Binding of the Herpes Simplex Virus Immediate-Early Gene Product ICP4 to Its Own Transcription Start Site

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A gel electrophoresis DNA-binding assay was used to detect proteins from herpes simplex virus type 1-infected and uninfected cells that specifically bind the upstream region of immediate-early (IE) gene 3. The assay is based on the altered electrophoretic mobility of DNA-protein complexes relative to that of free DNA in native gels. A series of end-labeled overlapping DNA fragments spanning a region from -272 to +27 (relative to the 5' terminus of the IE gene 3 mRNA) were used as probes. Two complexes were identified (referred to as A and B) which were driven by different protein factors. Formation of the A complex required (i) infected-cell proteins extracted at any time from 2 to 16 h postinfection; (ii) a 0.5 to 1 M NaCl extract of infected cells, and (iii) a DNA probe that contained the sequences from -4 to +27 (relative to the 5' terminus of IE gene 3 mRNA). The protein that drove the formation of the A complex is not related to transcription factors TFIIIA or Sp1 or their cognate binding domains since neither the 5S RNA gene nor the GC box of simian virus 40 could compete for proteins that induced formation of the A complex. Through the use of monoclonal antibodies, the complex was shown to contain the IE gene 3 product, ICP4. A more detailed localization of the DNA-binding site *in vitro* by using chemical footprinting revealed that binding occurs over the sequence from -10 to +3 relative to the mRNA terminus. The binding of ICP4 to its own transcription start site may explain the repression of IE gene transcription which attends the onset of early (β) gene expression and suggests an autoregulatory mechanism for gene control in herpes simplex virus type 1. The B complex was readily detected in uninfected cells (of a number of different cell lines), as well as in infected cells, with a probe containing the IE consensus sequence TAATGARATTC (where R is a purine) and two nested copies of the Sp1 binding motif GGGCGG; however, complexes were also detected with probes that lack the IE consensus sequence but contain Sp1 sites. These data suggest that the B complex contains the promoter-specific factor Sp1, and competition experiments with the clustered Sp1 binding domains from simian virus 40 confirmed this idea.

The developmental program of gene expression in herpes simplex virus type 1 (HSV-1) involves timed expression of three groups of genes, immediate-early (IE) (α), early (β), and late (γ) (4, 14, 17, 19). The sequential development to early and late gene expression requires expression of IE genes (15, 46). With the onset of early transcription, IE transcription is somehow repressed, and if this repression step is blocked, excessive amounts of IE mRNAs accumulate (4, 7, 17). These observations suggest that regulated expression of IE genes (activation as well as repression) is important in the overall developmental program. Studies of IE gene regulation using chimeric gene constructions have been used to elucidate IE regulatory sequences (3, 5, 20, 27, 37, 39), and it is known that some sites are recognized by *trans*-acting factors. For example, IE promoters and closely associated sequences contain multiple copies of the Sp1-binding sites (12, 16) and one or more copies of a conserved element, TAATGARATTC (where R is a purine) (26, 34), which has been implicated in *trans*-activation of IE gene transcription by a virion-associated protein (Vmw65) (3, 5, 6, 23, 25-27, 36, 37). The gene for Vmw65 has been identified and sequenced (6, 36); however, the protein does not appear to bind DNA (M. Muller, unpublished results). Thus, *trans*-activation is perhaps more complex than direct binding of Vmw65 alone to a DNA target sequence, and the basic mechanism remains an enigma.

Given the idea that upstream regions of IE genes contain sequences which respond to viral and perhaps cellular (40) *trans*-acting factors, a different approach was used to systematically search for factors which recognize and bind to

key regulatory regions of IE gene 3. The DNA-binding proteins were detected by a stoichiometric binding assay based on the altered electrophoretic mobility of DNA-protein complexes in native gels (9, 11). This technique has been particularly useful in the identification and purification of sequence-specific binding proteins in eucaryotic systems (42, 44, 45).

