoRI* site of the pMT2T vector (a gift from Dr. U. Siebenlist). pSG-hTFIIB and pSG-TFIID, which allow *in vitro* expression of human TFIIB (33 kDa) and TBP (38 kDa) from the T7 promoter, respectively, were provided by Dr. M. Muller (University of Liege, Liege, Belgium). *In vitro* translated p50 and p65 were obtained from Bluescript plasmids (47, 48). pALTER-IE4 was constructed by insertion of an *Nco*I/*Bam*HI fragment isolated from pGi4 into pALTER vector (Promega, Inc., Madison, WI). This plasmid was used for site-specific mutagenesis of IE4. Two COOH-terminal IE4 mutants were created. In pALTER-C426S, the cysteine codon at position 426 was mutated to a codon specifying a serine residue. Deletion of the COOH-terminal cysteine cluster was obtained by changing codon 393 to an opal STOP codon (TGA). The resultant clone was named pALTER-STOP393. The wild-type IE4 sequence as well as both mutated sequences were excised from pALTER by digestion with *Bam*HI and *EcoRI.* The 2-kilobase fragments were then cloned into the polycloning site of pCDNA3.1 (InVitrogen, Inc., Leek, The Netherlands). These pWTIE4, pC426S, and p393STOP constructs were used for both *in vitro* transcription/translation and eukaryotic expression, inasmuch as the genes are cloned under the control of T7 and CMV promoters. To create NH_2-terminal truncation mutants, a series of PCR reactions were designed to amplify IE4 coding sequences lacking the first 65, 81, and 182 codons, respectively. Three oligonucleotides carrying *NcoI* recognition sites at their 5' end were synthesized, so that a new ATG codon was incorporated at positions 65, 81, and 182. The oligonucleotide corresponding to the COOH terminus carried a *Bam*HI recognition site. The PCR mixture consisted of 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 2.5 units of *Taq* polymerase, 1 **μ**M each primer, and 200 ng of pALTER-IE4 in a total volume of 100 μl. The amplification procedure started with a 2-min preheating step at 94 °C followed by 35 cycles, each consisting of a 94 °C denaturation segment for 1 min, a 55 °C annealing segment for 1 min, and a 72 °C extension segment for 1 min 45 s; the whole set was followed by a final extension at 72 °C for 7 min. Amplified products (1.165, 1.115, and 813 base pairs) were resolved on a 1% agarose gel to analyze PCR yield and fragment size before purification was performed using the QIAquick PCR purification kit from Qiagen. Fragments were then cloned into the pNoTA/T7 shuttle vector of the Prime PCR Cloner cloning system (5 Prime → 3 Prime, Inc.) according to the manufacturer's instructions. Recombinant clones were tested for *in vitro* transcription/translation of truncated proteins with correct apparent M_r before the coding sequences were isolated by digestion with *Bam*HI. These DNA fragments were inserted into the *Bam*HI site of the pCDNA3.1 polylinker to obtain pΔ65, pΔ81, and pΔ182.
To express IE4 in bacteria, the ORF4-coding sequence was isolated from pGi4 by digestion with*Nco*I and*Hgi*AI, and both extremities of the fragment were blunt-ended and subcloned in the *SmaI* site of pUC19. A *KpnI-Bam*HI insert from pUC-IE4 was blunt-ended and cloned in phase with glutathione *S*-transferase (GST) in pGex-3X (Amersham Pharmacia Biotech, Uppsala, Sweden). Verification of the reading frame was performed by sequencing the DNA insert at the ligation sites. GST-TK carries the VZV thymidine kinase gene, which was amplified by PCR and cloned in frame with GST in pGex-5X1 (Amersham Pharmacia Biotech).

Gel Retardation Assays

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Nuclear proteins from untreated, phorbol myristate acetate-treated (100 ng/ml), or transfected cells were prepared by high salt extraction (49). Five micrograms of nuclear extracts were incubated for 20 min at room temperature with 0.2 ng of ^{32}P-labeled **κ**B oligonucleotide (45) in a volume of 10 **μ**l containing 20 mM Hepes-KOH, pH 7.9, 75 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5 mM $MgCl_2$, 1 mg of bovine serum albumin, 1.3 **μ**g of poly(dI-dC), and 1 mM dithiothreitol. DNA-protein complexes were analyzed on a low ionic strength 4% or 6% polyacrylamide gel. Following electrophoresis, gels were dried and submitted to autoradiography overnight at -80 °C. To quantify DNA-protein complexes, gels were further analyzed with a PhosphorImager scanner equipped with Image Quant software (Molecular Dynamics, Sunnyvale, CA). The specificity of the complexes was confirmed by competition experiments with a 50-fold molar excess of cold wild-type or mutant oligonucleotide (45). To characterize proteins in the electrophoretically retarded complexes, nuclear extracts were incubated for 15 min on ice with specific antibodies to p50, p65 (50), p52, c-Rel (51), and IE4 (52) before the ^{32}P-oligonucleotide was added.

Western Blot Analysis

Cells were rinsed three times in phosphate-buffered saline, then scraped in phosphate-buffered saline and lysed either in 1% (w/v) sodium dodecyl sulfate (SDS) buffer to extract total proteins or in high salt buffers to extract nuclear and cytoplasmic proteins (49). Ten micrograms of proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvi-nylidene difluoride membranes (Millipore, Inc., Bedford, MA). Membranes were blocked with 5% (w/v) milk powder in TBS-T buffer (20 mM Tris-HCl, pH 7.6, 500 mM NaCl, 0.1% (v/v) Tween 20). Incubation with the primary antibodies was performed for 1 h at room temperature under agitation in TBS-T buffer with 5% (v/v) milk blocking reagent (Amersham Pharmacia Biotech, Brussels, Belgium). The rabbit anti-sera used were a peptide-specific antiserum for either p50 (first 13 amino acids) (50) or p65 (amino acids 531-550, Santa Cruz Biotechnology, CA) and an antiserum directed against a fusion protein between GST and the full-length IκB-α (a gift from Dr. U. Siebenlist). Immuno-reactive proteins were detected by using anti-rabbit immunoglobulin G from pig conjugated to horseradish peroxidase (DAKO). After several washes, blots were prepared for enhanced chemiluminescence (ECL) detection as prescribed by the manufacturer (Amersham Pharmacia Biotech, Brussels, Belgium). Band intensities on the impressed films were quantified by photodensitometry (LKB, Sweden). To perform protein synthesis inhibition experiments, cells were exposed to cyclohexi-mide (50 μg/ml; Sigma).

