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Activation of the Human Immunodeficiency Virus Long Terminal Repeat by Herpes Simplex Virus Type 1 Is Associated with Induction of a Nuclear Factor That Binds to the NF-κB/Core Enhancer Sequence

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It has been previously shown that herpes simplex virus type 1 (HSV-1) infection of HeLa cells results in augmentation of gene expression directed by the human immunodeficiency virus (HIV) long terminal repeat (LTR). This effect is presumably mediated by protein interactions with the LTR. We have used two different assays of DNA-protein interactions to study the HSV-induced activation of the HIV LTR. Activation of the HIV LTR is associated with increased protein binding to LTR sequences in a region including the NF-κB/core enhancer and the Sp1 binding sequences as monitored by an exonuclease protection assay. Gel retardation assays demonstrated that HSV-1 infection resulted in the induction of a nuclear factor(s) that binds to the NF-κB/core enhancer sequence. In addition to the activation of the HIV LTR, HSV induction of NF-κB activity may be important for the regulation of HSV gene expression during a herpesvirus infection.

Human immunodeficiency virus (HIV) infection is clinically characterized by a long latency period between the time of infection and the onset of the acquired immune deficiency syndrome (9, 29). Thus, elucidation of the mechanisms that govern the spread and progression of HIV infection may have important clinical implications. Of particular interest are stimuli that can activate the expression of the HIV promoter within the long terminal repeat (LTR) sequences. Such stimuli have been hypothesized to induce HIV gene expression from latent proviruses (9) and thus increase viral replication and disease progression. Extracellular signals, including mitogens such as phytohemagglutinin (PHA) (33, 45, 49), cytokines such as GM-CSF (12), and differentiating agents such as phorbol esters (8, 21, 23, 33, 45, 49) and retinoic acid (27), may activate the HIV LTR. The HIV LTR may also be activated by a number of heterologous viruses (6, 17, 24, 30, 31, 34, 36, 40), including viruses frequently infecting patients with acquired immune deficiency syndrome such as members of the herpesvirus family, papovaviruses, and adenoviruses. The fact that these diverse viruses, each of which has *trans*-activating proteins, all have stimulatory effects on the HIV LTR suggests that activation of the LTR proceeds through a number of cellular regulatory factors (17, 36).

Within an infected cell, the expression and replication of HIV is regulated by a complex array of viral and cellular proteins. The virally encoded *tat* gene has a strong positive regulatory effect resulting in large increases in viral RNA and protein synthesis (5, 17, 32, 37, 41, 42, 51). The *art/trs* gene appears to regulate the processing, transport, or translation of mRNAs for viral structural proteins (10, 47). In addition, HIV encodes a negative regulator of its own expression, the B gene or 3′ *orf* (26).

Cellular transcriptional factors also contribute to the regulation of HIV gene transcription. Three binding sites for the cellular transcriptional factor Sp1 are present in the U3 region of the LTR, 45 to 78 base pairs (bp) 5′ to the RNA cap site (+1) (22). Two short segments homologous to the viral core enhancer sequence (48, 50), as well as to a sequence in the enhancer of the immunoglobulin κ gene (43), are also present in U3, 81 to 103 bp 5′ to the RNA initiation site. This sequence in the immunoglobulin κ gene binds a nuclear factor designated NF-κB (33, 43, 44), and in this paper the homologous sequence in the HIV LTR will be referred to as the NF-κB site. More recently, binding sites for cellular factors have been identified in the negative regulatory region of the HIV LTR (15, 16). In this study, we have sought to characterize changes in the interactions of cellular transcription factors and the HIV LTR following infection with herpes simplex virus (HSV) that may mediate the HSV-induced activation of the LTR. These studies have identified the induction of NF-κB activity following the infection of HeLa cells with HSV type 1 (HSV-1).

MATERIALS AND METHODS

Cells and viruses. HeLa-JW cells (gift of Barrie Carter) were grown in a 1:1 mixture of minimal essential medium and 199 medium containing penicillin G (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. BHM 23 cells (18) were grown in RPMI 1640 medium supplemented with penicillin, streptomycin, glutamine, and 15% fetal bovine serum. Stocks of HSV-1 strain KOS were propagated and titers were determined in Vero cells as previously described (11).

Plasmid constructions. The plasmid pBennCAT was constructed by ligation of the U3 and R regions of the HIV LTR

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to the chloramphenicol acetyltransferase (CAT) gene as previously described (17). The HIV *tat* gene was expressed under the control of the HIV LTR by the plasmid pAR, also previously described (17). A 236-bp *Ava*I-*Hin*dIII fragment from the HIV LTR, positions −155 bp to +81 bp with respect to the initiation of RNA transcription, was cloned into pUC8 and pUC19 for use in the exonuclease protection assays.

Transfections and CAT assays. Transfection of plasmid DNAs was carried out in 25-cm^2 screw-cap flasks on 60 to 70% confluent monolayers of HeLa cells, by the calcium phosphate coprecipitation method of Graham and Van der Eb (20). At 4 h posttransfection, cells were shocked with 15% glycerol in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline for 2 min, washed, and incubated in growth medium. At 24 h after transfection, cells were infected with 5 PFU of HSV-1 per cell. Cells were collected for CAT assays at 48 h posttransfection. Extracts were prepared and CAT assays were performed as described by Gorman et al. (19). Following incubation with 0.5 μCi of [^{14}C]chloramphenicol, extracts were extracted with ethyl acetate, dried, and analyzed by thin-layer chromatography as previously described (19).