MATERIALS AND METHODS

Restriction enzymes were from International Biotechnologies, New Haven, Conn., T4 polynucleotide kinase was purchased from U.S. Biochemical Corporation, Cleveland, Ohio, and yeast tRNA was obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Poly(dI-dC) · poly(dI-dC) was purchased from Pharmacia P-L, Piscataway, N.J. All labeled nucleotides were from New England Nuclear Corp., Boston, Mass., and electrophoresis reagents were purchased from Bio-Rad Laboratories, Richmond, Calif.

Cells and viruses. BHK cells were grown in Dulbecco modified Eagle medium (Flow Laboratories, Inc., McLean, Va.) supplemented with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.), 100 μ g of streptomycin per ml, 100 U of penicillin per ml, and 0.075% NaHCO₃ in closed vessels or 0.225% NaHCO₃ in open vessels. Stocks of HSV-1 KOS were prepared from low-multiplicity passage as described previously (33).

Preparation of extracts. BHK cells growing in roller bottle cultures were infected with HSV-1 at an input multiplicity of 5 PFU per cell, and cultures were harvested at 12 to 16 h

FIG. 1. Binding of factors from HSV-1-infected cells to IE gene 3 region containing Sp1 and IE consensus sequences. (A) HSV-1 genome showing in detail the 5' region of IE gene 3. In the detailed map of IE gene 3, the known regulatory sequences important in this study are indicated. All coordinates are based on the mRNA cap site defined by Mackem and Roizman (25). The essential promoter region, from -50 to -108, corresponds to that defined by Cordingley et al. (5). Sp1 sites (16) and TAATGARATTC (indicated by AT box) sequences are also indicated. The 2-kilobase *Bam*HI-*Hind*III fragment from pGX58 (containing the *Xho*I C fragment cloned into the *Xho*I site of pMK16) was subcloned into Blue Scribe (Vector Cloning Systems, San Diego, Calif.) to produce pJD1. The identity of pJD1 was verified by restriction mapping and DNA sequencing (30). Restriction sites correspond to DNA probes used to map complexes by gel mobility shift assays. (B) Mobility shift assays. Probes used for binding assays were prepared by 5'-end labeling as described in Materials and Methods followed by digestion with a second restriction enzyme to generate singly end-labeled fragments (29). Binding reaction mixtures contained 3 μ g of nuclear extract in a final volume of 20 μ l. Nuclear extracts were prepared from BHK cells infected with HSV-1 and harvested at 16 h postinfection as described in Materials and Methods. The *Sau*3A-*Nco*I probe (from -272 to -208) (lanes A through E), which includes the IE consensus sequence and two Sp1 sites in pJD1, and a 74-bp probe from pSP64 (lanes F through J) were used. The following

postinfection. The following operations were performed at 4°C. The cells were scraped into medium and pelleted at 800 $\times$ g for 3 min. The pellet was washed twice with 20 pellet volumes of buffer A (10 mM Tris hydrochloride [pH 8], 150 mM NaCl), and the final pellet was suspended in 5 pellet volumes of sterile water containing 0.2 mM EDTA. After addition of an equal volume of buffer B (20 mM Tris hydrochloride [pH 8.2], 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 M NaCl), the lysate was vortexed and placed on ice for 30 min. The extract was then centrifuged at low speed (1,000 $\times$ g for 10 min), and the supernatant was centrifuged at 100,000 $\times$ g for 60 min. The supernatant was then dialyzed overnight against several changes of buffer B with the NaCl reduced to 50 mM. The extract was centrifuged (12,000 $\times$ g for 5 min) to deposit a small amount of insoluble material and then stored in aliquots at -80°C. The activities were stable for 6 months at -80°C and survived numerous freeze-thaw cycles. Extracts from MSB-1 cells (2) were prepared exactly as described by Manley et al. (28). Extracts were prepared from purified virions by using Nonidet P-40 exactly as described previously (33).