In Vitro Analysis of Protein-Protein Interactions

GST, GST-IE4, GST-TK, GST-p50 (50), and GST-TFIIB (Dr. M. Mu¨ller, University of Liege, Belgium) were expressed in bacteria following classical induction with 0.1 M isopropyl-1-thio-β-D-galactopyranoside) for 1-3 h at 37 °C. Lysates were prepared using the anionic detergent *N*-laurylsarcosine (Sarcosyl) 1.5% (v/v) in STE (10 mM Tris-HCl, pH 8,150 mM NaCl, 1 mM EDTA). Following three cycles of sonication, bacterial debris were removed by centrifugation. Proteins were then purified on glutathione-Sepharose 4B affinity beads (Amersham Pharmacia Biotech) in STE-Triton 4% (v/v). Protein-coupled Sepharose beads were washed eight times in phosphate-buffered saline before the interactions were performed. Proteins were labeled with [^{35}S]methionine (ICN, Brussels, Belgium) using the *in vitro* coupled transcription/translation system from wheat germ extracts or reticulocyte lysates (Promega Inc., Madison, WI). A control for *in vitro* transcription is provided by production of the firefly luciferase (61 kDa). Equal amounts of ^{35}S-labeled proteins were incubated with 50 **μ**l of protein-coupled Sepharose beads in 400 **μ**l of NETN (20 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 1.5% (v/v) Nonidet P-40). Binding reactions were allowed to take place for 1 h at room temperature, and the beads were then washed six times in NETN. Bound proteins were eluted by boiling for 2 min in 1**X** SDS sample buffer, followed by loading on 12% SDS-PAGE. Gels were subsequently dried and autoradiographed. To ensure optimal washing conditions, 5 **μ**l of ^{35}S-labeled luciferase were incorporated into each interaction reaction and disappearance of the corresponding band was followed.

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Results

NF-κB Binding Motifs Are Required for HIV-1 LTR Activation by IE4

Transactivation of the HIV-1 LTR by the IE4 protein has been detected in Vero cells and human A3.01 T-cells (2, 12, 13). In the A3.01 cells, IE4 inducibility was dependent on the presence of Sp1 and/or NF-κB binding motifs. We set out to analyze LTR transactivation by IE4 in HeLa cells, because it has been shown that VZV infection induces LTR activation (42) and because NF-κB induction is well documented in these cells (53). To characterize the role of NF-κB, a full-length LTR-CAT construct (pHIV-CAT) or a promoter harboring point mutations in both **κ**B elements (pHIV*-CAT) (Fig. 1*A*) was transfected in the presence or absence of an IE4-expressing vector, pGi4. Transfection of the pHIV-CAT construct resulted in a basal level of CAT activity, whereas pHIV*-CAT elicited no detectable levels of CAT activity under the same conditions (data not shown). In the presence of IE4, pHIV-CAT was trans-activated up to 58-fold, but no increase in CAT activity was seen with pHIV*-CAT (Fig. *1B).* To further analyze the role of NF-κB, a minimal construct pκB-CAT containing both HIV-1 **κ**B sites upstream of the *c-fos* TATA box was used (Fig. 1*A*). The activity of this minimal promoter has been shown to be entirely associated with NF-κB (46, 50). pκB-CAT displays a higher basal activity than pHIV*-CAT. Transactivation of this promoter by IE4 reached a 10-fold activation, lower than with the full-length LTR (Fig. *1B).* These experiments were also performed in Vero cells. In these cells, pHIV-CAT and pκB-CAT were stimulated by IE4 (1.5 **μ**g and 2.5 **μ**g of expressing vector) as in HeLa cells but a clear activation was also seen with pHIV*-CAT (data not shown), suggesting that a NF-κB-independent mechanism co-existed with the NF-κB-dependent mechanism.
Because NF-κB activity is controlled mainly by IκB-α, the influence of this inhibitory molecule was tested in the IE4-mediated transactivation. In HeLa cells, co-transfection of increasing concentrations of a plasmid expressing IκB-α with the three reporter constructs was first performed. Presence of ectopic IκB-α resulted in a clear shut-down of the activities of pHIV-CAT and pκB-CAT but, as expected, had little effect on pHIV*-CAT (data not shown). Interestingly, the presence of IκB-α at the lowest concentration (1 μg/ml) required for down-regulating promoter CAT activity in our system prevented transactivation by the IE4 protein in HeLa cells with a maximum 2-fold stimulation (Fig. *1B).* In Vero cells, transactivation of pHIV-CAT in the presence of IκB-α was not affected, whereas transactivation of pκB-CAT was almost completely blocked (data not shown). These findings suggested that IE4-mediated transactivation of the HIV-1 LTR in HeLa cells requires NF-κB factors.
Influence ofIE4 on NF-κB Induction in HeLa Cells—Having established that the NF-κB-responsive elements are essential to IE4-driven transactivation of the HIV-1 LTR in HeLa cells, we wanted to determine whether IE4 could induce NF-κB in this cell line. Therefore, HeLa cells were transiently co-trans-fected by the IE4-expressing vector (pCMV-4) or by a vector without insert (pCMV) and pHIV-CAT. Because the HTLV-1 Tax protein has the capacity to activate members of the NF-κB family (54, 55), the effect of a Tax-expressing vector (pCMV-Tax) was tested in parallel. Whole cell and nuclear protein extracts were prepared from these cells. Fig. 2*A* shows the dose-dependent stimulation of the HIV-1 LTR by both IE4 and Tax. -Fold stimulation of CAT activity was of the same order of magnitude in the presence of the two proteins. To detect NF-κB activation, gel retardation assays were performed using a probe encompassing the **κ**B sites present in the HIV-1 LTR.