Nuclear extracts. For the preparation of nuclear extracts, HeLa cells were grown as monolayer cultures in 850-cm^2 roller bottles. When cells reached confluence, they were harvested or infected with HSV-1 strain KOS (5 PFU per cell). Cells were harvested 20 h postinfection at a time point when HSV-induced cytopathic changes were evident in the culture. Cells were removed from the monolayer by treatment with 1 mM EDTA and washed in phosphate-buffered saline. BHM 23 cells were grown in suspension in 750-ml flasks. They were harvested by centrifugation at 1,000 × *g* and washed in phosphate-buffered saline. Nuclear extracts were prepared by the procedure of Dignam et al. (7) modified by Quinn et al. (39) such that buffer C contained 20% glycerol–0.45 M NaCl and buffer D contained 20% glycerol–80 mM KCl. Protein concentrations were determined by the method of Bradford (4).

Exonuclease protection assays. A 646-bp *Hae*II fragment was purified from the pUC8 and pUC19 subclones of the *Ava*I-*Hin*dIII LTR fragment by agarose gel electrophoresis and elution onto DEAE paper (Schleicher & Schuell, Manchester, N.H.). The fragments (500 ng) were 3′ end labeled with ^{32}P by incubation with Klenow fragment DNA polymerase (Amersham Corp., Arlington Heights, Ill.) in the presence of [^{32}P]dATP and [^{32}P]dCTP (3,000 Ci/mmol; Amersham) as previously described (18). Labeled DNA fragments were subjected to the immunobead-based exonuclease assay as previously described (18, 39). This assay allows thorough washing of protein-DNA complexes bound to immunobeads; thus, irrelevant nuclear proteins and excess nonspecific competitor DNA [poly(dI-dC)] are removed prior to exonuclease treatment, allowing more complete digestion to occur. Briefly, the ^{32}P-labeled *Hae*II fragments (10 ng) were prebound to form an immunobead-DNA complex (25). A β-galactosidase-*lac* repressor fusion protein (750 ng) (13) was allowed to bind to the *lac* repressor binding site of the pUC fragment and to rabbit anti-β-galactosidase antibodies (Promega Biotec, Madison, Wis.). This complex was bound to immunobeads coated with mouse anti-rabbit immunoglobulin antibodies (Bio-Rad Laboratories, Richmond, Calif.). Binding of nuclear proteins to the DNA-immunobead complex was performed by the addition of 20 to 50 μg of nuclear protein and incubation for 30 min at room temperature in a 15-μl volume containing 8 μg of poly(dI-dC) in 80 mM KCl–20 mM HEPES (pH 7.9)–0.2 mM EDTA–20% glycerol. For competition experiments, 10 ng of unlabeled, double-stranded competitor oligonucleotide was included in the protein binding reaction. The beads were centrifuged for 2 min at 4°C, suspended in exonuclease buffer (18), and digested with 3 to 4 U of λ exonuclease (Bethesda Research Laboratories, Gaithersburg, Md.) for 3 min at 30°C. The reaction was stopped and DNA was removed from the immunobeads by the addition of an equal volume of exonuclease stop buffer (18). The DNA was then extracted with an equal volume of phenol, ethanol precipitated, suspended in formamide loading buffer, and electrophoresed on a 6% polyacrylamide–7 M urea gel. DNA sequence reactions were used as size markers (28). The gel was autoradiographed for 1 to 3 days at −70°C with an intensifying screen.

Gel retardation assays. Gel retardation assays were performed as described previously (39). Double-stranded oligonucleotides were made by annealing complementary oligonucleotides synthesized on a 380A DNA synthesizer (Applied Biosystems, Foster City, Calif.). They were then ^{32}P labeled by incubation with Klenow fragment of DNA polymerase in the presence of all four [^{32}P]deoxynucleoside triphosphates. Radiolabeled oligonucleotides (3,000 to 7,500 cpm) were incubated with 8 μg of nuclear protein and 3.0 μg of poly(dI-dC) in a total volume of 6 μl. Nonradioactive DNAs were added for competition experiments. DNA-protein complexes were analyzed by electrophoresis on 4% acrylamide gels run in 0.5× Tris-borate buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA).

Synthetic oligonucleotides used for these studies and for exonuclease protection assays were as follows: NFκB, 5′-GCTACAAGGGACTTTCCGCTGGGGACTTTCCAG-3′, 5′-GCCTGGAAAGTCCCCAGCGGAAAGTCCCTTGTA-3′; NF-κB*, 5′-GCTACAACTCACTTTCCGCTGCTCACTTTC CAG-3′, 5′-GCCTGGAAAGTGAGCAGCGGAAAGTGAGT TGTA-3′; Sp1, 5′-GGGAGGCGTGGCCTGGGCGGGACT GGGGAGTGGCG-3′, 5′-CCCGCCACTCCCCAGTCCCGCC CAGGCCACGCCTC-3′; Tar, 5′-CTCTGGCTAACTAGGGA ACCCACTG-3′, 5′-CAGTGGGTTCCCTAGTTAGCCAGAG AGCT-3′; ST, 5′-AGCTTCGAGCCCTCAGATGCTGCA-3′, 5′-AGCTTGCAGCATCTGAGGGCTCGA-3′.

RESULTS

Activation of the HIV LTR by HSV-1 infection of HeLa cells. The ability of HSV to *trans* activate HIV LTR-directed expression of heterologous indicator genes such as CAT, as well as augment HIV replication, has been demonstrated in a variety of cell types (17, 30, 31, 36, 40). To confirm that the cells we were using for biochemical studies exhibited the HSV-induced activation of the LTR, we performed the CAT assays shown in Fig. 1. Cells were transfected with pBenn-CAT and infected with HSV-1 (middle lane) or cotransfected with the HIV *tat* gene. Even in the absence of the *tat* gene, HSV infection augments CAT activity 50-fold as quantitated by scintillation counting of acetylated and unacetylated forms of [^{14}C]chloramphenicol, separated as shown in Fig. 1.

