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Role for DNA-Protein Interaction in Activation of the Herpes Simplex Virus Glycoprotein D Gene

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On the basis of experiments with mutant virus and transfection with isolated genes, the herpes simplex virus immediate-early gene product ICP4 is known to positively regulate the transcription of viral early and late genes and negatively regulate expression from its own promoter. Binding of ICP4 to DNA sequences in several viral genes has been reported, yet the significance of ICP4-DNA interaction in transcriptional activation remains unclear. We have studied this problem by using the early glycoprotein D (gD) gene, which possesses a binding site at approximately −100 relative to the RNA initiation site. We linked this promoter and various mutant constructs to the chloramphenicol acetyltransferase gene in order to measure promoter activity in transient transfections both in the presence and in the absence of an ICP4-encoding plasmid. The natural promoter was activated 3.3-fold, and a deletion construct lacking the binding site was activated minimally (1.7-fold). Constructs containing multiple tandem repeats of the binding site (three or five inserts) demonstrated higher expression in the presence of ICP4 than did the natural promoter while retaining low levels of expression when unstimulated. Gel mobility shift assays and DNase I footprinting analyses indicated that ICP4 associated with multiple binding sites. In vitro transcription from a gD promoter construct containing multiple binding sites showed increased RNA synthesis in the presence of partially purified ICP4. These data provide the first direct evidence that binding of ICP4 to a specific DNA sequence in the gD gene contributes to activation of transcription.

Infection of cells with herpes simplex virus (HSV) results in the sequential expression of viral genes (31, 32). These genes, which are transcribed by the host RNA polymerase II (7), are regulated at the level of transcription (28, 58, 59). *trans*-Acting viral proteins and *cis*-acting nucleotide sequences appear to play a significant role in the regulatory scheme (4, 6, 16, 17, 24, 34, 35, 38, 44, 49, 50, 58, 59). The first set of viral genes expressed during infection are those of the immediate-early, or alpha, class. Of the five immediate-early genes, ICP4 (α4) is essential for the synthesis of mRNA from early and late genes (60). Results from transfection experiments indicate that ICP4 can activate the transcription of early and late genes and repress transcription from its own gene (10, 15, 26, 45–47). ICP4 is a phosphoprotein of 175,000 apparent molecular weight which demonstrates DNA-binding properties (20–22, 31, 36, 37, 41, 42). Faber and Wilcox have shown that ICP4 binds tightly to a consensus binding sequence, ATCGTCNNNNYCGRC, present in some but not all viral genes that it regulates (20). Beard et al. have demonstrated that an ICP4-enriched preparation (fraction VIII) affects the initiation step of transcription from the glycoprotein D (gD) gene (1); however, the relationship between DNA binding and modulation of transcription remains unclear.

In the case of the ICP4 gene, the consensus binding sequence is important for autoregulation (26; J. DiDonato and M. Muller, Abstr. 12th International Herpesvirus Workshop 1987, p. 293). The binding sequence, however, is not critical for down regulation of the ICP27 gene by ICP4 (26). These disparate findings may reflect the significance of position and context of an ICP4 binding site as well as the complexity of ICP4 action. The ICP4 gene contains a binding site at the RNA initiation site, which when occupied by the protein could block transcription of the gene. Functional studies with a deletion mutant in the binding site support this hypothesis (DiDonato and Muller, Abstr. 12th International Herpesvirus Workshop 1987). Transfection experiments addressing the role of *cis*-acting DNA sequences in the positive regulation of the gD gene were inconclusive in assigning a functional importance to the sequences in the 5′ region of the gene, including the consensus binding site located around −100 (16). These experiments, however, used viral superinfection as a means of introducing viral *trans*-activating genes into transfected cells. The introduction of other viral regulatory proteins by this means may explain the inability to reveal a *cis*-acting site responsible for *trans* activation by ICP4.

In an effort to simplify the analysis, we have cloned the gD promoter-upstream region in front of the bacterial chloramphenicol acetyltransferase (CAT) gene and used the gDCAT construct in cotransfection experiments with an ICP4-encoding plasmid. Similarly, constructs lacking the consensus binding site at −100 or containing multiple tandem repeats of the binding site were made so that we could examine the functional importance of the consensus binding site. The insertion constructs were tested by gel mobility shift assays for ability to bind ICP4 from infected cell extracts. The results indicated that the presence of multiple binding sites conferred the ability to form multiple complexes with ICP4, and these results were confirmed by DNase I footprinting. Transfection of the CAT constructs indicated that deletion of the consensus binding site in the gD promoter diminished but did not abolish *trans* activation by ICP4. The insertion of multiple binding sites (three or five inserts) restored the ability of the gene to respond to ICP4, to a degree somewhat greater than that of the natural promoter. In vitro transcription with mutant DNA as the template and partially purified ICP4 indicated that the presence of multiple binding sites, or

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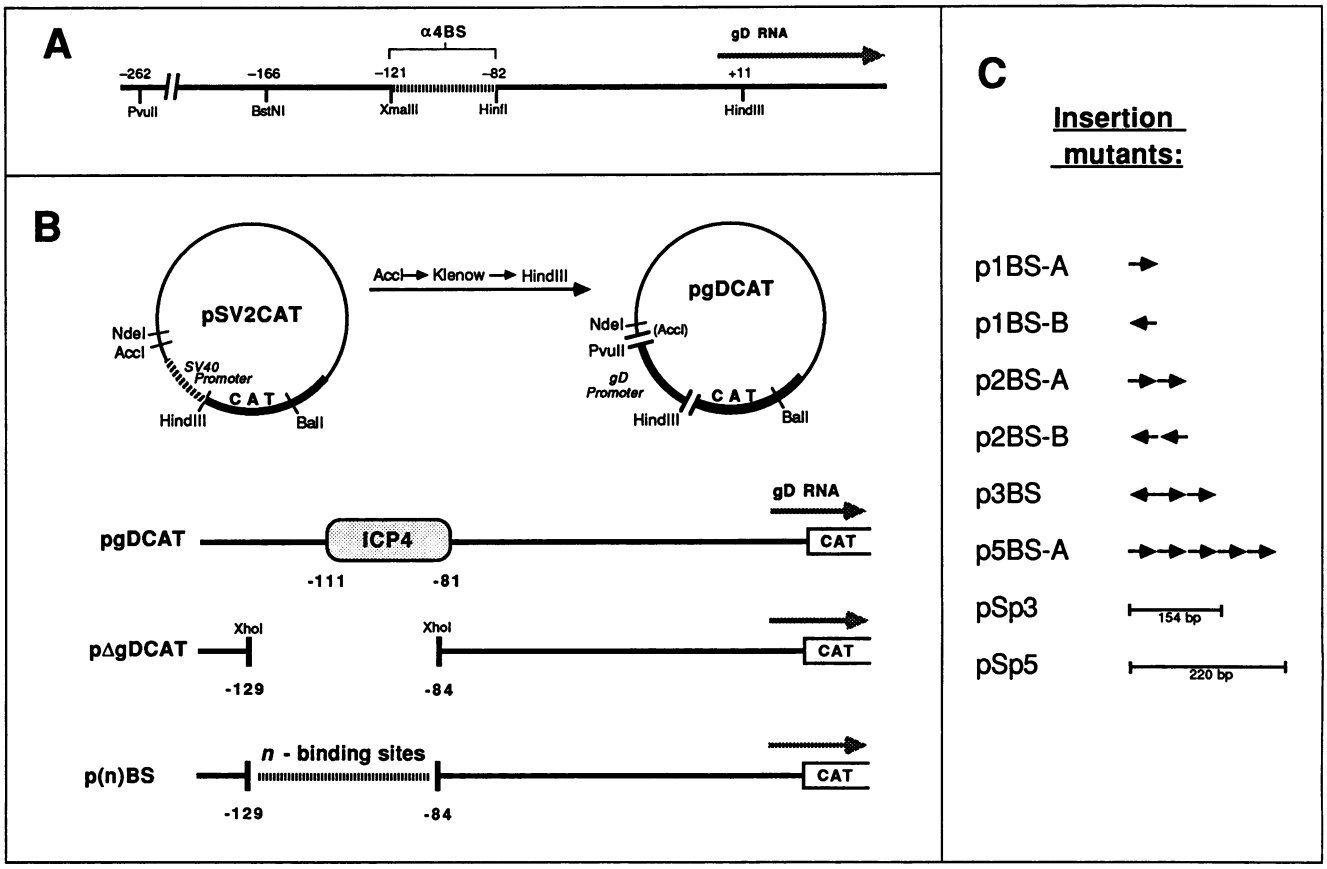