Plasmids and DNA probes. A plasmid containing the *Xho*I C fragment (pGX58) was provided by C. Preston. The *Bam*HI-*Hind*III fragment of pGX58 was subcloned into the polylinker of Blue Scribe (Vector Cloning Systems, San Diego, Calif.). This construction (pJD1) has a 2-kilobase insert of HSV-1 DNA, and the identity of the subcloned fragment was confirmed by restriction analysis and DNA sequencing. The pJD1 construct contains the transcription initiation site, as well as upstream sequences, of IE gene 3 (Fig. 1A). The complete sequence of the short repeat region has been reported by McGeoch et al. (30). Probes were selected from a 300-base-pair (bp) region 5' of IE gene 3 and were end labeled with [³²P]ATP by using T4 polynucleotide kinase (29). In most instances, fragments were cut with a second restriction enzyme, and the uniquely end-labeled fragments were isolated from preparative polyacrylamide gels.

Detection of DNA-protein complexes. The assay for detection of DNA-protein complexes was a modification of that described by Singh et al. (44). Reaction mixtures (volume, 20 μ l) were assembled to contain 10 mM Tris hydrochloride (pH 7.6), 1 mM EDTA, 0.1% Nonidet P-40 (vol/vol), 10 to 20 mM NaCl (contributed from the extract), 2 μ g of poly(dI-dC) $\cdot$ poly(dI-dC), and 20,000 dpm (1 to 2 ng) of end-labeled DNA fragment as probe. Reactions were initiated by the addition of the protein extract; incubation was at 30°C for 15 min. In some instances, incubations were performed at room temperature (24°C) with identical results. At the end of the incubation interval, 0.1 volume of sample dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 50% glycerol, 25 mM EDTA) was added, and the samples were loaded on a 4% polyacrylamide gel (acrylamide-bis weight ratio, 30:1) containing 6.7 mM Tris hydrochloride (pH 7.5) and 3.3 mM sodium acetate. The gel was prerun for 1 h at 20 mA with constant buffer recirculation. After loading of the samples, electrophoresis was performed at 20 mA until the bromophenol blue had migrated 9 to 14 cm. The gel was dried and exposed to SB-5 film with an intensifying screen, usually overnight.

DMS interference assays. A singly end-labeled DNA probe

amounts (μ g) of poly(dI-dC) $\cdot$ poly(dI-dC) were used: lanes A and F, 0; lanes C and H, 0.4; lanes D and I, 1; lanes E and J, 2. The position of the B complex is marked on the right.

(45 bp) was treated with dimethyl sulfate (DMS) (20°C for 8 min) by standard sequencing methods described by Maxam and Gilbert (29) and then repurified by standard methods by using tRNA as a carrier (10 µg/ml). The methylated probe was pretreated with RNase and incubated with extracts from infected cells under conditions described above to allow formation of complexes. Free DNA and DNA-protein complexes were then separated on preparative low-ionic-strength gels, and the DNA was recovered from the gel in the following way. The gel fragments were inserted into the well of a 0.8% agarose gel (Tris acetate buffer containing 0.1% sodium dodecyl sulfate), and 100 V was applied for 30 min to drive the fragments onto a DEAE membrane placed 1 cm from the well. The fragments were recovered by washing the membrane with 0.25 ml of 1 M NaCl followed by alcohol precipitation and cleavage by piperidine (29). The cleavage products were analyzed on 12% DNA sequencing gels.

RESULTS

Formation of DNA-protein complexes in extracts of infected and mock-infected extract cells. The genome of HSV-1 and the locations of the various IE genes are shown in Fig. 1A. To search for sequence-specific DNA-binding proteins, end-labeled probes were prepared that span the region from -272 to +27 relative to the 5' terminus of the mRNA of IE gene 3 as mapped by Mackem and Roizman (25) (Fig. 1A). These probes were used in a mobility shift assay to detect DNA-protein complexes with altered electrophoretic mobility

FIG. 2. Effect of ionic strength on complex formation with *AvaII-BamHI* probe. The *AvaII-BamHI* probe (from -233 to +27) was uniquely end labeled at the *BamHI* site and incubated with 3 µg of infected- or mock-infected-cell extracts under standard conditions described in Materials and Methods except that the monovalent ionic strength was varied as indicated. The concentration of $MgCl_2$ was varied in mock-infected and infected-cell extracts, although only the mock reactions are shown (lanes G and H). In the infected cell extracts, the probe was completely degraded by inclusion of $MgCl_2$. The positions of the A and B complexes are marked on the right.