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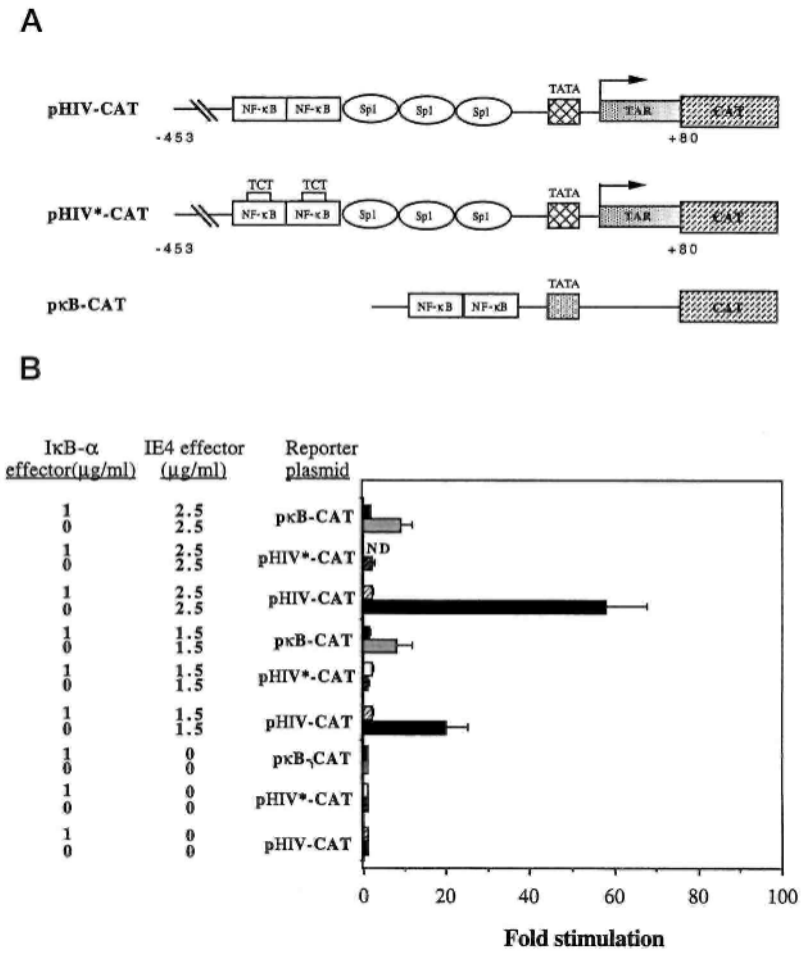

FIG. 1. Stimulation of HIV-1 LTR by IE4 is dependent on NF-κB. A, Schematic representation of the CAT reporter plasmids. LTR sequences extending from -453 to +80 relative to the transcription initiation site were cloned in both HIV-CAT and HIV-CAT constructs; the point mutations introduced in the κB sites of pHIV*-CAT are indicated. The NF-κB enhancer and Sp1 elements are depicted as well as the TAR element. The TATA box is the cross-hatched box, and the broken arrow locates the initiation site. pκB-CAT contains the minimal κB promoter with the c-fos TATA box (stippled box) (57). B, HeLa cells were transfected by lipofection and CAT activity of reporter plasmids was determined 44 h after transfection as described under "Materials and Methods." Each reporter CAT construct (0.5 μg) was co-transfected with 1.5 and 2.5 μg of IE4-expressing vector (pGi4). Fold stimulation of CAT activity was calculated relative to the basal level of each reporter plasmid in the presence of carrier DNA alone, arbitrarily set at 1. To test for inhibition of NF-κB activity, co-transfection with 1 μg of IκB-α effector plasmid was performed. In this case, the stimulations by IE4 were also expressed relative to the activity of each reporter plasmid in the presence of the pRSV-IκB-α vector alone, which was set at 1. Results from four independent experiments are presented with standard errors of the mean.*

Incubation of nuclear extracts from non-transfected cells (used as an uninduced control) with the **κ**B probe exhibited a very low level of the specific NF-κB band (Fig. *2B, lane 1).* Transfection of pCMV or pCMV-4 had little effect on the amount of the NF-κB complex (Fig. *2B, lanes 2* and *3),* whereas a strong induction was seen with pCMV-Tax (Fig. *2B, lane 4).* The electrophoretic mobility of these DNA-protein complexes was identical to that induced by phorbol myristate acetate, a powerful NF-κB inducer (19). These complexes specifically competed with an excess of unlabeled **κ**B oligonucleotides, but an excess of mutant oligonucleotides, which are devoid of NF-κB binding activity, had no effect (data not shown). The immunological characterization of these complexes demonstrated the presence of p50 and p65 and the absence of p52, c-Rel, and IE4 (data not shown). Therefore, IE4 does not bind to the **κ**B sites. Under these experimental conditions, expression of IE4 is not associated with a nuclear translocation of the p50-p65 NF-κB complex, whereas expression of Tax induces such a translocation. Having established the effect of IE4 and Tax on the nuclear translocation of NF-κB, we next

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investigated the effect of these proteins on the stability of IκB-α, because the classical pathway of NF-κB activation proceeds through degradation of this cytoplasmic inhibitor. Nuclear and cytoplasmic extracts from the cells that were used in the gel retardation assay were used for Western blot analysis. No significant variations in intrinsic IκB-α levels were detected when IE4 was present, whereas a clear cytoplasmic diminution was seen with Tax (data not shown). No modification in the levels of p50, its cytoplasmic precursor p105, or p65 were detected after transfection of the IE4 vector (data not shown). In order to ensure that an effect of IE4 was not masked by IκB-α neosynthesis, we next compared the stabilities of IκB-α in transfected cells exposed to cyclohex-imide to prevent *de novo* protein synthesis. As shown in Fig. 2*C*, expression of Tax led to a significant reduction in the cytoplasmic levels of IκB-α relative to that observed in pCMV-transfected cells. In contrast, expression of IE4 had apparently no effect on the IκB-α cytoplasmic levels. These results indicate that the requirement for NF-κB in the IE4-mediated transac-tivation of the HIV-1 LTR is not associated with a nuclear translocation of NF-κB/Rel family members concomitant with a degradation of cytoplasmic IκB-α.