FIG. 1. The gD promoter-upstream region. (A) Location of the DNA fragment, designated α4BS, which was subcloned into pACYC177. The ends of this fragment were blunted by using the Klenow fragment followed by addition of *Xho*I linkers, and the α4BS fragment was cloned into the *Xho*I site of pACYC177. (B) Rationale for constructing the gDCAT chimera. *Pvu*II (−262)-*Hin*dIII (+11) fragments from clones pRED2 and pERD7.119 of Everett (16) were subcloned into an *Acc*I (Klenow blunted)-*Hin*dIII-cut pSV2CAT vector to produce pgDCAT and pΔgDCAT, respectively. To create multiple-binding-site insertion constructs, pΔgDCAT was digested with *Xho*I at the −129 to −84 junction and ligated with an excess of α4BS fragments. (C) Nomenclature and orientation of gDCAT insertion constructs. Arrows indicate that α4BS fragments were inserted at the *Xho*I site in pΔgDCAT; arrows in the left-to-right direction indicate the natural orientation of the binding site in the gD promoter. pSp3 and pSp5 are spacer mutants created by ligating 154-bp or 220-bp *Hin*fI fragments of pBR322 to which *Xho*I linkers had been attached into pΔgDCAT.

the absence of a site, correlated with the rate of RNA synthesis. These data demonstrate that although ICP4 may *trans* activate the gD gene to a limited extent in the absence of a consensus binding site, one or multiple binding sites increase the ICP4 response.

We have recently identified another consensus site to which ICP4 binds in the natural gD gene downstream of the RNA start site, in the coding region (unpublished data). The presence of these two sites in the natural gene and their interaction may be significant in conferring an adequate response to ICP4 during viral infection.

MATERIALS AND METHODS

Cells and viruses. Vero cells and HeLa cells were maintained in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Hazleton, Lenexa, Kans.), 100 μg of streptomycin per ml, and 60 μg of penicillin per ml. Procedures for preparing HSV type 1 (HSV-1) have been described previously (52, 55). HSV-1 (KOS) was used to infect cells for preparation of nuclear extracts.

Plasmids and DNAs. The ICP4 binding-site region from −82 to −121 in the gD promoter was subcloned from pJB3 (33) by excision with *Xma*III and *Hin*fI (Fig. 1A) and preparation of the ends according to the following procedure: (i) filling in of the ends with deoxynucleoside triphosphates, using the Klenow polymerase fragment; (ii) ligation to *Xho*I linkers; (iii) digestion with *Xho*I; (iv) removal of excess linkers by passage through a Centricon 30 column (Amicon Corp., Danvers, Mass.); and (v) ligation of the gel-purified fragment into *Xho*I-cut pACYC177 (5) that had been treated with calf intestine alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). This construct was designated pα4BS. The promoters for gDCAT constructs were derived from *Pvu*II (−262)-*Hin*dIII (+11) fragments of pRED2 and pERD7.119 (16) to yield pgDCAT and pΔgDCAT, respectively (Fig. 1B). The promoter fragments were ligated into pSV2CAT (43) which had been blunted at the *Acc*I site by using the Klenow fragment and cut at the *Hin*dIII site to remove the simian virus 40 early promoter. Multiple-insertion constructs were made by ligating *Xho*I α4BS fragments (47 base pairs [bp]) into *Xho*I-cut,

dephosphorylated pΔgDCAT. Recombinants were screened by restriction enzyme analysis, and orientation was determined by sequencing with Sequenase-modified T7 DNA polymerase (United States Biochemical Corp., Cleveland, Ohio) (57). Similarly, spacer mutants were made by cloning 154-bp or 220-bp *Hin*fI fragments of pBR322, which had been blunted with the Klenow fragment and the ends of which had been converted to *Xho*I, using linkers, into pΔgDCAT.

The ICP4-encoding plasmid used for transfections, p175 (provided by R. Everett), has been previously described (19). The transcription of ICP4 in this construct is driven by the simian virus 40 early promoter and is not subject to autoregulation by the ICP4 gene product.

Template DNAs for in vitro transcription experiments were purified from low-melting-temperature agarose as previously described (48). pΔgDCAT and p5BS-A were cleaved at the *Nde*I site in the vector DNA and the *Bal*I site in the CAT gene. The resulting fragments contained gD promoter sequences from *Pvu*II to *Hin*dIII, with 231 bp of pBR322 vector DNA to the left of the promoter and 528 bp of the CAT sequence downstream of the RNA initiation site.

Preparation of extracts. Nuclear protein extracts used in gel mobility shift assays were prepared according to the method of Dignam et al. (12). HeLa cells were infected with HSV-1 (KOS) at a multiplicity of infection of 10 and harvested 5 h postinfection. Protein determinations by the Bradford assay (2) showed that extracts typically contained 5 mg of protein per ml of extract.

Gel mobility shift assays. The method for detection of DNA-protein complexes was a modification of that described by Singh et al. (53). End-labeled probe (3 to 5 ng) was added to each 20-μl reaction mixture containing 10 mM Tris hydrochloride (pH 7.6), 0.1% (vol/vol) Nonidet P-40, and nonspecific competitor DNA (5 μg of sonicated salmon sperm DNA or 5 μg of 40-bp *Escherichia coli lac* operator DNA [51]), as indicated. In addition, contributed from the extract were the following: 5 to 10 mM KCl, 10 to 20 μM EDTA, 1 to 2 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (pH 7.9), 2.5 to 5.0 μM dithiothreitol, and 1 to 2% glycerol. Reactions were initiated by the addition of nuclear protein extract or ICP4 (fraction VIII) (20). The fraction VIII partially purified ICP4 contained approximately 5% ICP4 (1). In this study, it is referred to as ICP4 (fraction VIII), and all determinations of protein concentration refer to the quantity of protein in fraction VIII. Incubations were performed at room temperature (24°C) for 70 min. In some cases, monoclonal antibodies (2 μg of protein per reaction) were added after a 30-min incubation with extract and incubated for an additional 40 min. One-tenth volume of sample dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 50% glycerol, 25 mM EDTA) was added to reactions prior to electrophoresis on a 4% polyacrylamide gel (acrylamide-BIS, 29:1) containing 6.7 mM Tris hydrochloride (pH 7.5) and 3.3 mM sodium acetate. The gel was prerun for 30 min at 20 mA with constant buffer recirculation before the samples were loaded. After electrophoresis of the samples at 20 mA, the gel was dried and exposed to X-ray film at −70°C with an intensifying screen.

The monoclonal antibodies to ICP4, H944, H942, and H950 have been described previously (37) and were a gift of Lenore Pereira.