FIG. 3. Target sites for factors from infected and uninfected cells that bind sequences upstream of IE gene 3. The following 5'-end-labeled probes were prepared from pJD1 (coordinates relative to the mRNA terminus): *NcoI-Sau3A* (-208/-4, lanes A through E), *Sau3A-NcoI* (-272/-208, lanes F through J), and *AvaII-BamHI* (-233/+27, lanes P through T). Control reactions (lanes K through O) contained a plasmid DNA probe (a 74-bp *EcoRI-HpaII* fragment from pSP64). Binding reaction conditions were as described in the legend to Fig. 1B. Reactions without protein (lanes A, F, K, and P) served as reference markers for free fragments. Lanes B, C, G, H, L, M, Q, and R contained protein from mock-infected BHK cells (M at gel origin), and lanes D, E, I, J, N, O, S, and T contained extracts from infected BHK cells (I at gel origin). In each set of paired lanes (M and I), the left lane contained 3 µg of protein and the right lane contained 6 µg. The positions of the A and B complexes are marked on the right.

compared to that of free DNA (9, 11, 44, 45). This is a stoichiometric DNA-binding assay that can be used with crude extracts provided that a large excess of heterologous DNA polymer is included to prevent nonspecific aggregation between basic proteins and the small amount (1 to 2 ng) of radioactive target sequence. A titration with poly(dI-dC) · poly(dI-dC) and either an HSV-1 *Sau3A-NcoI* probe (-272/-208) or a control probe of plasmid DNA was performed using a crude extract of HSV-1-infected cells. In the absence of the heterologous polymer, the probe was degraded (Fig. 1B, lanes A and F), whereas it was stabilized in the presence of polymer and a distinct complex was detected (lanes D and E). This complex, designated B, was not seen with the probe derived from plasmid DNA (lanes F through J). Moreover, the B complex was specifically eliminated by challenge with 5 to 10 ng of nonradioactive *Sau3A-NcoI* DNA (see Fig. 5A).

An *AvaII-BamHI* probe of 260 bp that spans the region from -233 to +27 of IE gene 3 was first used to differentiate factors from mock-infected and infected cells (Fig. 2). To establish optimal conditions for detecting complexes, the ionic strength of the binding reaction mixture was varied. The data show that in extracts of mock-infected cells a single complex (designated B) formed (lanes B through F) which gradually dissociated with increasing ionic strength. In in-

FIG. 4. Further localization of DNA target sites. DNA from pJD1 (described in the legend to Fig. 1) was 5' labeled at the *Nco*I site, purified, and redigested with *Sau*3A or *Eco*RI to produce *Sau*3A-*Nco*I (–272/–208) (lanes A through D) or *Nco*I-*Eco*RI (–208/–108) (lanes E through H). pJD1 was also labeled at the *Eco*RI site and then digested with *Bam*HI to produce the *Eco*RI-*Bam*HI probe from –108 to +27 (lanes M through P). From this probe a derivative was prepared by digestion with *Sau*3A to yield a probe from –108 to –4 (lanes I through L). Therefore, the only difference between these two fragments is the unlabeled *Sau*3A-*Bam*HI sequence from –4 to +27. The various probes were challenged with proteins from infected cells (3 μ g) (lanes I), mock-infected cells (3 μ g) (lanes M), or Nonidet P-40 washes of purified virions (0.25 μ g) (lanes ϕ). Control reactions (lanes P through T) contained a 74-bp probe derived from plasmid DNA. The positions of the A and B complexes are marked on the left.

fectected-cell extracts, a complex with identical mobility was detected (lanes I through M). This observation coupled with the fact that B complex formation in infected- and mock-infected-cell reactions showed an essentially identical response to ionic strength (cf. the B complex in lanes B through F with that in lanes I through M) indicates that the B complexes derived from both extracts were most likely the same. Inclusion of $MgCl_2$ in the binding reaction mixture resulted in extensive degradation of the probe in mock-infected- (Fig. 2, lanes G and H) and infected-cell (data not shown) extracts. A second complex (designated A) was reproducibly seen in infected extracts with this probe. This complex was well resolved electrophoretically from the B complex and was more sensitive to increasing concentrations of the monovalent cation. For example, there was a 15- to 20-fold reduction in A complexes over the range of KCl tested (Fig. 2) compared with a 5-fold reduction for B complexes. These observations suggest that the factors that drive the A and B complexes are likely to be different.