IE4 Has No Effect on the Cytoplasmic Retention of Ectopic p50 and p65 by the IκB-α and p105 Inhibitors

To ascertain the absence of NF-κB induction, we looked at the degradation of the cytoplasmic inhibitors IκB-α and p105 in cells co-expressing various members of the Rel/NF-κB family *(i.e.* p50, p105, p65, and IκB-α) with IE4. This methodology was applied previously in the molecular characterization of NF-κB activation (54). Multiple transient co-transfections involving p50 and p65; p50, p65 and IκB-α; and p50, p65, IκB-α, and IE4 were therefore conducted. Every transfection condition was analyzed by Western blotting and immunofluorescence to determine the intracellular repartition and expression levels of each protein. To conduct the immunoblotting experiments, nuclear and cytoplasmic proteins were first extracted from transfected cells prior to resolution on SDS-PAGE. As shown in Fig. 3, the anti-p50 antibody detected the intrinsic p50 and p105 proteins as well as a third band on the gel which corresponds to the ectopic p50. The molecular weight difference between trans-fected and endogenous p50 is a consequence of the pMT2T-p50 construction (45). Ectopic p50 is as predominantly nuclear as the intrinsic p50; upon expression of IκB-α, a significant reduction in nuclear levels of ectopic p50 could be observed (Fig. 3).

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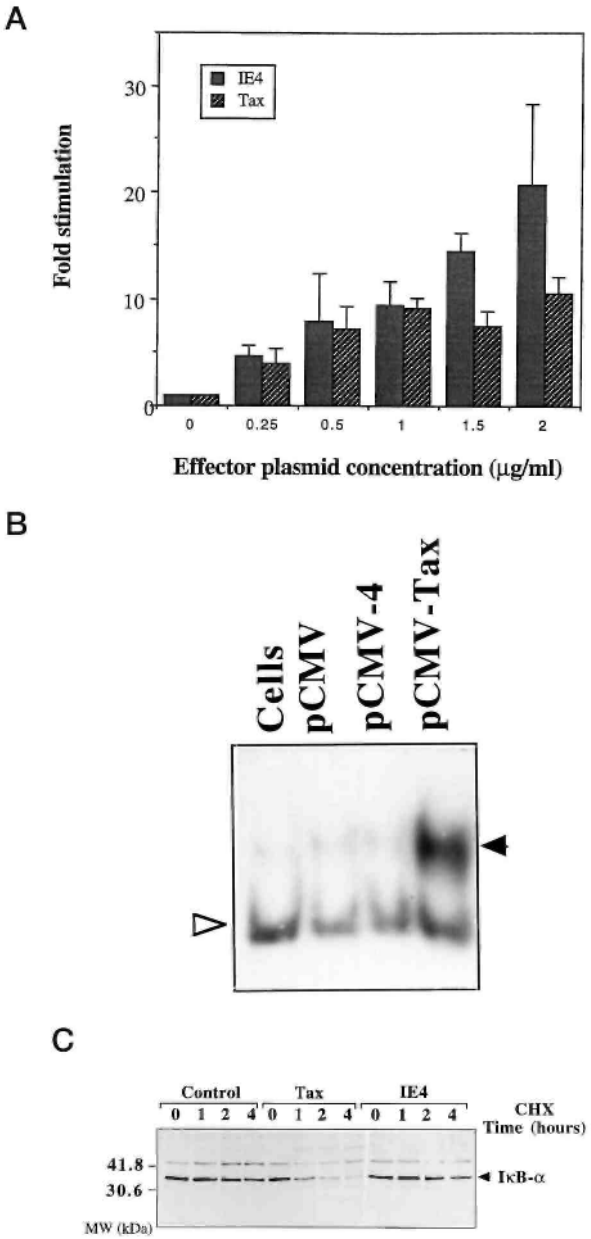

FIG. 2. NF-κB binding activity in IE4-transfected cells. A, HeLa cells were transfected by lipofection with increasing concentrations of IE4 (pCMV-4)- and Tax (pCMV-Tax)-expressing vectors as indicated. Total input DNA was normalized by using the pCMV vector. -Fold stimulation of CAT activity was determined as described in the legend to Fig. 1 and presented as means of three independent experiments (± standard error of the mean). B, nuclear extracts were prepared from non-transfected cells and from cells transfected with 2.5 μg of pCMV (vector without insert), 2.5 μg of pCMV-4 (expressing IE4), and 2.5 μg of pCMV-Tax harvested 24 h after transfection. These extracts were then incubated with the ^{32}P-labeled probe encompassing the κB elements of the HIV-1 LTR. DNA-protein complexes (specified by the filled arrow) were resolved on a 6% polyacrylamide gel. The open arrow refers to a nonspecific band. Seven independent experiments were performed to determine induction of binding activity. C, IκB-α expression was monitored in cytoplasmic extracts prepared from cells transfected with the pCMV vector (control), the pCMV-Tax vector, or the pCMV-4 vector. Forty-eight hours after transfection, cytoplasmic extracts were prepared after treatment with cycloheximide (CHX) for the indicated times and immunoblotted with an IκB-α-specific antiserum. The positions of prestained molecular weight markers are indicated.

No clear effect of IE4 on these levels could be evidenced when the IE4-expressing vector was included.

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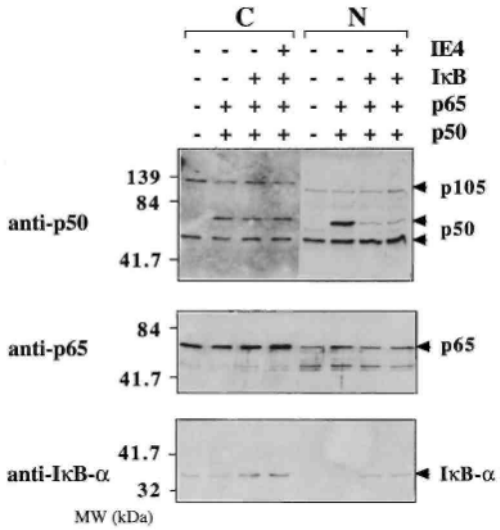

FIG. 3. Influence of IE4 on ectopic IκB-a degradation in HeLa cells. p50, p65, and IκB-α expression was followed in cells co-trans-fected with the p50-, p65-, IκB-α-, and IE4-expressing vectors (1 μg each). Twenty-four hours after transfection, nuclear and cytoplasmic extracts were fractionated and immunoblotted with antisera specific for p50, p65, and IκB-α. Two arrows specify the p50 subunits, the upper arrow corresponding to the transfected p50 and the lower arrow indicating the endogenous protein. Quantification of band intensities was performed by photodensitometry analysis of the autoradiograms.