DNase I footprinting. DNA footprint probes were prepared by digestion of pgDCAT or p5BS-A with *Hin*dIII, treatment with alkaline phosphatase, and end labeling with [γ-^{32}P]ATP, using T4 polynucleotide kinase. A secondary cut was made with *Bst*NI, and the probes were separated on a polyacrylamide gel and electroeluted. Binding reactions were carried out in a 50-μl volume containing about 3 ng of probe (15,000 cpm). Conditions were identical to those used in the gel mobility shift assay; however, 100 ng of poly(dI-dC) was used as a nonspecific competitor. Reactions were initiated by adding 5 μg of ICP4 (fraction VIII) and incubated for 60 min at 24°C. Before DNase treatment, 50 μl of 5 mM $CaCl_2$–10 mM $MgCl_2$ was added, and then samples were digested for 60 s at room temperature with 0.5 to 6.0 μl of a freshly diluted 5-μg/ml solution of DNase I (Sigma Chemical Co., St. Louis, Mo.). DNase was diluted in 50 mM Tris hydrochloride (pH 7.9)–0.1 M KCl–12.5 mM $MgCl_2$–1.0 mM EDTA–1 mM dithiothreitol–20% glycerol (14). Digestion was terminated with the addition of 12 μl of 3 M ammonium acetate, 0.25 M EDTA, and 1 μl of pronase (20 mg/ml). Samples were then incubated for 30 min at 30°C, extracted with phenol-$CHCl_3$, ethanol precipitated, and loaded on an 8% 8 M urea sequencing gel. Gels were dried and subjected to autoradiography. G+A Maxam-Gilbert reactions were performed for each of the probes (39).

Transfections and CAT assays. Vero cells at 70% confluency in 10-cm dishes were transfected by the calcium phosphate method (30) followed by glycerol shock 5 h posttransfection. For each plate of cells, 10 μg of a CAT plasmid was cotransfected with either 2 μg of p175 (18) or 2 μg of Bluescribe M13+ (Stratagene, San Diego, Calif.). Cells were harvested 44 h after transfection, sonicated, and incubated 10 min at 60°C. CAT assays were carried out with modifications of the procedure of Gorman et al. (29). Assay reactions (180 μl) contained 140 mM Tris hydrochloride (pH 7.5), 0.2 μCi of [^{14}C]chloramphenicol, 0.88 mM acetyl coenzyme A, and 1.5 mg of cell lysate. Reactions proceeded for 3 h at 37°C and were then extracted with ethyl acetate. Radioactive chloramphenicol and acetylated forms were separated by thin-layer chromatography, and enzyme activity was quantitated by counting the spots in a scintillation spectrophotometer.

In vitro transcription. The procedures for in vitro transcription reactions have been described previously (1, 48). Nuclear extracts were obtained from suspension cultures of uninfected HeLa cells. The procedure for preparing partially purified ICP4 (fraction VIII) has been described by Faber and Wilcox (20, 21).

RESULTS

Association of ICP4 with a specific DNA sequence in the gD promoter-upstream region. Figure 1A shows the subcloned DNA fragment (α4BS) that encompassed the identified ICP4 consensus binding site. The α4BS fragment included virtually all of the reported region (−81 to −111) that was resistant to DNase I digestion in the presence of partially purified ICP4 (fraction VIII) as well as 10 bp to the left of the reported binding site (20). The subcloned fragment to which *Xho*I linkers were attached was excised from plasmid pα4BS with *Xho*I, labeled at both ends, and used in gel mobility shift assays with nuclear extracts from HSV-1-infected or mock-infected cells. Several nonspecific competitor DNAs, including salmon sperm DNA and poly(dI-dC), were tested to eliminate nonspecific interaction of proteins with the probe (unpublished data); however, the best results were obtained with a 40-bp fragment of the *E. coli lac* operator (51). Incubation with extract from infected but not uninfected cells resulted in formation of a specific slower-migrating complex with the α4BS 47-bp probe (Fig. 2A, lane C).

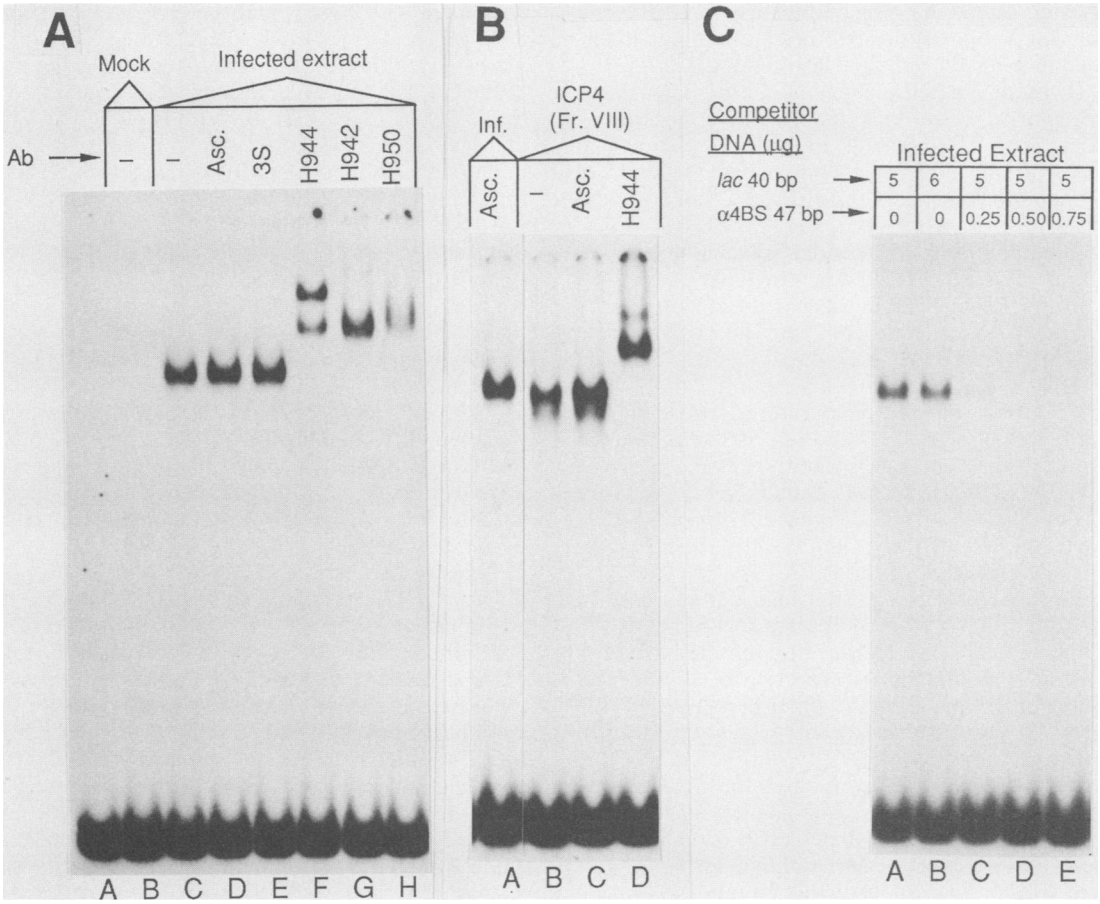

FIG. 2. Binding of ICP4 to the 47-bp cloned DNA fragment (α4BS) containing the consensus binding sequence. The 47-bp α4BS fragment was obtained by cutting pα4BS with *Xho*I, and the ends were labeled by using the Klenow fragment and [α-^{32}P]dTTP. (A) Binding of protein from HSV-1-infected cell extracts to the α4BS fragment. Lanes: A, migration of the free probe (typically, gel mobility shift assays performed with the α4BS probe contained 5 μg of *E. coli lac* operator 40-bp DNA as nonspecific competitor); B, reaction with mock-infected extract; C through H, reactions with infected extracts. (B) Binding of ICP4 from fraction VIII to the α4BS fragment; reactions with infected cell extract (lane A) and with ICP4 (fraction VIII) (lanes B through D). (C) Competition of the complex with unlabeled α4BS or *E. coli lac* operator DNA. Each reaction contained 5 μg of *lac* DNA as a nonspecific competitor. In all cases in which cell extracts or ICP4 (fraction VIII) was used with the α4BS probe, 5 μg of protein was used per reaction. Where antibody or mouse ascites fluid (Asc.) was used, 2 μg of protein was added per reaction. 3S, Monoclonal antibody against glycoprotein C.