Delineation of DNA target sites for the A and B complexes.

The following overlapping probes were used to define the sequence elements involved in complex formation in the region 5' to IE gene 3: *Sau*3A-*Nco*I (–272/–208), *Ava*II-*Bam*HI (–233/+27), and *Nco*I-*Sau*3A (–208/–4). Control reactions with a plasmid DNA probe derived from pSP64 were also carried out. Extracts were prepared from mock-infected or HSV-1-infected BHK cells and assayed individually with various probes. Complexes were not detected with a control plasmid probe (Fig. 3, lanes L through O), showing that the complexes are specific for HSV DNA. The HSV DNA probes yielded B complexes of essentially identical mobility with both infected- and mock-infected-cell extracts. Thus, the identity of B complexes was based on electrophoretic mobility which was always the same for DNA probes in the size range of 45 to 260 bp (data not shown). Other investigators have also noted that the mobility of complexes is independent of probe length (for DNA fragments in this size range) and, in addition, that shorter probes give less background and sharper bands (44).

As shown above, the *Ava*II-*Bam*HI probe with infected-cell extracts yielded a distinct A complex (Fig. 3, lanes S and T). The A complex was a major band that was detected only with infected-cell extracts and by using probes that extend (in a 3' direction) past the *Sau*3A site located at –4 relative to the transcription start site of IE gene 3 (25). The results were confirmed with additional DNA fragments that span this region. Extracts were prepared from infected and uninfected BHK cells, as well as from purified virions, and were tested against the probes indicated in Fig. 4. Several conclusions can be drawn from the data. First, the *Sau*3A-*Nco*I (–272/–208) probe produced a very strong B complex in infected- and mock-infected-cell extracts compared with other probes tested; other probes formed the B complex, although to a lesser extent (Fig. 3, lanes C through E). Therefore, these data suggest that the DNA target sequence for the B complex is unrelated to the TAATGARATTC sequence, because probes lacking this sequence were capable of entering into a B complex. Second, the Nonidet P-40 extracts of virions which contain the virion-associated *trans*-inducing factor Vmw65 (as detected by Western blots with the monoclonal antibody LP1 [31]) did not form a specific complex with any probes tested, although aggregation was observed with all probes including a plasmid control. Third, a pair of probes uniquely labeled at the 5' *Eco*RI site but differing only in their 3' ends (*Eco*RI-*Sau*3A and *Eco*RI-*Bam*HI; Fig. 4, lanes I through P) displayed a marked difference in ability to enter into the A complex. These data clearly show that removing sequences from *Sau*3A (–4) to *Bam*HI (+27) eliminated key DNA target sites required for the A complex. Complexes were not detected in reactions treated with proteinase K or sodium dodecyl sulfate (0.5%) before loading the gel (data not shown); therefore, one or more protein factors form these complexes. In addition, the target site for the A complex was close to (within about 30 bp) the 3' end of the restriction fragment probe. It is known that some DNA-binding proteins, such as *Escherichia coli* RNA polymerase holoenzyme, bind preferentially to the ends of restriction fragments (32); however, the A complex was readily detected with a probe containing the target site placed more toward the middle of the fragment (Fig. 5B). Thus, high-affinity binding to DNA ends can be excluded.