HSV-1 infection is associated with alterations in exonuclease-protected domains in the HIV LTR. Experiments with deletion mutants derived from LTR-CAT constructions (30, 36) or defined fragments from the enhancer region of the HIV LTR (30) have led to the identification of a region of the LTR including both the NF-κB and the Sp1 binding sites as being important in HSV activation. To determine whether HSV-induced activation of the LTR might be mediated by

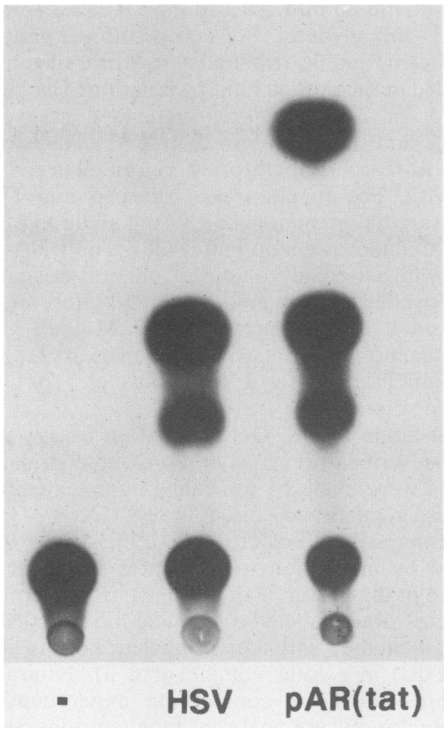


FIG. 1. Effects of HSV-1 and *tat* on the HIV LTR. HeLa cells were transfected with 5 μg of HIV LTR CAT plasmid DNA alone (lanes - and HSV) or cotransfected with HIV LTR CAT and 10 μg of pAR(*tat*) DNA [lane pAR(tat)]. At 24 h posttransfection, cells were infected with HSV-1 (lane HSV). Cell extracts were harvested at 48 h posttransfection.

alterations in protein binding to Sp1 or NF-κB sites, we have used an exonuclease protection assay developed by Levens and co-workers (18, 25, 39). This assay has been useful for rapidly assessing DNA-protein interactions over DNA segments of several hundred base pairs in length. In this assay, the binding of nuclear proteins to radiolabeled DNA fragments blocks the processive digestion of the DNA by λ exonuclease. Discrete species of DNA are generated following nuclease treatment in the presence of nuclear factors, allowing the determination of sites in the DNA protected from digestion by protein binding. For these assays, a 236-bp *Ava*I-*Hin*dIII fragment of the HIV LTR (positions −155 to +81 with respect to the start of transcription) was subcloned into pUC8 and pUC19 (Fig. 2). The two plasmids contain the same LTR insert cloned in opposite orientations relative to the *lac* operator in pUC. By using both constructs, the 5′ (pUC19) and 3′ (pUC8) boundaries of protected domains due to nuclear protein binding within the insert can be determined. For the assays shown in Fig. 3, a 646-bp *Hae*II fragment containing both the HIV LTR insert and the adjacent pUC vector *lac* operator sequence was end labeled with ^{32}P, bound to the immunobeads, incubated with nuclear protein extracts, and then treated with λ DNA exonuclease. Digestion products were resolved by electrophoresis on denaturing polyacrylamide gels.

Figure 3 shows an exonuclease protection assay performed with nuclear extracts derived from BHM 23, a human B-cell line known to have an abundance of NF-κB binding protein (18) and from HeLa cells with and without HSV infection. Both the pUC8 and pUC19 constructs were examined. As controls, immunobeads were incubated without λ exonuclease or without nuclear extracts. A prominent band can be seen near the top of each gel in all lanes digested with exonuclease, representing the binding of the *lac* repressor to the *lac* operator. With the pUC8 construct mapping the 3′ boundary of binding domains, prominent exonuclease stop sites were identified in the BHM 23 extract at −35, −50, and −60 bp relative to the start of RNA transcription. In the extract from uninfected HeLa cells, the −60 stop site produced an intense band. Repeated experiments failed to show the −35 stop site and showed only faint protection at the −50 site in uninfected HeLa cells. In extracts of HSV-infected HeLa cells, each of the three exonuclease stop sites