Migration of the complex was further retarded in the presence of a specific monoclonal antibody against ICP4 (lanes F through H) but not in the presence of mouse ascites fluid (lane D) or a monoclonal antibody (3S) directed against HSV-1 glycoprotein C (lane E). It is interesting that the H944 monoclonal antibody formed two bands (lane F), which may have resulted from the presumed homodimer of ICP4 involved in the DNA-protein complex (40). If in fact a homodimer was involved, the H944 monoclonal antibody may have formed two different complexes, depending on whether one antibody molecule was bound to one ICP4 molecule in the dimer or whether two antibody molecules were bound, one to each molecule of ICP4 in the dimer. Titration experiments (unpublished data) in which antibody is added in increasing amounts indicate that the upper band is favored when the antibody concentration is high and that the lower band is favored when the antibody concentration is low. These data are consistent with the binding of an ICP4 homodimer to DNA. The fact that H944 yielded two bands and H942 (lane G) yielded only one may reflect the difference in recognition epitopes of these antibodies. H950 has been reported to inhibit the formation of DNA-ICP4 complexes (37), which is also evident with the α4BS probe by comparing the amount of the probe in the retarded complex in lane H versus the amount in lane C or G. Figure 2B shows the results with ICP4 (fraction VIII) in the gel mobility shift assay. Migration of the ICP4-DNA complex was quite similar to that observed with infected cell extract. The small difference in mobility of the bands could have resulted from protein(s) in the extract adding to the ICP4-DNA complex. Some smearing beneath the main bands may have been caused by degradation of the ICP4 or by monomer forms of ICP4 forming the complex.

To examine the specificity of the interaction between ICP4 and the α4BS DNA probe, competition experiments were performed with either unlabeled specific competitor DNA (α4BS 47 bp) or nonspecific competitor DNA of similar size (*E. coli lac* operator 40 bp). Competitor DNAs were added to binding reactions before the addition of cell extract. Addition of 1 μg of *lac* 40-bp DNA above the 5 μg used as a standard nonspecific competitor had no effect on the amount of probe involved in a complex with ICP4 (Fig. 2C, lane B). However, when 250 ng of unlabeled α4BS 47-bp DNA was used as a competitor, virtually all of the probe remained

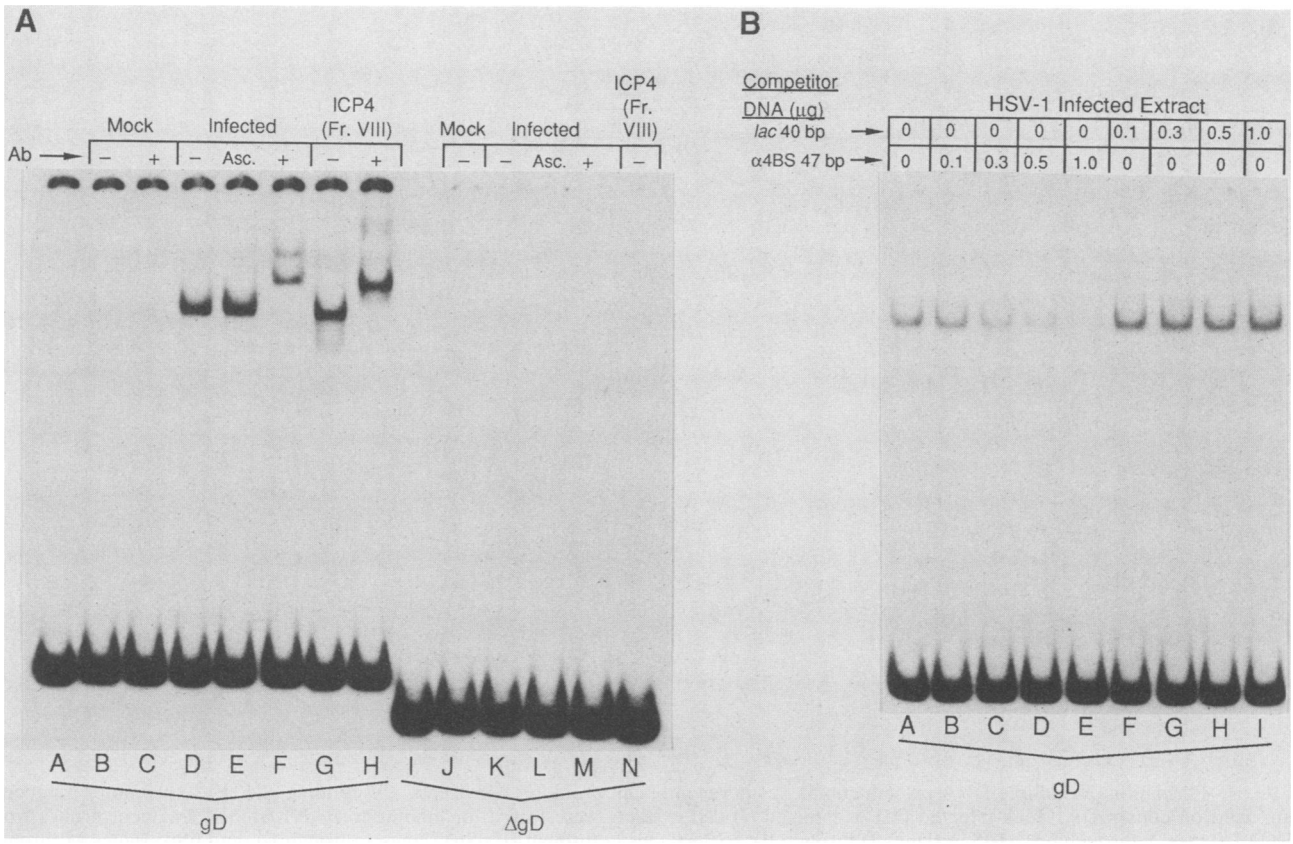

FIG. 3. Gel mobility shift assays showing that deletion of the consensus binding site in the gD gene abolished ICP4 binding. (A) ICP4 formed a complex with the gD promoter fragment but not with the fragment that had been deleted between −129 and −84. Promoter probe fragments were prepared from pgDCAT or pΔgDCAT as described in Materials and Methods. The *Bst*NI-*Hin*dIII gD probe was used in lanes A through H; the *Bst*NI-*Hin*dIII ΔgD probe was used in lanes I through N. (B) Competition of ICP4 binding to the gD promoter, using α4BS or *lac* operator competitor DNA. The promoter probe fragment from pgDCAT was used in the competition experiment (lanes A through I). In all cases in which promoter fragment probes were used in gel mobility shift assays, 5 μg of sonicated salmon sperm DNA was used as nonspecific competitor DNA with 10 μg of protein from cell extracts or 5 μg of protein from ICP4 (fraction VIII). Asc., Mouse ascites fluid.

unbound (lane C). A 250-ng amount of competitor DNA represented a 50-fold molar excess of competitor DNA, since 5 ng of probe DNA was used in each reaction. These results demonstrate that a subfragment of the gD promoter which encompassed the region of binding located by Faber and Wilcox (20) was able to form a specific complex with ICP4.

To examine the binding of ICP4 to the consensus binding site in the context of the entire gD promoter, gel mobility shift assays were performed by using *Bst*NI-*Hin*dIII fragments from pgDCAT or pΔgDCAT (Fig. 1A and B). These fragments, which extended from −166 to +11, were singly end labeled at the *Hin*dIII site. The promoter fragment from pgDCAT formed a specific complex in the presence of infected cell extract or ICP4 (fraction VIII) (Fig. 3A, lanes D and G). Furthermore, the monoclonal antibody H944 retarded migration of the complex, which indicated the presence of ICP4 in the complex (lanes F and H). The probe fragment derived from pΔgDCAT, which lacked a consensus binding site, did not yield complex formation with extracts from infected or mock-infected cells (lanes J through N). In the gel mobility shift assays with promoter fragment probes, the optimal nonspecific competitor DNA was sonicated salmon sperm DNA rather than the *lac* 40-bp fragment (data not shown); salmon sperm DNA was used in these experiments. The nonspecific competitor DNA that works most effectively may depend on the size of the probe and the extent to which nonspecific DNA binding occurs.