Similarly, IE4 had no influence on the nuclear p65 levels in the presence of IκB-α (Fig. 3). These observations were connected with the analysis of IκB-α levels. As expected, ectopic IκB-α was predominantly cytoplasmic (Fig. 3). Presence of IE4 in the transfected cells did not lead to a modification in either cytoplasmic or nuclear levels of IκB-α. The second pathway of investigation, which was to follow intracellular localization of the NF-κB/Rel proteins by immunofluorescence, confirmed that expression of IE4 had no significant effect on the intracellular repartition of p50 and p65 (data not shown).
It has been demonstrated that the p105 precursor of the p50 subunit of NF-κB also exhibits IκB-like activity (54, 56). Because IE4 appears incapable of overriding cytoplasmic retention of p50 and p65 by IκB-α, we investigated whether it could exert its action on p105. No effect was detected on the intrinsic p105 levels (data not shown). Multiple cotransfections were performed with p105-, p65-, and IE4-expressing vectors followed by immunoblotting and immunofluorescence. Under these conditions, IE4 was not capable of influencing p105 or p50 levels, or p65 intracellular repartition (data not shown). These data suggest that the ability of IE4 to activate transcription through **κ**B motives is not corroborated with a capacity to override the inhibitory activities of p105.

IE4 Interacts with Several Transcription Factors in Vitro—

The results obtained from the gel shift assays and the Western blot analysis strongly suggest that the NF-κB-dependent activation of the LTR by IE4 must depend on other mechanisms than the translocation of NF-κB to the nucleus. Stimulation of transcription by activators results from multiple direct and indirect interactions between these activators, the co-activators and components of the basal transcription complex (57). Thus, p65, which possesses an acidic transactivation domain, was shown to interact with the general transcription factors TBP and TFIIB (58, 59) as well as with the p300 co-activator (60). Due to the lack of information concerning the molecular properties of IE4, its capacity to bind *in vitro* to TBP and TFIIB was investigated. A fusion protein between GST and IE4 was expressed in *E. coli* and purified on glutathione-Sepharose beads.

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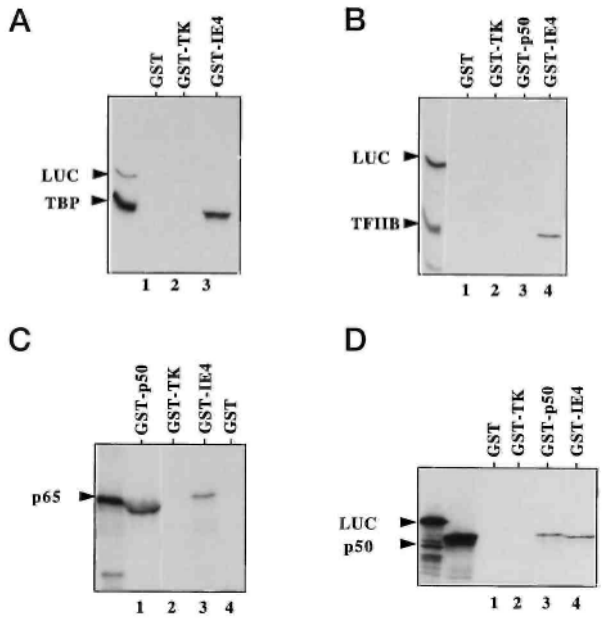

FIG. 4. In vitro interaction of IE4 with TBP, TFIIB, p50, and p65. Equal amounts (5 μl) of in vitro translated [^{35}S]methionine-labeled TBP (A), TFIIB (B), p65 (C), and p50 (D) were incubated with the fusion proteins GST, GST-TK, GST-IE4, and GST-p50, which were coupled to Sepharose beads, as indicated at the top of each panel. A fixed amount of [^{35}S]methionine-labeled luciferase was incorporated in each binding reaction. The beads were extensively washed in NETN and eluted in SDS sample buffer before proteins were resolved by 12% SDS-PAGE. Experiments were repeated three times.