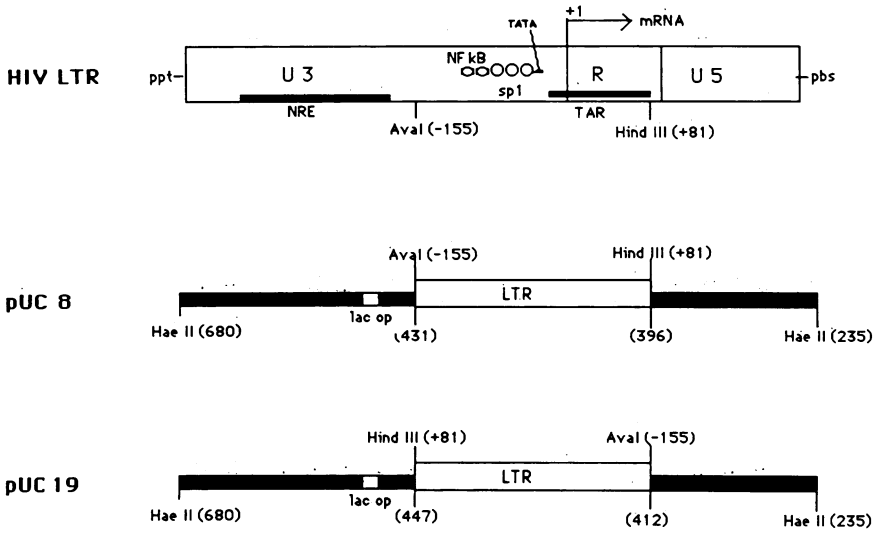


FIG. 2. Construction of plasmids used for exonuclease protection assays. A 236-bp *Ava*I-*Hin*dIII fragment of the HIV LTR was subcloned into pUC8 and pUC19 as shown. These two clones placed the LTR sequences adjacent to the *lac* operator in pUC in opposite orientations. The positions of the *Ava*I and *Hin*dIII sites are indicated with respect to the start of RNA transcription in the HIV LTR. The numbers in parentheses below the line for each pUC construct indicate the position of each restriction site with respect to the nucleotide sequence of the pUC vector.

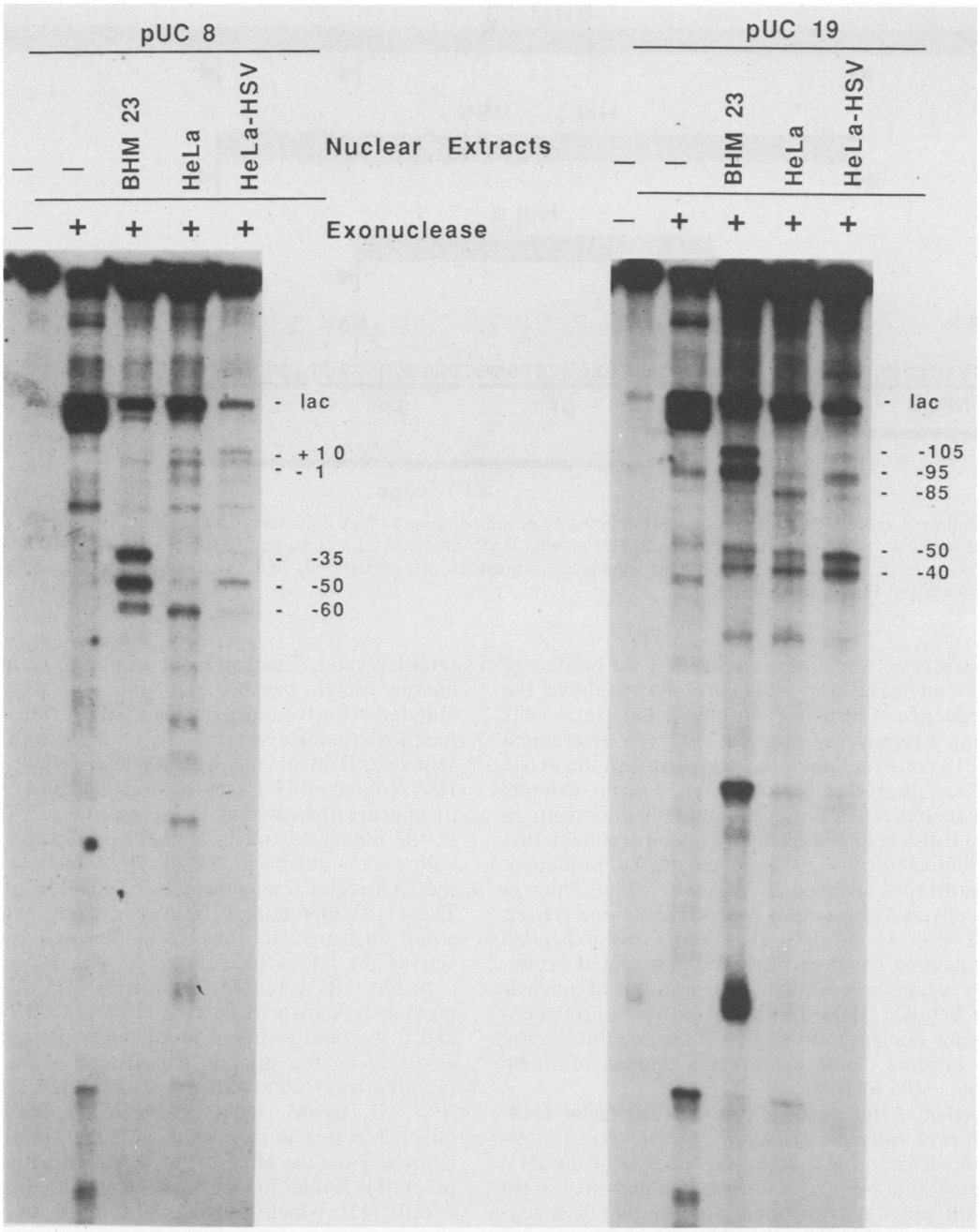


FIG. 3. λ exonuclease protection assays. Exonuclease protection assays were performed by using nuclear extracts of human B cells (BHM 23), HeLa cells, and HeLa cells infected with HSV. Both the pUC8 (3′ boundaries of exonuclease stops) and pUC19 (5′ boundaries of the exonuclease stops) constructs were examined. Nuclear extracts and λ exonuclease were added as shown in the lane headings. The stop band due to the *lac* operator is shown. The numbers to the right of the bands represent the distance in nucleotides of that stop site relative to the RNA cap site in the LTR.

observed in BHM 23 cells was present, with the −50 site being most prominent.