Binding of ICP4 to the gD promoter fragment probe was specific (Fig. 3B). Complex formation was diminished as the concentration of α4BS 47-bp unlabeled competitor increased (lanes A through E). Competition was nearly complete at approximately 100-fold molar excess of the specific competitor DNA fragment. The addition of up to 200-fold molar excess of the *lac* operator 40-bp fragment competitor did not eliminate complex formation (lanes F through I). In fact, at all concentrations of the *lac* DNA used, complex formation was slightly better than in its absence. This result was quite reproducible and may be explained by the ability of the *lac* DNA to compete for a nonspecific binding protein(s) present in extracts, which the salmon sperm DNA was unable to bind. Thus, in the presence of these two types of competitor DNAs, the ICP4 binding sequence had the best opportunity to interact with ICP4 without interference from nonspecific protein interactions.

Evidence that multiple ICP4 binding sites allow the formation of multiple DNA-protein complexes. To test whether the multiple-binding-site mutants (Fig. 1C) could bind multiple ICP4 molecules, gel mobility shift assays were performed by using infected cell extracts and ICP4 (fraction VIII). The results with the gD probe (Fig. 4A, lanes A through C) were similar to those shown in Fig. 3A and served as a base-line

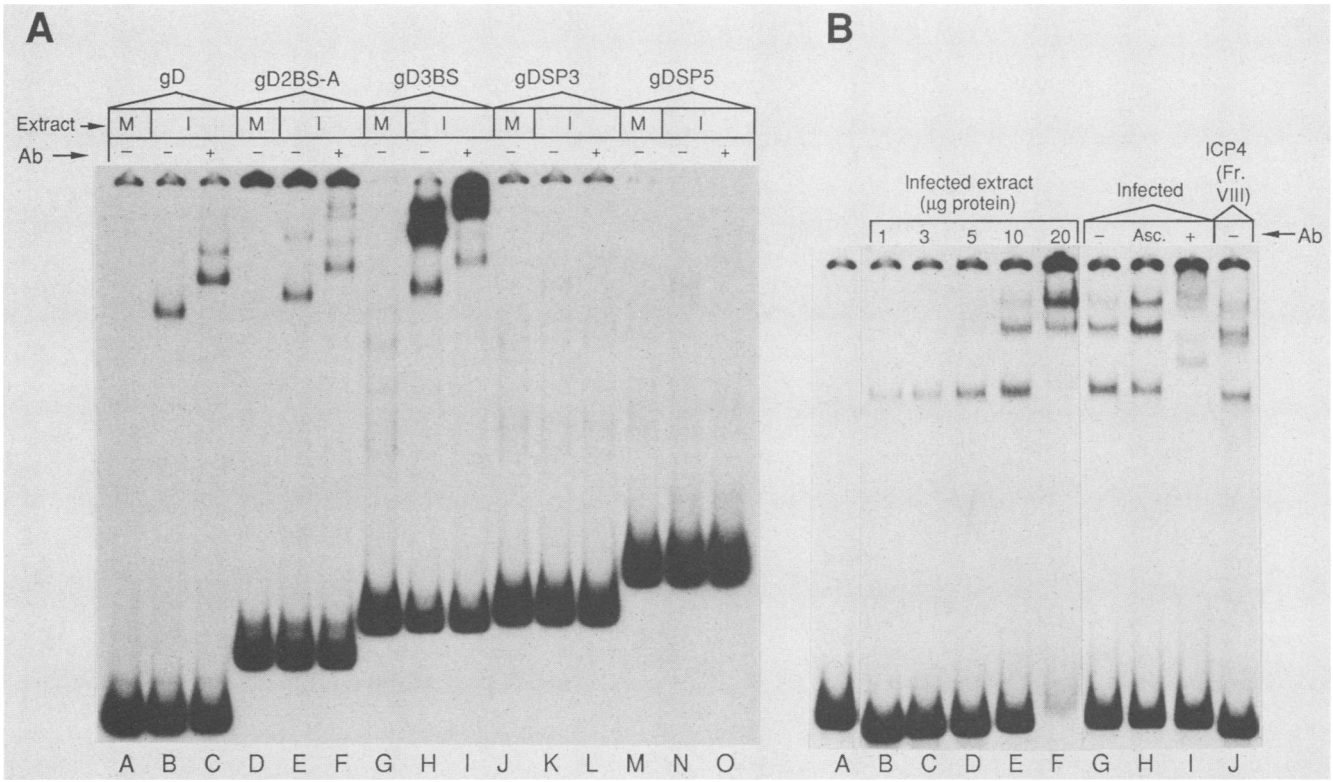

FIG. 4. Formation of multiple DNA-protein complexes in recombinant promoter constructs. (A) Binding of ICP4 to promoter fragments from insertion constructs. Mock-infected (M) or infected (I) cell extracts were tested for binding to *Bst*NI-*Hin*dIII fragment probes from pgDCAT (lanes A through C), p2BS-A (lanes D through F), p3BS (lanes G through I), pSp3 (lanes J through L), and pSp5 (lanes M through O). The H944 monoclonal antibody (2 μg per lane) was included in the binding reactions in lanes C, F, I, L, and O as described in Materials and Methods. Each lane contained 10 μg of protein extract. (B) Titration of protein on gD5BS-A. Lanes: A, free p5BS-A *Bst*NI-*Hin*dIII probe; B through F, gels illustrating the effect of increasing protein concentrations of infected cell extracts on mobility of the probe. In lanes G through I, 10 μg of infected cell extract was used. The monoclonal antibody H944 was used to identify the presence of ICP4 in the multiple complexes in lane I. Binding of ICP4 from fraction VIII (5 μg of protein) to the p5BS-A probe is shown in lane J.

control. The promoter fragment from p2BS-A yielded two different complexes with infected extract (Fig. 4A, lane E). The lower band presumably represented one of the two sites occupied by ICP4, and the upper band represented a complex in which both binding sites were occupied. The addition of H944 antibody produced four bands which migrated more slowly (lane F), indicating that ICP4 was part of both complexes, and the number of bands may be explained by the number of antibody molecules in the complex (as discussed above). Likewise, in the presence of infected cell extract, the p3BS probe fragment yielded three bands whose mobilities were further retarded by the addition of H944 antibody (lanes H and I). It is interesting that the p3BS probe consistently yielded strong complex formation in gel mobility shift assays, which may relate to the unusual orientation of the binding sites in this mutant (Fig. 1C). Figure 4A (lanes J through O) depicts the mobility shift assays of spacer mutants containing inserts of pBR322 DNA roughly equivalent in size to three or five α4BS fragments. A band specific to infected cell extract was barely detectable.

Figure 4B shows the multiple complexes obtained by using a promoter fragment probe from p5BS-A. In the presence of infected cell extract or ICP4 (fraction VIII), three bands were prominent (lanes G, H, and J). Lanes B through F show a titration of infected cell extract on the p5BS-A probe, which demonstrates that the different complex species presumably represented one- or multiple-site occupation by ICP4 molecules, depending on the protein concentration. The relationship between mobility of a complex and the ratio of DNA to protein required to form the complex remains unclear. Considering the limitations of the gel system in the high-molecular-weight range, however, the complexes to which the greatest number of ICP4 molecules were bound may have remained in the well (lane F).