A GST-TK protein fusion that carries VZV thymidine kinase was purified according to the same procedure and used as a negative control in addition to GST alone. p50 fused to GST also served as a ligand in this experiment. Equal amounts of *in vitro* translated [^{35}S]methionine-labeled TBP and TFIIB were incubated with GST, GST-TK, GST-p50, and GST-IE4-coupled Sepharose beads. A definite amount of ^{35}S-labeled luciferase protein was also included in each interaction reaction. After extensive washing, which was monitored by disappearance of the luciferase signal, bound proteins were eluted in 1**X** SDS sample buffer and analyzed by SDS-PAGE. Most of the TBP and TFIIB interacted specifically with GST-IE4 (Fig. 4, *A* and *B, lanes 3* and *4),* whereas they were not retained by the control GST and GST-TK-coupled Sepharose beads (Fig. 4, *lanes 1* and *2* in both *A* and *B).* No interaction of TBP and TFIIB with GST-p50 could be evidenced (Fig. *4B, lane 3;* data not shown). VZV IE63 (in a GST-IE63 fusion protein), another VZV regulatory protein, was also tested for its ability to bind to TBP and TFIIB under the same conditions; no definite interactions could be demonstrated (data not shown). Because NF-κB is required for the IE4-mediated stimulation of the HIV LTR, we also assessed the binding of IE4 to p50 and p65. As shown in Fig. 4 *(C* and *D),* GST-IE4 could associate *in vitro* with p65 and p50 *(lanes 3* and *4,* respectively). No interaction was detected with the GST or GST-TK controls *(lanes 2* and *4* and *lanes 1* and *2,* respectively). As expected, radiolabeled p65 or p50 interacted with GST-p50 (Fig. *4C, lane 1;* Fig. *4D, lane 3).* To ensure that interactions were not an artifact due to the GST-IE4 construct, reciprocal experiments involving GST-p50 and GST-TFIIB fusion proteins and *in vitro* translated IE4 were conducted. The GST-p50 and GST-TFIIB ligands were able to bind efficiently to the [^{35}S]methionine-labeled IE4, because the IE4 protein was detected in the GST-p50 and GST-TFIIB eluates but not in that from the GST and GST-TK immobilized on glutathione-Sepharose beads (Fig. 5*A*). Binding was detected when the GST-IE4 construct was used as a ligand for IE4, suggesting that the IE4 protein could oligomerize *in vitro.*
To determine which regions of IE4 are involved in these interactions, mutant IE4 proteins were engineered. STOP393 encodes a truncated protein of 393 amino acids that lacks the cysteine-rich COOH-terminal cluster (13), whereas C426S encodes an IE4 mutant in which cysteine residue at position 426 has been changed to a serine. As shown in Fig. *5A,* the COOH-terminal mutants STOP393 and C426S still bound to GST-IE4, GST-p50, and GST-TFIIB, although with relatively less affinity than the wild-type (WT) IE4 protein. When assayed in transient transfections, both mutations totally abrogated transactivation of the HIV LTR as illustrated in Fig. *5B.* These results were in accordance with previous data (13) on the SV40 promoter. The NH_2-terminal half of the IE4 polypeptide possesses

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an acidic-rich region in the first 65 amino acids as well as arginine-rich stretches interspersed from amino acids 67 to 182. These regions could potentially be important in the properties of IE4. Therefore, three truncation mutants were tested in the GST-chromatography binding assay. *In vitro* translated Δ65 and Δ81, which lack the acidic region and both the acidic region and an arginine-rich run respectively, bound to GST-IE4, GST-p50, and GST-TFIIB with affinity similar to WT IE4, although signals are weaker. In contrast, an IE4 mutant protein which lacks the entire arginine-rich region, Δ182, failed to interact with all GST ligands (Fig. 5*A*). Very interestingly, when their functional properties were studied *in vivo*, both Δ65 and Δ81 mutants elicited unmodified transactivating properties on the HIV LTR, whereas Δ182 failed completely to stimulate the HIV-CAT target (Fig. *5B).* The arginine-rich region appeared critical for the activation of the HIV-1 LTR, whereas it could not be excluded that this lack of transactivation reflected a major structural change of IE4 induced by the truncation of the first 182 amino acids. These data indicate that IE4 was capable of interacting *in vitro* with itself, TBP, TFIIB, p50, and p65. These interactions were likely dependent on an argi-nine-rich region present in the NH_2-terminal half of the molecule. The COOH-terminal end and the cysteine at position 426 could also be important for the binding activity, as the interaction was diminished when the COOH-terminal end was absent or mutated.

Discussion

The molecular mechanisms underlying VZV IE4 regulatory properties are still greatly misunderstood, although it now seems clear that IE4 is a strong transactivator whether expressed alone or in synergy with VZV IE62 (2, 4, 5, 12, 43). In the past, interesting evidence has been presented concerning the identification of specific upstream elements involved in IE4 inducibility (13). Moreover, the same study established that IE4 could act transcriptionally in A3.01 cells using the HIV-1 LTR as a model system for investigation. Our previous work based on mRNA steady-state level analysis had led us to conclude that IE4 could fulfill its functions through transcriptional and post-transcriptional mechanisms (11, 12). Because the transcriptional effect was also studied using the HIV-1 LTR as experimental system, the present work was focused on the pathway of LTR activation.
The first main observation from the transfection assays is that the κB-responsive elements are essential to IE4-mediated activation in HeLa cells, inasmuch as an LTR mutated at both **κ**B sites is refractory to the action of IE4. Interestingly, this mutant construct was still transactivated in Vero cells, indicating that the other *cis*-acting sequences present in the LTR contribute to IE4 inducibility in these cells. Co-expression of the cytoplasmic inhibitor IκB-α prevented transactivation of the HIV LTR by IE4, which clearly confirmed that NF-κB is required in HeLa cells.

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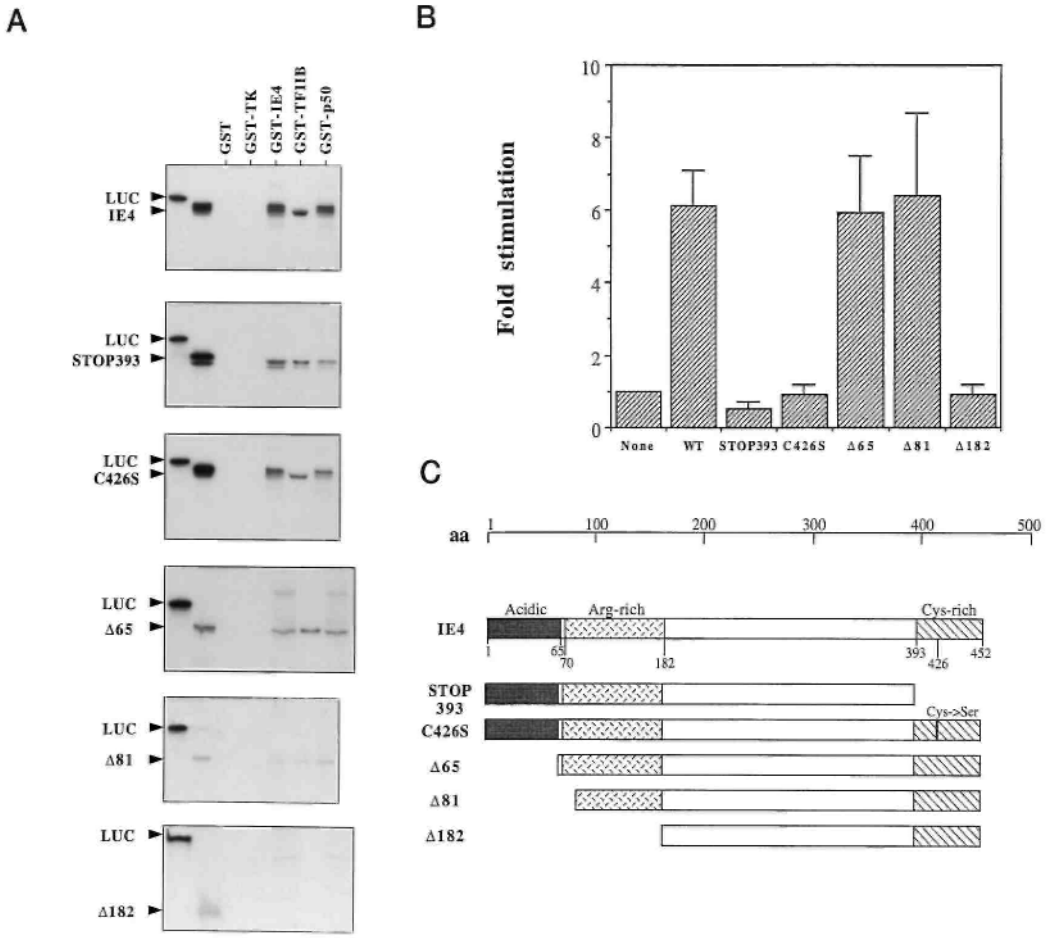