Characterization of factors responsible for complexes. The factor responsible for the B complex was observed in both infected- and uninfected-cell extracts; however, a variety of other cell lines contain factors which produced B complexes, such as MSB-1 cells (2) (a chicken T-cell line), chicken

FIG. 5. Competition binding experiments with A and B complexes. (A) Competition of B complex with Sp1-binding sites of SV40. Binding reactions were carried out using the *Sau3A-NcoI* (–272/–208) probe and 12 μ g of infected-cell extracts (lanes B through P) or MSB-1 S100 extracts (lanes Q through S). For lanes B through H, 0, 20, 40, 80, 100, 200, or 300 ng, respectively, of pBR322 was used, and for lanes I through P, 0, 20, 40, 80, 100, 200, 300, or 400 ng, respectively, of pXHO(p+i)² was used. The latter has the 77-bp GC box of SV40 (nucleotides 37 to 114) cloned into pBR322. For lanes R and S, 200 ng of pBR322 or pXHO(p+i)², respectively, was used as competitor DNA. The additional bands (just below the B complex) were often seen with higher concentrations of crude extract and were apparently nonspecific since it was not possible to compete with a nonradioactive probe. (B) Competition of A complex. Binding reactions as in Fig. 1B contained 3 μ g of HSV-1-infected-cell extracts and an *EcoRI-EcoRI* probe which contains the *EcoRI-BamHI* fragment (–108/+27) and an additional 20 bp of plasmid DNA (to the *EcoRI* site in the polylinker). For lanes A and F, there was no challenge with competitor. For lane B, 25 ng of unlabeled *EcoRI-EcoRI* competitor fragment was used, and for lanes C through E, 100, 400, or 800 ng, respectively, of pXHO(p+i)² was used. For lanes G through J, challenge was with 50, 100, 400, or 800 ng, respectively, of pXS-15. This plasmid contains an SV40 genome (cloned into pBR322) in which all six GC box repeats have been deleted (10).

embryo fibroblasts, and HeLa cells (data not shown). The probes that contain the Sp1-binding consensus sequence GGGCGG (12, 16) were found to yield the B complex, suggesting that Sp1 may be responsible. To test this idea, a competition experiment was performed using plasmid pXHO(p+i)², which contains the 77-bp GC box of simian virus 40 (SV40) (nucleotides 37 to 114) cloned into pBR322. The B complex was relatively unaffected by addition of pBR322 competitor in infected-cell and MSB-1 extracts (Fig. 5A). In both extracts, it is clear that the addition of pXHO(p+i)² specifically competed for binding sites in the *Sau3A-NcoI* target sequence. An additional band, located below the B complex, was an artifact (see the legend to Fig. 5A). These data strongly suggest that the B complex was a result of Sp1 binding, in agreement with the results of Jones and Tjian (16), who mapped Sp1 binding to this region of the HSV-1 DNA by using different methods.

A competition experiment was also performed using HSV-1-infected-cell extracts and an *EcoRI-EcoRI* probe containing the region from –108 to +27, with an additional 20 bp of vector DNA on the 3' end. The vector sequence did not affect formation of complexes (data not shown). The resulting A complex was challenged with either pXHO(p+i)² or pXS-15. The latter is a plasmid containing SV40 DNA deleted for the six Sp1-binding sites (10). Neither of these DNAs could compete for binding of factors that constitute the A complex (Fig. 5B); furthermore, a control (lane B) showed that 25 ng of the unlabeled *EcoRI-EcoRI* fragment effectively eliminated the A complex. Based on these data, it appears that the target sequence for the A complex (–4 to

+27) is not shared by any sequence in SV40 DNA. In addition, the *Xenopus laevis* 5S RNA gene, which is a target sequence for the transcription factor, TFIIA (24), did not compete for binding of the A factor(s) to the –4 to +27 target (data not shown). Thus, it appears that the A factor is unrelated to TFIIA or its cognate binding domain.

Recent reports have indicated that IE gene expression is regulated by the IE gene 3 product (ICP4) (13, 18, 20, 35). This raised the possibility that ICP4 may be a component of the A complex. To test this idea, the assay reported by Kristie and Roizman (21, 22) was used to determine whether addition of a monoclonal antibody to ICP4 (H640) (1) imparts an additional shift in the mobility of the A complex. Binding reactions were designed with a probe (*AvaI-BamHI*, from –17 to +27) that would yield A complexes but lacks Sp1 sites. Two control monoclonal antibodies against viral proteins were tested: LP1, directed against Vmw65, the *trans*-inducing virion factor (31); and 6898, directed against an HSV-1 65-kilodalton DNA-binding protein (a gift from H. Marsden). The two control antibodies did not affect the mobility of the complex (Fig. 6A, lanes A, B, and C). On the other hand, the H640 antibody produced a clear shift upward (lane D, A'). Additional controls showed that this group of antibodies did not affect the B complex mobility (data not shown). These data show that the mobility of the A complex was specifically affected by anti-ICP4 antibody, which strongly suggests that ICP4 is at least a component of the complex.