In the assays which used the pUC19 construct, the 5′ boundaries of protein binding sites in the LTR can be determined. With BHM 23 extracts, λ exonuclease digestion was stopped at approximately −105 and −95 bp (based on restriction fragment size markers). Two additional smaller fragments are seen; one maps at the junction of LTR and pUC sequences, and the smaller band corresponds to a site within the pUC vector. In uninfected HeLa cells, a stop site occurred at −85 with a weak stop site at −95. HSV-infected HeLa cell extracts produced a strong stop site at −95 with a fainter band at −105. In all cell lines, additional stop sites occurred further 3′ at positions −50 and −40. These did not appear to vary in any of the cells examined and may represent binding to more 3′ regulatory sequences such as the TATA box.

The positions of the exonuclease stop sites identified in the region of the NF-κB and Sp1 sites for each of the three nuclear extracts are shown schematically in Fig. 4. The

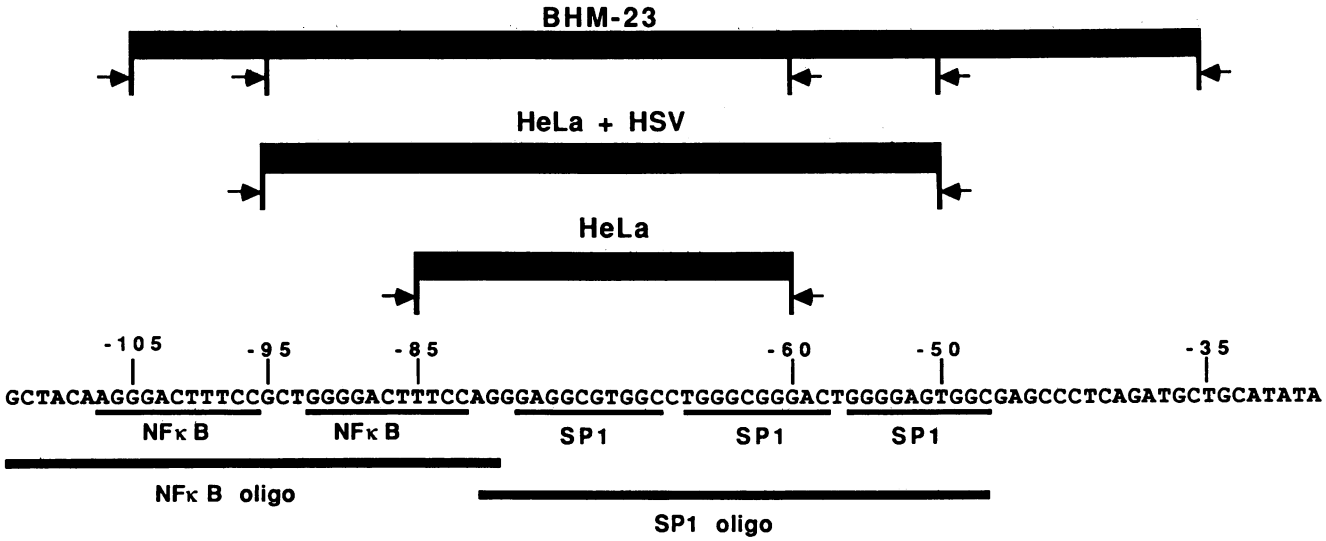


FIG. 4. Location of exonuclease protection sites induced by protein binding to core enhancer. The sequence of the HIV LTR is indicated. The boundaries of the strongly protected domains of HeLa cells, HSV-infected HeLa cells, and BHM 23 cells are indicated by arrows. The Sp1 recognition sequences and the NF-κB core enhancer sequences are underlined, and the regions used to prepare the specific oligonucleotides used in Fig. 5 are indicated.

extent of the protected domains, as defined by the borders of the prominent nuclease stop sites, are shown above the nucleotide sequence. Extracts from uninfected HeLa cells produce strong exonuclease stops at −85 (as determined from the pUC19 construction) and −60 (seen with the pUC8 fragment). Thus, in HeLa cells a 25-bp domain exhibits strong exonuclease protection. The protected domain in HSV-infected HeLa cells is expanded as compared with that seen in the uninfected cells. In the infected cells, prominent exonuclease stop sites are seen at −95 and −50 bp. Thus, in the infected cells, a 45-bp region encompassing one NF-κB and two Sp1 sites is strongly protected. This expanded domain of protection has been observed in repeated exonuclease assays using independent preparations of nuclear proteins (see below). BHM 23 nuclear extracts produce a series of strong nuclease protection stops in this region whose distal borders define a protected domain of 70 bp extending from −105 to −35.