FIG. 5. Role of the COOH-terminal and NH_2-terminal regions of IE4 in its regulatory functions and its interaction with transcription factor IIB and p50. A, in vitro protein-protein binding by IE4 mutants. Equal amounts of in vitro translated [^{35}S]methionine-labeled wild-type IE4, STOP393, C426S, and Δ65 were incubated with bacterially expressed GST, GST-TK, GST-IE4, GST-TFIIB, and GST-p50, which were coupled to Sepharose beads, as indicated at the top of each panel. [^{35}S]Methionine-labeled Δ81 and Δ182 were added in larger amounts because the proteins were labeled in vitro with less efficiency and signals were weaker. A fixed amount of [^{35}S]methionine-labeled luciferase was also incorporated in each binding reaction. The beads were extensively washed in NETN and eluted in SDS sample buffer before proteins were resolved by 12% SDS-PAGE. Experiments were repeated three times independently. B, stimulation of the HIV-1 LTR by IE4 requires both cysteine-rich COOH-terminal and arginine-rich regions. HeLa cells were co-transfected by lipofection with 0.25 μg of pHIV-CAT and 0.5 μg of IE4 expression vectors (WTIE4 or mutant proteins) as indicated. CAT activity was determined 44 h after transfection. -Fold stimulation of CAT activity was calculated relative to the basal level of reporter plasmid in presence of control plasmid alone, arbitrarily set at 1. The results shown are representative of three independent experiments, and error bars represent standard error values. C, schematic representation of wild-type IE4 and mutant proteins. The wild-type IE4 contains a highly acidic amino-terminal region (dark stippled area), an arginine-rich region (stippled area), and a cysteine-rich carboxyl-terminal cluster (hatched area). The positions are denoted under the bar. The mutation STOP393 is an insertion of a STOP codon at position 393, which leads to the expression of a truncated protein lacking the cysteine-rich cluster. The mutation C426S is the substitution of a serine residue for a cysteine residue at position 426. The mutation Δ65 is a deletion of amino acids 1-65 removing the acidic region. The mutation Δ81 is a deletion of residues 66-81 removing an arginine-rich stretch. The mutation Δ182 is a deletion of residues 82-182 completely removing the arginine-rich region.

Despite some controversy as to the nature of the HSV-1 IE proteins implicated in the transactiva-tion of the LTR by HSV-1, it is currently thought that ICP27, the IE4 homolog in HSV-1, has no effect on

HIV-1 LTR expression (32, 39). Therefore, our study demonstrates another difference in the properties of IE4 and ICP27, inasmuch as IE4 is able to activate the HIV-1 LTR.

Several viral proteins (LMP1, Tax, IE1, etc.) are capable of triggering NF-κB induction (35, 36, 38, 61). Transactivation of the HIV-1 LTR by LMP1 of EBV or IE1 of CMV is associated with an increase in NF-κB binding to the κB-responsive elements (35, 36). In HeLa cells, no significant NF-κB induction was observed after transient transfection of the IE4 expression vector, whereas an important induction was seen with Tax. It should be mentioned here that LTR activity was stimulated to similar if not higher levels by IE4 than by Tax. Moreover, the strong NF-κB induction seen with Tax indicates that our experimental conditions allow for detection of such an effect and that the poor signal seen with IE4 is not an artifact due to a low transfection efficiency. Therefore, IE4 is not a strong inducer of NF-κB, unlike Tax, LMP, or IE1. Recent lines of evidence indicate that Tax promotes translocation of NF-κB from the cytoplasm into the nucleus by liberating NF-κB from several distinct cytoplasmic complexes (54, 55, 61, 62). We detected the IκB-α degradation induced by Tax but, under the same experimental conditions, IE4 was not capable of triggering IκB-α proteolysis. Moreover, the Western blot analysis of transfected IκB-α levels in the presence of IE4 corroborated the CAT assays, illustrating the absence of IE4 transactivation in the presence of ectopic IκB-α. It should be noted that experiments with transiently transfected Tax have previously allowed detection of a variation in levels or repartition of NF-κB/Rel proteins by Western blot or immunofluorescence (54, 56). The degradation of IκB-β, another isoform of IκB, is frequently associated with agents that elicit a persistent NF-κB response, including bacterial lipopolysaccharide and interleukin-1 (63).

Similarly, the constitutive activation of NF-κB in Tax-expressing cells could possibly be maintained by the chronic down-regulation of IκB-β protein expression (55). IκB-β turnover in IE4-transfected cells could also be investigated in the future; however, this hypothesis seems unlikely as no NF-κB induction is observed with IE4.