DNase footprinting of the interaction of fraction VIII with single or multiple binding sites. Figure 5A shows the protection of the ICP4 binding region in the gD promoter and the specificity of binding to the consensus binding site. Similar to the findings of Faber and Wilcox (20), protection in the gD promoter between −115 and −75 relative to the RNA start site was observed. Nonspecific *lac* operator 40-bp DNA was unable to compete against this binding even at 100-fold molar excess of the labeled probe (Fig. 5A, lane 3). When the α4BS 47-bp fragment was used as a competitor, however, binding to the footprint region was greatly diminished at 100-fold molar excess of the competitor (lane 8). In addition to the footprint observed over the consensus binding region, some weak protection from nuclease digestion was detectable from −48 to −66, over a very G-rich region in the promoter. Figure 5B illustrates a similar experiment, using a probe from p5BS-A, to define the interaction of ICP4 (fraction VIII) with the five direct repeats of α4BS in this construct. There were five regions of nuclease protection (lane 2), which were apparently competed against equally by the α4BS competitor fragment (lanes 4 through 6) but not at all by the *lac* operator 40-bp DNA (lane 3). Each 33-bp pro-

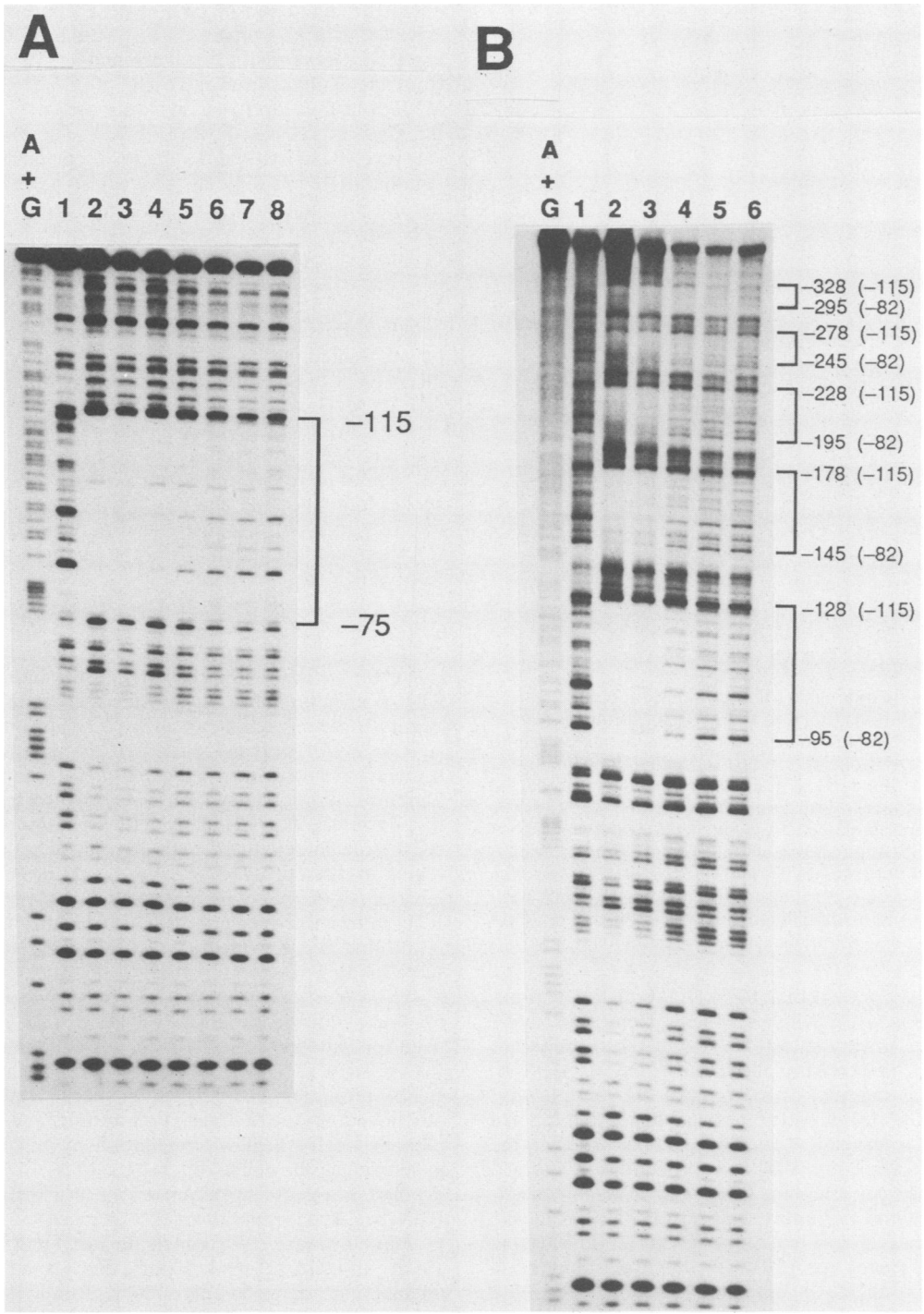

FIG. 5. DNase I footprint of ICP4 (fraction VIII) on the pgDCAT and p5BS-A promoter fragments. (A) Footprint of the *Bst*NI-*Hin*dIII fragment from pgDCAT. Lane 1, Ladder formed by DNase I treatment alone; lane 2, ICP4 (fraction VIII) footprint. Lanes 3 through 8 contained ICP4 (fraction VIII) and various competitor DNAs. Lane 3 contained 200 ng of *lac* 40-bp competitor DNA. Lanes 4 through 8 contained 25, 50, 100, 150, and 200 ng, respectively, of α4BS 47-bp competitor DNA. (B) Footprint of the *Bst*NI-*Hin*dIII fragment from p5BS-A. Lane 1, Ladder resulting from DNase I treatment. Lanes 2 through 6 contained ICP4 (fraction VIII); lane 3 contained 150 ng of *lac* 40-bp competitor DNA; lanes 4 through 6 contained 50, 100, and 150 ng, respectively, of α4BS 47-bp competitor DNA. Numbers on the right indicate the regions of protection relative to the RNA initiation site. Numbers in parentheses refer to the sequence locations as they would exist for the α4BS region in the natural gD promoter.

tected region was similar in size to the protected region in the natural gD promoter (40 bp). Because of inherent resistance to DNase at the junction regions of the inserts, however, the precise boundaries of the right-hand ends are not known for p5BS-A. These results correlate with the data obtained from gel mobility shift assays.

Effect of fraction VIII on in vitro transcription of mutant DNA. Using a nuclear extract from uninfected HeLa cells,

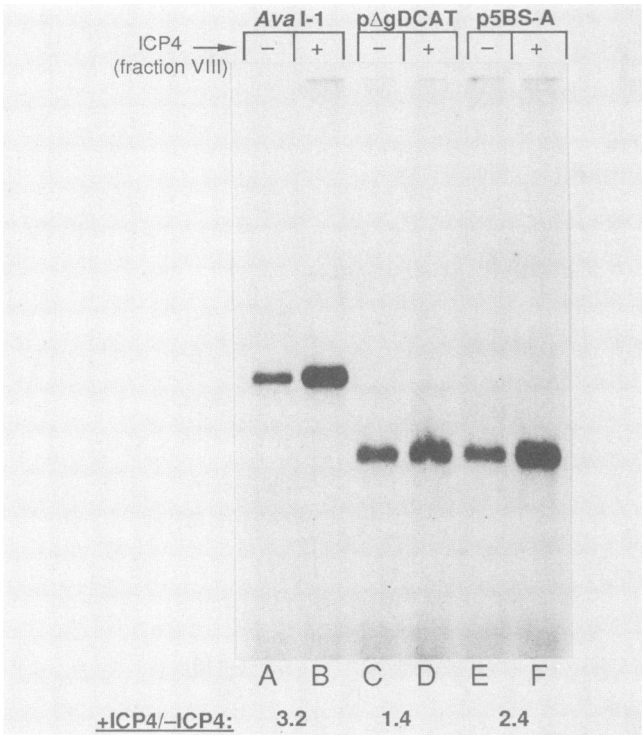

FIG. 6. Stimulation of in vitro transcription from pΔgDCAT and p5BS-A templates by ICP4 (fraction VIII). Each reaction contained 100 ng of template DNA plus nuclear extract from uninfected HeLa cells as described previously (1). The 1.55-kilobase-pair *Ava*I-1 template, obtained from pJB3 as previously described (1), yielded the expected 0.84-kilobase transcripts (lanes A and B). The 530-nucleotide transcripts produced from the *Nde*I-*Bal*I templates of pΔgDCAT and p5BS-A are shown in lanes C and D and in lanes E and F, respectively. In lanes B, D, and F, 8 μg of ICP4 (fraction VIII) was included during the transcription reactions. Bands were cut out and counted in a scintillation spectrophotometer to determine the level of stimulation (indicated at the bottom).

we have previously shown that ICP4 (fraction VIII) stimulates the rate of in vitro RNA synthesis from viral DNA templates (1, 48). In this study, we tested the response to ICP4 (fraction VIII) with templates from p5BS-A, pΔgDCAT, and a fragment of the natural gD gene, *Ava*I-1 (33). Figure 6 shows the runoff transcripts generated in vitro from these DNA templates in the presence and in the absence of ICP4 (fraction VIII). All reactions contained equivalent amounts of template DNA and nuclear extract. As we have previously reported, transcription from the *Ava*I-1 template was increased with ICP4 (fraction VIII), in this case 3.2-fold (lanes A and B). The level of stimulation was lower for the pΔgDCAT template, 1.4-fold (lanes C and D). The p5BS-A template, however, was stimulated 2.4-fold (lanes E and F).