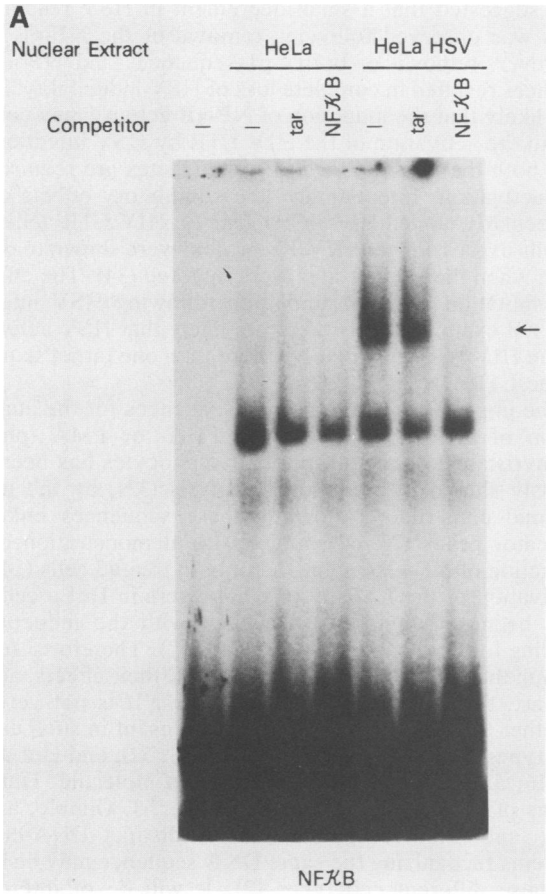
HSV-1 infection of HeLa cells induces nuclear factor binding to the κB/core enhancer sequence. The observed alterations in exonuclease protection in the segment of the HIV LTR containing the NF-κB and Sp1 binding sites were consistent with previous functional studies that had suggested that the target of HSV activation of the LTR mapped to this region (30, 36). To analyze which DNA sequences in this region might be exhibiting increased binding to nuclear proteins following HSV infection, we performed gel retardation assays with oligonucleotides that spanned the region of exonuclease protection. Two oligonucleotides were used (Fig. 4), one containing the two NF-κB sites and a second containing the three Sp1 binding sites. These oligonucleotides were end labeled with ^{32}P, incubated with nuclear extracts derived from HSV-infected and uninfected HeLa cells, and electrophoresed on polyacrylamide gels. Autoradiographs of these gel retardation experiments are shown in Fig. 5.

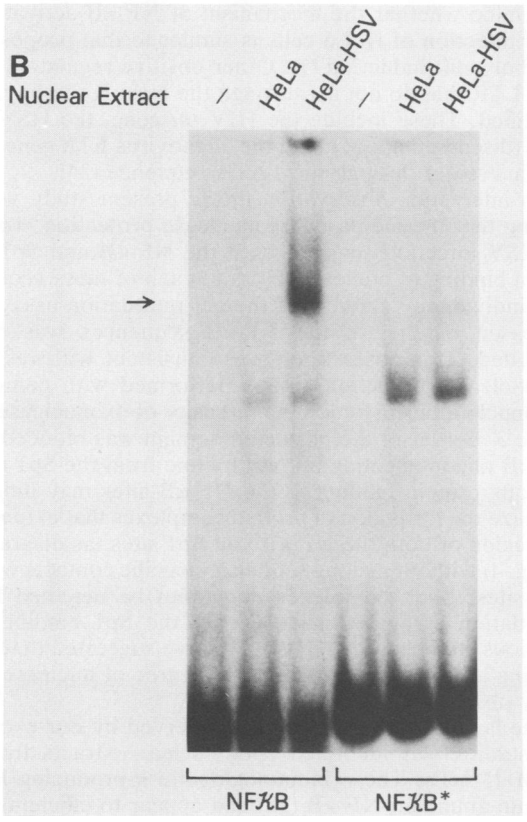
The results of a gel retardation experiment using the NF-κB oligonucleotide are shown in Fig. 5A. Incubation of the NF-κB probe with nuclear extracts derived from uninfected HeLa cells results in the production of a single retarded band. This band may represent an abundant DNA-binding protein capable of binding both to NF-κB and to a mutated NF-κB sequence (Fig. 5B) previously shown to be functionally inactive (33). In contrast, when the NF-κB sequence is incubated with nuclear extract prepared from HSV-1-infected HeLa cells, a second band of slower mobility appears (indicated by the arrow in Fig. 5A). The intensity of the induced band is markedly reduced by competition with excess unlabeled NF-κB DNA but not by DNA from the TAR region (map positions +35 to +54) of the HIV LTR. Thus, HSV infection of HeLa cells results in the induction of a new nuclear factor that has binding activity to the NF-κB sites of the HIV LTR.

In Fig. 5B, a mutant form of the HIV NF-κB sequence previously shown to be devoid of NF-κB binding activity and to be nonfunctional in enhancer assays (33) has been analyzed by the gel retardation assay. The HSV-induced retarded band seen with the radiolabeled NF-κB sequence (Fig. 5B, arrow) is not present when the mutant NF-κB oligonucleotide is incubated with the HSV-infected HeLa extract. Thus the HSV-induced NF-κB activity has binding properties similar to that observed in activated human Jurkat T cells (33), which also fails to bind to the mutant NF-κB site.

The binding properties of the oligonucleotide containing the HIV LTR Sp1 sites are shown in Fig. 5C. Nuclear extracts derived from both uninfected and HSV-infected HeLa cells produce similar patterns of DNA retardation. Both extracts result in the appearance of a specific retarded band (Fig. 5C, arrow) of similar intensity and mobility. It is interesting that the band seen in HSV-infected cells may be of slightly faster mobility; however, the significance of this slight change is unclear. Certainly, HSV infection of the HeLa cells does not result in an obvious alteration of Sp1 binding comparable to that seen for the NF-κB sequence.