Our *in vitro* binding experiments shed new light on the mechanism by which IE4 could cooperate with NF-κB, thereby activating transcription of the HIV-1 LTR. An interaction between IE4 and the TBP or TFIIB components of the basal complex of transcription was evidenced in our GST pull-down experiment. A significant binding of IE4 to itself and to the p50 and p65 NF-κB subunits was also observed. The use of mutant proteins provided interesting data on the properties of IE4. Deletion of the COOH-terminal region rich in His/Cys residues led to a total loss of transactivation, although the capacity to interact with itself or with p50 or TFIIB was not totally diminished. Therefore, the 59 COOH-terminal amino acids are not sufficient in themselves to mediate the physical interaction between IE4 and the GST ligands. The point mutation at residue 426 showed that this Cys is not a critical residue for the binding of IE4 *in vitro* but is very critical for the transactiva-tion of the HIV-1 LTR. Two NH_2-terminal truncated mutants that are fully functional *in vivo* are still capable of interacting with IE4, TFIIB, and p50. The amino-terminal acidic region is thus not required for the transactivation of the HIV-1 LTR. This observation is in accordance with a previous report proposing a role for this region in the transactivation of reporter genes carrying minimal poly(A) signals (14). Removal of the arginine stretch from amino acids 70 to 82 did not influence either the binding or transactivating capacities of IE4. In contrast, the Δ182 mutant, which had lost the property to interact with IE4, TFIIB, and p50, displayed an abrogated transactiva-tion capacity. The region between amino acids 82 and 182, which contains arginine- and serine-rich clusters, appears crucial to the regulatory functions of IE4. Although this mutant protein turns out to be as stable as IE4, we cannot exclude that the lack of biological activity of this mutant reflects a major structural alteration. Deletion or mutation of the COOH-ter-minal region prevents transactivation without completely abolishing binding, possibly by disrupting a critical contact point. Despite the COOH-terminal modifications, the interaction could still be mediated by the amino-terminal region. Indeed, a pull-down experiment carried out between a GST-IE4 (1–402) and an *in vitro* translated IE4 containing a stop codon at position 393 demonstrates that these molecules can still dimer-ize (data not shown), suggesting that the amino-terminal part of IE4 plays an important role in dimerization. This dimeriza-tion hypothesis will be tested in the future with the analysis of mutants in a two-hybrid system. Meanwhile, computer analysis of the amino-terminal part of IE4 reveals the presence of a bZIP-basic domain at position 120, within the arginine-rich region. Because this domain has been shown to be required for the dimerization of many transcription factors (64), this observation reinforces the potential importance of the arginine-rich region in dimerization. The arginine-rich motives that have been identified in ICP27 have been implicated in the properties of the protein (65). Mutant proteins deleted in these regions cannot complement infection with an HSV-1 virus deficient in ICP27. The arginine residues are required for correct HSV-1 late gene expression. They could be important in the post-transcriptional functions of ICP27, given that they are involved in RNA binding activity, as shown for RGG motifs found in numerous RNA-binding proteins (66). Our present data suggest that the arginine regions of IE4 could

be involved in physical protein-protein interactions. Deleting this region either removes a critical domain or disrupts the overall structure of the protein in such a way that binding is prevented. Experiments are currently being conducted to investigate the role of these regions in the transcriptional and post-transcriptional properties of IE4.

It has been shown that a GAL4-IE4 chimera transactivates a promoter carrying GAL4-binding sites, suggesting that once IE4 is brought to the vicinity of the promoter it can activate transcription (13). The interactions between IE4 and p50-p65 on the one hand and between IE4 and TBP and TFIIB on the other suggest that IE4 could be targeted to the vicinity of the basal transcription complex through protein-protein interactions with DNA-binding proteins tethered to their sites and could therefore stabilize or enhance processivity of the multim-eric transcription complex. This activity is reminiscent of a co-activator, which stimulates activator-dependent transcription (67), and of Bcl-3 activity, which could tightly associate to p52 homodimers to transactivate through the **κ**B motif (51). p65 also binds to TBP and TFIIB, which contact next to each other the promoter site (58, 59). It could therefore be possible that the redundant binding of IE4 to both general transcription factors and NF-κB increases initiation of transcription. There is accumulating evidence that interactions with multiple transcription factors, activators, and co-activators exist for viral transactivators such as Tax of HTLV-1 or E1A of adenovirus. E1A has been shown to bind to TBP and p65 to promote LTR transactivation. E1A is also capable of binding to p300, a co-activator recruited to a number of unrelated promoters by protein-protein interactions, although this interaction is not involved in LTR transactivation (68). Recently, Tsukada *et al.* (69) have established that Tax stimulates human pro-interleu-kin-1β promoter by direct interactions with the transcription factors NF-IL6 and Sp-1. It is therefore possible that IE4 represents a critical co-activator used by VZV to divert cellular transcription factors and enhance viral-specific gene expression. If so, IE4 could act as an adaptator/bridging factor for the VZV IE62 protein. It is interesting that a co-activating fraction termed USA (upstream factor stimulatory activity) purified from HeLa cells is required for NF-κB activation of the HIV-1 LTR in cell-free transcription systems (70). A PC1 co-factor originating from this USA fraction was shown to enhance p65-dependent activation in this cell-free reconstruction of transcription (59). This USA fraction is also required for activation by USF (upstream stimulatory factor), which is a helix-loop-helix regulatory factor (70). Now, this USF cooperates with IE62 to activate expression of VZV genes 28 and 29 (71). We know that IE4 and IE62 act synergistically to stimulate expression of genes unresponsive to IE4 alone (2, 4, 12, 43), whereas IE62 induces nuclear translocation of IE4 in transfected cells (52). This suggests very strongly that the increase in IE62-mediated transactivation by IE4 could potentially involve a collaboration of IE4 with this USA and USF/IE62, which might be involved in the LTR transactivation. Various experiments are currently under way in our laboratory to further define the interplay between IE4, IE62, activators, co-activators, and transcription factors both *in vitro* and *in vivo.* Direct effects of IE4 on transcription will be envisaged using *in vitro* transcription assays. These should help to elucidate the molecular mechanisms implicated in transcriptional activation by the multifunctional IE4.

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