Figure 7 shows the kinetics of RNA synthesis in the presence of ICP4 (fraction VIII) with pΔgDCAT and p5BS-A as the template. Reactions were stopped at 15, 30, 45, and 60 min, and the RNA products were analyzed on a 2% glyoxal gel. The expected transcripts of approximately 530 nucleotides in length were detected in all reactions during 60 min. RNA synthesis increased with time with the template from p5BS-A, whereas with the pΔgDCAT template RNA synthesis increased only slightly after 30 min.

Effect of ICP4 binding sites on gD gene expression in vivo. To test the hypothesis that multiple ICP4 binding sites may affect the regulation in vivo of gene expression by ICP4, a series of gDCAT constructs was transfected into Vero cells in the presence of p175, a plasmid which expresses ICP4 from the simian virus 40 early promoter (18). Bluescribe M13+ plasmid DNA was used as a control. The results of the CAT assays are shown in Fig. 8. The fluctuations in the low-level base-line values made direct comparison of fold stimulation levels difficult. The wild-type promoter containing one binding site, pgDCAT, produced reasonable base-line activity and was stimulated 3.3-fold with p175. The deletion construct, pΔgDCAT, demonstrated similar base-line activity, while the fold stimulation was reduced to 1.7-fold. The insertion of one or two binding sites into the deletion construct did not lead to an increase in the base-line or stimulated activities above those of pΔgDCAT. The insertion of three or five binding sites restored the stimulated activity levels to that of the wild-type pgDCAT. The results of six transfections with plasmid p5BS-A showed that the base-line CAT activity was low and that cotransfection with p175 produced approximately eightfold stimulation. The spacer mutants pSp3 and pSp5, which corresponded in size to p3BS and p5BS-A, respectively, responded minimally to ICP4 stimulation, much as did the parent clone, pΔgDCAT, from which they were derived.

DISCUSSION

We report that the direct or indirect interaction of ICP4 with a specific DNA nucleotide sequence influences the degree to which ICP4 can *trans* activate the gD promoter. Assays performed to measure ICP4 interactions with the sequences upstream of the gD promoter confirm the observations of Faber and Wilcox that a binding region from −115 to −75 interacts specifically with partially purified ICP4 or ICP4 from infected cell extracts (20). The insertion of this region in three or five copies in the gD promoter at the −84 position confers an increased positive responsiveness to ICP4 in transfections and in vitro transcription assays. This is the first report to assign a functional significance to the ICP4 binding site in activation of the gD gene.

To date, the association of ICP4 with the consensus binding sequence proposed by Faber and Wilcox (20) has been reported in the six HSV genes encoding gD, ICP0 (37, 41), ICP4 (21, 42), ICP42 (41), ICP25 or αTIF (41), and ICP27 (36). The ICP4 binding site in the ICP4 gene is positioned at the transcriptional start and appears to be responsible for negative regulation by ICP4, yet the ICP0 gene can also be negatively regulated by ICP4 (25) although it possesses a site upstream of the RNA start site at −60. ICP4 has also been shown to *trans* activate genes such as thymidine kinase (23), which do not bind ICP4 with high affinity in vitro (21, 36). Failure to observe strong binding under these conditions may result from low-affinity binding in vitro under the conditions used or binding to regions of the gene which have not been examined.

Our contribution to explain the mechanism of ICP4 action is to show that the association of ICP4 with the consensus binding site(s) in the gD gene results in increased expression in vitro and in vivo. Whether the consequence of ICP4 binding to the consensus site is a negative or a positive change in gene expression probably depends not only on the position of the site but also on the surrounding *cis*-acting elements to which cellular factors may bind. For example, just to the right of the natural binding site in the gD gene we have identified a G-rich region which is bound by cellular proteins from uninfected cells (unpublished data). Perhaps

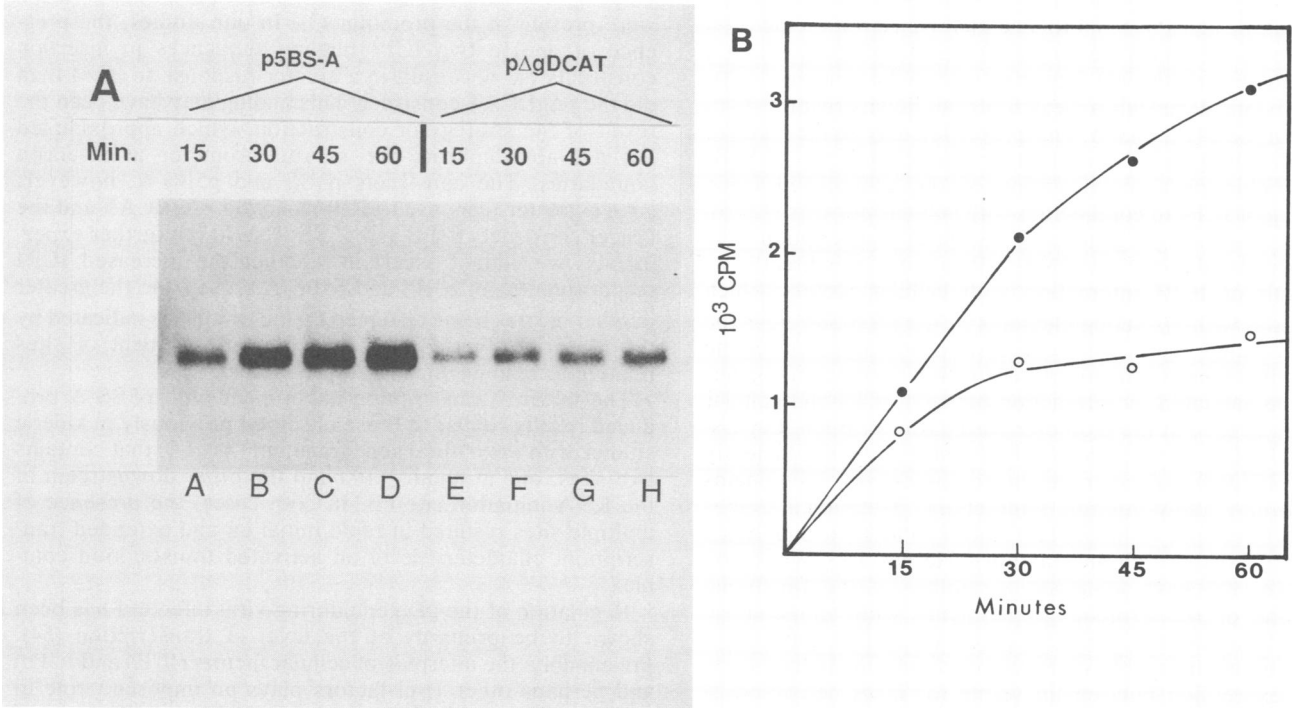

FIG. 7. Kinetics of in vitro transcription from pΔgDCAT and p5BS-A with ICP4 (fraction VIII). (A) *Nde*I-*Bal*I fragments of p5BS-A (lanes A through D) or pΔgDCAT (lanes E through H) were used as templates for in vitro transcriptions. All reactions were carried out in the presence of 8 μg of ICP4 (fraction VIII). Reactions were stopped at the indicated time points and analyzed on a 2% glyoxal agarose gel as previously described (1). A 100-ng amount of template DNA was used in each reaction. (B) Plot of the data obtained from A. Radioactivity in each band was determined by excision of the bands and counting in a scintillation spectrophotometer. The data are from incubation with (●) p5BS-A DNA (lanes A through D) and (○) pΔgDCAT DNA (lanes E through H).