It was of interest that although the exonuclease protection assays demonstrated protection over the Sp1 sites, the gel retardation assays only revealed induction of binding to the NF-κB sequences and not to the adjacent Sp1 oligonucleotide. We therefore repeated the exonuclease protection





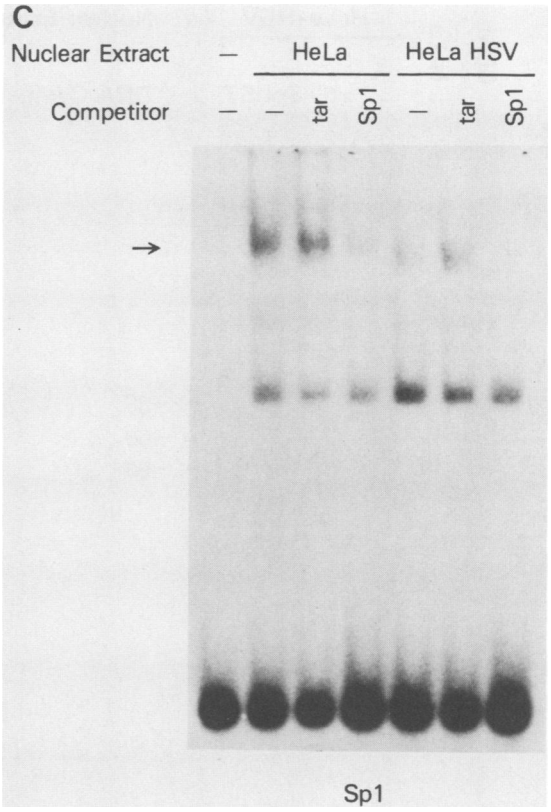


FIG. 5. Gel retardation analysis of the induction of protein binding to the HIV LTR by HSV infection. (A) Analysis of binding to the NF-κB sequence. A ^{32}P-labeled oligonucleotide (Fig. 4) based on the sequence of the NF-κB site was incubated with nuclear extracts derived from uninfected (HeLa) or HSV-1-infected (HeLa HSV) HeLa cells. Competition experiments were performed by the addition of 10 ng (greater than 10-fold molar excess) of cold double-stranded oligonucleotides to the binding reaction. Competitor oligonucleotides were the unlabeled NF-κB oligonucleotide and a sequence derived from the HIV TAR (tar) region. (B) Analysis of binding to a mutant NF-κB sequence. In addition to the NF-κB oligonucleotide used in panel A, a mutant NF-κB sequence (NF-κB*) previously shown to lack functional enhancing activity was used in gel retardation assays. (C) Gel retardation analysis of the HIV LTR Sp1 binding sites. An oligonucleotide corresponding to the three Sp1 binding sites of the HIV LTR was labeled with ^{32}P and used for gel retardation assays. Competitor DNAs were cold Sp1 oligonucleotide and the HIV TAR oligonucleotide used in panel A.

assays with the pUC8 construct in the presence of competitor oligonucleotides derived from the NF-κB and Sp1 sequences as well as from a sequence mapping between the Sp1 sites and TATA box in the HIV LTR (−46 to −30, synthesized with flanking *Hin*dIII adaptors), denoted as ST. The results of these competition experiments are shown in Fig. 6. As previously seen, infection of HeLa cells by HSV resulted in a shift in the major protected band from −60 to −50 bp. Incubation of the pUC8 construct with HeLa-HSV nuclear extract in the presence of the NF-κB oligonucleotide resulted in decreased exonuclease protection at the −50 and −60 positions. Incubation with the Sp1 and ST oligonucleotides did not reduce exonuclease protection at these positions. Thus, as suggested by the gel retardation assays, the 3′ expansion of the exonuclease-protected domain induced by HSV infection is the result of sequence-specific DNA binding of factors to the NF-κB sites.

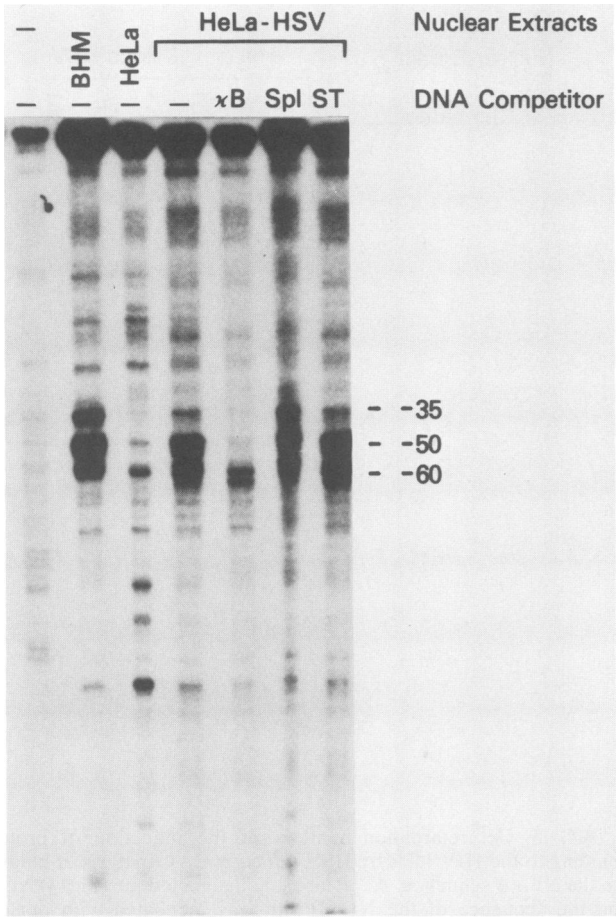


FIG. 6. Exonuclease protection assay with competitor oligonucleotides. Exonuclease protection assays were performed on the HIV LTR pUC8 construct as described in Materials and Methods. Nuclear extracts used are indicated in the lane headings. For the DNA competition experiments, 10 ng of the indicated double-stranded oligonucleotides was coincubated with the radiolabeled pUC8 LTR fragment and the HeLa-HSV nuclear extract prior to exonuclease digestion. The positions of the exonuclease stop sites relative to the RNA start site in the LTR are indicated to the right of the bands.