Footprinting the A complex by DMS interference assays. A chemical footprinting technique (42, 43) was used to further

FIG. 6. Reaction of complexes with antibodies. Binding reactions were carried out as described below with 2 μ g of synthetic polymer, 1 ng of *Ava*I-*Bam*HI (-17/+27) probe, and 6 μ g of infected-cell extract. Additionally, for lane B, 2 μ g of LPI antibody to the virion *trans*-inducing factor (31) was used, for lane C, 2 μ g of 6898 antibody to a 65-kilodalton DNA-binding protein was used, and for lane D, 2 μ g of H640 antibody to ICP4 (1) was used. The complexes were allowed to form for 20 min at room temperature followed by incubation with antibody for 45 min. Identical results were obtained if the extract was preincubated with antibody for 45 min followed by addition of the probe. The shift in the position of the A complex in reactions containing H640 is marked A'.

define the DNA-binding domain associated with the A complex. The technique involves pretreatment of the probe with limited quantities of DMS and then determining at what positions base alkylation inhibits complex formation. Briefly, the probe described in the legend to Fig. 6 (*Aval* BamHI, -17/+27), containing the target sequence, was lightly methylated and used as substrate for complex formation. ICP4-bound DNA and free DNA fragments were then recovered by preparative low-ionic gel electrophoresis and cleaved at G residues. Since addition of methyl group inhibits formation of DNA-protein complexes, a footprint is derived by determining the efficiency of G-residue cleavages (a quantitative measure of methylation) in complexes as opposed to free DNA. In other words, any G residues that interfere with formation of the A complex would be selectively depleted in the corresponding G ladder since DNA molecules methylated at particular sites would be excluded from entering into the A complex. The sequence data are shown in Fig. 7. Comparing strand cleavage efficiency in the complex-associated DNA and free DNA, it is clear that DNA fragments that were methylated at G residues marked with an asterisk less effectively entered into the A complex. Therefore, G-residue methylation at these sites impeded complex formation. Based on these data, a binding sequence was derived (Fig. 7) which spans from -10 to +3 relative to the start site of transcription. Similar data were obtained by exonuclease III mapping (47) with a different fragment (data

not shown). Finally, the sequence derived includes the critical *Sau3A* site defined by mobility shift assays and is in close agreement with the consensus sequence recognized by ICP4, as shown by Faber and Wilcox (8).

DISCUSSION

Based on mobility shift assays using a variety of DNA probes that span the 5' side of IE gene 3, two complexes were identified and characterized. The B complex was detected by using extracts from infected or uninfected cells and was readily seen with probes containing clustered Sp1 sites. The results of competition experiments using SV40 Sp1 sites strongly suggest that the B complex is driven by Sp1, in agreement with a previous report (16). The probe that gave the strongest B complex (-272/-208) also has the IE consensus sequence 5'-TAATARATCC. Attempts to identify DNA-binding proteins, either in virion extracts (Fig. 4) or in cellular extracts, that bind the IE consensus sequence at -257 (using mobility shifts) were uniformly negative; however, IE consensus sequences located elsewhere in the HSV-1 genome have not yet been evaluated.