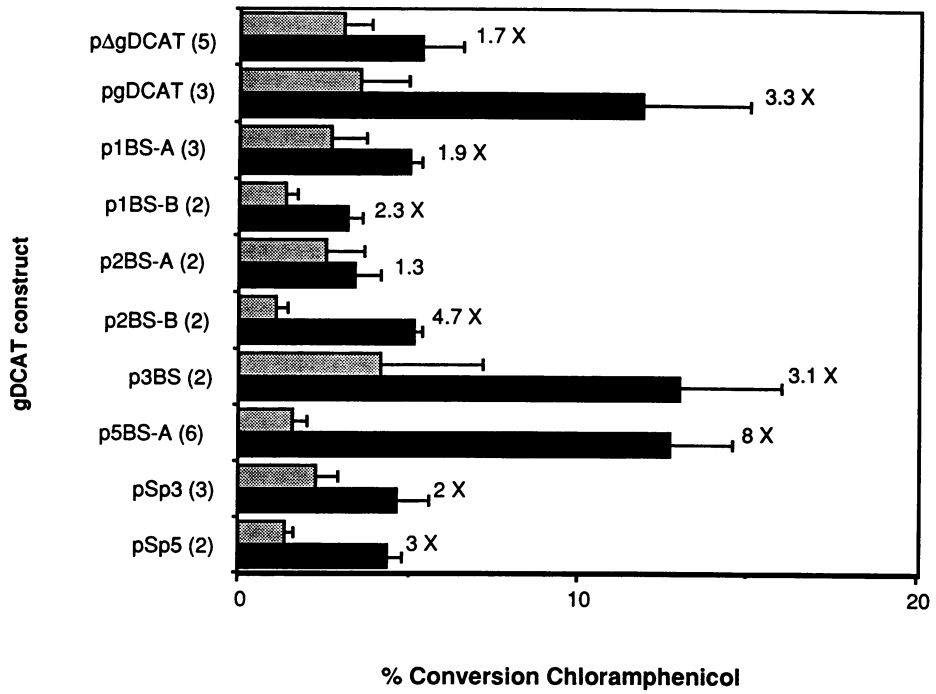

FIG. 8. CAT conversion of gDCAT constructs in the presence and absence of ICP4. Bar values indicate the mean percent conversion of [^{14}C]chloramphenicol to acetylated forms for transfections of the indicated gDCAT constructs cotransfected with either Bluescribe M13+ DNA (▨) or p175 (■). Values in parentheses indicate the number of times each transfection was performed in separate experiments. Error bars indicate standard error of the mean. Fold activation values for each construct are indicated to the right of each data set.

the juxtaposition of ICP4 to a cellular transcription factor(s) is necessary for maximal expression of the gD gene. Careful examination of the gD footprint with ICP4 (fraction VIII) (Fig. 5, lane 2) reveals a weak footprint downstream (−48 to −66) of the α4BS region. This protection may be the result of contaminating cellular proteins in the ICP4 (fraction VIII) preparation or a manifestation of the generalized DNA binding of ICP4 in conjunction with cellular proteins (22). In the case of the gD gene, modulation of transcription may occur either directly, by DNA-ICP4 action, or indirectly, as a result of ICP4 interaction with cellular factors which associate with *cis*-acting elements in the promoter near the ICP4 binding region. As Coen et al. have suggested for the thymidine kinase gene (6), the activation of some viral genes may be mediated by interaction between ICP4 and cellular factors needed at the promoter during transcription, bypassing ICP4-DNA interactions. The ICP4 protein is sufficiently large (175,000 molecular weight) to possess several domains which may allow multiple interactions. Mutational studies by DeLuca and Schaffer (11) and T. Paterson and R. D. Everett (Abstr. 12th International Herpesvirus Workshop 1987, p. 88) have identified a number of the functional domains of the protein. Experiments with temperature-sensitive mutants in the ICP4 gene have shown that certain mutants are incapable of down-regulating immediate-early gene expression but remain viable for *trans* activation of early genes (8). These data support the hypothesis that multiple domains in ICP4 are responsible for the observed effects on gene expression.

All evidence suggests that ICP4 in fraction VIII is responsible for the strong footprints seen in the gD promoter and p5BS-A. The competition footprinting experiments in conjunction with the gel mobility shift assays support the proposal that ICP4 binds specifically to the α4BS fragment and that this interaction is responsible for the observed footprints. These findings do not exclude the involvement of accessory proteins in ICP4-DNA complexes or in the effects observed on levels of gene transcription. We found that the addition of mouse ascites fluid to gel mobility shift reactions with ICP4 (fraction VIII) improved ICP4-DNA complex formation (Fig. 2B, lane C), indicating assistance from auxiliary proteins in the formation of stable complexes. This result is consistent with data reported by P. Kattar-Cooley and K. W. Wilcox, who used a highly purified form of ICP4 that gave rise to enhanced complex formation in the presence of a variety of protein and nonprotein substances (Abstr. 12th International Herpesvirus Workshop 1987, p. 3).

In the competition gel mobility shift assays, a considerable excess of specific competitor DNA was needed (50- to 100-fold molar excess) for competition of ICP4-DNA complexes. It is most likely that the quantity of ICP4 in the reactions was in excess of that needed to detect minimal complex formation. Thus, the competition experiments did not demonstrate 1:1 competition when an unlabeled homologous competitor was used. Binding of ICP4 to the α4BS sequence was specific; strong complexes were detected in the presence of nonspecific competitor DNA in at least 1,000-fold molar excess (*E. coli lac* operator 40-bp fragment or salmon sperm DNA).

The study of promoters containing multiple copies of *cis*-acting regulatory elements has been informative in other viral systems (3, 27, 56). For example, Brady et al. demonstrated increased responsiveness of the human leukemia T-cell virus type 1 long terminal repeat to the *trans* activator p40x when multiple copies of a 21-bp regulatory sequence were present in the promoter (3). In our studies, the presence of one to two ICP4 binding sequences in insertion constructs did not result in a greater response to ICP4 than did the pΔgDCAT construct. This finding may have been the result of the strategy of construction, which approximated but did not duplicate the natural promoter at insertion boundaries. The constructs p3BS and p5BS-A, however, gave a greater response to ICP4 than did pΔgDCAT and the spacer constructs p3BS and p5BS-A. Without further experiments, we cannot ascertain whether the increased ICP4 responsiveness of p3BS and p5BS-A arose from the greater number of sites being occupied by the protein as indicated by the footprint (Fig. 5B) or from the arrangement of sites relative to binding sites for other factors.

The in vitro transcription experiment with p5BS-A produced results similar to those observed previously in kinetic studies with the natural gene fragment (*Ava*I-1) that contains two sites, one at about −100 and the other downstream of the RNA initiation site (1). In both cases, the presence of multiple sites resulted in rapid initiation and extended transcription, characteristic of an activated transcription complex.

Regulation of the gD gene during viral infection has been shown to be primarily at the level of transcription (54). Presumably, the interplay of cellular factors (ICP4 and ICP0) and perhaps other viral factors plays an important role in regulating the level of gene expression in vivo. The use of viral mutants in the ICP4 gene showed that a functional ICP4 protein was essential for the appropriate expression of later gene classes (9, 13, 60). The experiments presented in this study indicate that binding of ICP4 to a specific DNA sequence in the gD gene plays a role in positively affecting transcription of the gene. This regulatory scheme may share elements with the mechanisms of regulation by other HSV genes containing ICP4 binding sites and contribute to an understanding of the mechanism of regulation of viral genes by ICP4.

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