DISCUSSION

The activation of the HIV LTR induced by HSV is apparently a complex process. At least two different HSV immediate early gene products, ICP0 and ICP4, have been shown in transfection experiments to enhance the expression of HIV LTR-CAT constructs (30, 34, 36) and the synthesis of virus by an infectious clone of HIV (36). These stimulatory effects appear to require contributions from the cellular environment as the effects of different cloned HSV genes are variable in different cell types. Several adjacent regions of the HIV LTR may play a role as the *cis*-responding elements to the HSV-induced activation. In this study, we have shown that the activation of the HIV LTR by HSV-1 infection is associated with an enlargement of a nuclease-protected domain that overlaps both the NF-κB and Sp1 sites and with the induction of previously absent NF-κB activity in HeLa cells. The segment containing the NF-κB and Sp1 sites of the HIV LTR (−104 to −30) was shown to be able to confer HSV inducibility on a heterologous promoter (30). Deletion mutagenesis within this region (36) suggested that a small decrement in HSV responsiveness was observed following removal of the NF-κB sites; however, removal of both Sp1 sequences and NF-κB sequences resulted in complete loss of HSV inducibility. Thus, it is likely that the induction of NF-κB activity may contribute to the activation of the HIV LTR by HSV infection and that both the NF-κB and Sp1 binding sites are required for full activation. Interestingly, the stimulatory effects of the molecularly cloned HSV ICP0 gene on HIV LTR activity in T cells expressing the HIV Tat protein were shown to persist even when the NF-κB sites were mutated (34). The effect of this mutation on LTR activation following HSV infection was not examined. It is therefore likely that HSV activation of the HIV LTR may involve more than one target sequence in the LTR DNA.

The importance of the NF-κB sequences for the augmentation of HIV LTR activity in PHA- or PMA (phorbol 12-myristate 13-acetate)-treated lymphocytes has been previously shown by mutational analysis (33), by the use of plasmid constructs containing these sequences linked to indicator genes (23, 33), and by the demonstration of the induction of NF-κB binding activity in treated cells (14, 33). Activation of the LTR by phorbol esters in HeLa cells has also been shown to be associated with the induction of binding to these same sequences (8, 52). Therefore, several stimuli that activate the HIV LTR exert their effects, at least in part, through the NF-κB sequences. It is not yet clear whether the NF-κB binding activity present in such diverse cell types as B cells (43), T cells (14, 33, 52), and HeLa cells (8, 16, 52) is due to the same protein molecule. Different forms of this protein (2, 46; D. Recker, J. Gimble, and E. Max, submitted for publication) or distinct DNA-binding proteins recognizing the same DNA sequence may be found in these different cell types (2). It will be of interest to determine whether the mechanism of NF-κB activation by HSV infection of HeLa cells is similar to that proposed for phorbol ester induction (1). Other positive regulators of the HIV LTR that do not act through the NF-κB site have been identified. These include the HIV *tat* gene, the HSV ICP0 gene (discussed above), and the adenovirus E1A gene which appears to act through the TATA sequence (34).

An interesting observation in the present study was the finding that the increased exonuclease protection produced by HSV infection spanned both the NF-κB and Sp1 sites. When binding of nuclear factors to each of these sequences was individually studied by the gel retardation assay, only increased binding to the NF-κB sequences was readily identified. This observation was consistent with results of exonuclease protection assays performed with competitor oligonucleotides in which the intensity of exonuclease stops at the 3′ border of the protected domain was reduced by an NF-κB oligonucleotide but not by one from the Sp1 region. Perhaps protein binding to the NF-κB sites may induce or stabilize the formation of protein complexes that extend over the region of both the NF-κB and Sp1 sites (as diagrammed in Fig. 4) without making sequence-specific contacts with the Sp1 sites. Such complexes might not be detected by gel retardation assays which use only the Sp1 binding sites. Previous studies of the HIV LTR have suggested that factor binding at one site may affect the degree of nuclease sensitivity observed at distal sites (16).

The largest protected domain observed by our exonuclease studies was identified with nuclear extracts from the BHM 23 cells. These immunoglobulin κ-producing B cells contain abundant NF-κB (18) and appear to efficiently support the function of the HIV LTR, as they have recently

been shown to be infectible by HIV (W. Maury and A. Rabson, manuscript in preparation).

The finding that HSV-1 infection of HeLa cells induces NF-κB binding activity may have important implications for understanding the regulation of HSV gene expression. It is tempting to speculate that HSV may activate the expression of its own genes by inducing cellular factors such as NF-κB, which might be required for the HSV transcription.

Although we have not excluded the possibility that the increased protein binding to the HIV LTR in the HSV-infected cells is due to a direct interaction of HSV proteins with the LTR DNA, it seems likely that the changes we have observed are, at least in part, mediated by cellular factors. The activation of HSV immediate early genes by the HSV protein Vmw 65 has recently been shown to be associated with the induction of a DNA-binding activity containing both cellular and viral proteins (38) that is similar or identical to the octamer-binding protein that interacts with sequences present in many cellular and viral promoters (35). A role for the induction of the cellular factor NF-κB in the activation of herpes virus gene expression has not yet been identified. A computer homology search of the HSV-1 ICP0 gene region sequence identified the sequence GGGACTT (identity of 7 of 8 bp with part of the NF-κB sequence) twice within the ICP0 gene (J. Ostrove and A. Rabson, unpublished observation). The functional significance of these sequences is not known. It is of interest that an identity of 10 of 11 bp with the NF-κB site is also present in the promoter of the cytomegalovirus IE gene (3). Thus, the induction of NF-κB activity by HSV infection in HeLa cells may contribute to the regulation of HSV gene expression in these cells, and this DNA-binding protein may have a more generalized role in the control of gene expression for other members of the herpesvirus family.

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