Another complex, the A complex, was found only with infected-cell extracts by using certain DNA sequences. Notably, only the probes that span across the start site of transcription entered into the A complex. A key restriction site in elucidating the binding domain by mobility shifts was the *Sau*3A site located at -4, since probes singly labeled 5' upstream of this site were competent in formation of the A complex before but not after truncation with *Sau*3A. Further definition of the binding domain by chemical footprinting

FIG. 7. Mapping with DMS interference. The probe described in the legend to Fig. 6 was methylated and incubated with 12 μ g of infected-cell proteins under the standard conditions. The reaction mixtures were loaded on a preparative (thickness, 3 mm) low-ionic-strength gel, and free and A complex DNAs were identified by a 4-h exposure of the wet gel. Complexes which were methylated at sites which interfere with binding of protein were thereby separated from the bulk of randomly methylated probe in the free DNA. The DNA was recovered, repurified, and cleaved with piperidine (29) followed by analysis on a 12% sequencing gel. Shown are cleavages at G residues in free DNA (lane 1) and in the A complex (lane 2). The latter cleavages gave a footprint (bracket) of the protein interactive sites in the DNA sequence (*).

verified that the binding site included the following sequence:

5'-CGCCCCGATCGTC-3'
3'-GCGGGGCTAGCAG-5'

To identify HSV-1 gene products in the A complex, monoclonal antibodies were used in conjunction with mobility shift assays as described by Kristie and Roizman (21, 22). One protein component of the A complex was identified as the IE gene 3 product, ICP4; however, I cannot rule out the presence of ancillary factors that may confer specificity in the binding. Thus, the significant finding in this work was that ICP4 bound specifically to its own transcription start site. The actual target sequence deduced by DMS footprinting fits the consensus sequence reported recently by Faber and Wilcox (8). In that work, ICP4 was shown to bind to a site approximately 100 bp upstream of the transcription start site in the gene for glycoprotein D, which is a late gene; furthermore, these investigators suggested that binding of ICP4 to this promoter may be involved in stimulation of transcription. Kristie and Roizman (21, 22) found binding of ICP4 to various promoter-regulatory regions by using the same biochemical approaches described in the present report; however, their results differ from mine in two respects. First, these investigators did not report binding to the transcription start site in IE gene 3, and second, I was unable to detect ICP4 binding in the -135 to -194 region of IE gene 3 (22). The reasons for the second difference are not clear, although the -135 to -194 region lacks the ICP4 DNA-binding consensus site derived by Faber and Wilcox (8).

Binding of ICP4 in a complex with its own transcription start site implies a negative autoregulatory scheme, as suggested by previous reports that IE gene expression is regulated by ICP4 (13, 18, 20, 35). The responder sequence involved in autoregulation has recently been reported to extend from the ICP4 transcriptional start site to approximately +30, i.e., very close to the target site for the A complex derived in the present work (P. O'Hare and G. S. Hayward, Abstr. 11th Int. Herpesvirus Workshop, p. 1, 1986). Moreover, the ICP4-containing A complex cannot be detected in crude extracts before about 2 h postinfection (unpublished observations), which is about the time when IE gene 3 is being repressed; therefore, it is proposed that formation of the A complex is related to repression of IE gene 3 transcription. If the same binding consensus sequence exists in promoter regions of genes which are activated by ICP4, such as early and late genes (8, 21, 22), it is reasonable to propose that activation and repression by ICP4 may be unrelated to the actual DNA sequence in the binding site and perhaps more related to the influence of ancillary proteins which exert control over transcription by acting in concert with ICP4. This interaction may be in the form of protein-protein cooperation in formation of stoichiometric complexes with ICP4. As noted above, the fact that the A complex cannot be detected at times when ICP4 is present in infected cells (1 to 2 h postinfection) further suggests that ICP4 is necessary but not sufficient alone to down regulate its own transcription, in agreement with other reports (13, 41). Alternatively, ICP4 may be posttranslationally modified (or processed) (38) in such a way that DNA recognition and binding affinities are altered. Finally, the accessibility of ICP4 DNA-binding sites to different genes in viral chromatin may be modified at various times after infection. Ultimately, greater understanding of DNA-protein organization in tran-

scribing HSV DNA is required to understand the mechanism behind activation and repression of IE genes.

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ADDENDUM IN PROOF

A recent report by Pizer et al. (L. I. Pizer, D. G. Tedder, J. L. Betz, K. W. Wilcox, and P. Beard, *J. Virol.* 60:950-959, 1986) directly supports the idea that an interaction between ICP4 and DNA can result in an inhibition of transcription of an immediate-early gene or stimulation of an early or late gene.

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