JOURNAL OF VIROLOGY, June 1989, p. 2510–2520
0022-538X/89/062510-11$02.00/0

Vol. 63, No. 6

ICP4-Binding Sites in the Promoter and Coding Regions of the Herpes Simplex Virus gD Gene Contribute to Activation of In Vitro Transcription by ICP4

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Received 17 November 1988/Accepted 16 February 1989

The herpes simplex virus immediate-early gene product ICP4 activates the transcription of viral early and late genes. We characterized the DNA sequence elements of the early glycoprotein D (gD) gene that play a role in the response to ICP4 in vitro. Using gel mobility shift assays and DNase I footprinting, we identified three ICP4-binding sites, two 5′ to the mRNA start site and a third within the coding region. Site II, which gave a footprint between nucleotides −75 and −111 relative to the RNA start site, was previously identified by Faber and Wilcox and contained the reported consensus ICP4-binding site. Site III, which was located between nucleotides +122 and +163, was very similar to the site II sequence, including a core consensus binding sequence, TCGTC. The site I sequence (nucleotides −308 to −282), however, did not share significant homology with either site II or site III. In vitro transcription experiments from mutant constructs of the gD promoter indicated that all three ICP4-binding sites contribute to the stimulation of transcription by ICP4. DNase I footprinting of the gD promoter with uninfected nuclear extracts of HeLa cells showed protection of two very G-rich sequences between nucleotides −33 and −75. We propose that optimal transcription of the gD gene depends on the interaction of ICP4 with multiple binding sites across the gene and cellular factors that recognize specific sequence elements in the promoter.

Herpes simplex virus (HSV) is a large virus with a double-stranded DNA genome. The viral genome encodes approximately 70 proteins, and after infection of a susceptible cell the genes for these proteins are expressed in a temporal order (30, 42). Based on measurements of RNA levels, regulation of transcription plays an important role in controlling viral gene expression (6, 34, 61). Immediately after infection, a subset of five viral genes are transcribed into mRNA. These immediate-early or α RNAs encode proteins, among which ICP4, ICP0, and ICP27 have demonstrated regulatory activity (18, 25). The synthesis of the immediate-early proteins is followed by the synthesis of a second class of proteins, termed early or β proteins, which include enzymes involved in viral DNA replication. With the onset of viral DNA replication, the synthesis of a third class of genes, late or γ genes, is initiated or amplified. The late genes encode many of the proteins that are involved in assembling the virion structure. The orderly expression of the three classes of genes has been shown to involve nucleotide sequences 5′ to the coding regions of the genes and regulatory proteins that interact with these sequences (7, 15–17, 26, 29, 32, 33, 40, 52, 55). How these interactions take place is a central theme in investigations of control of transcription. A substantial amount of information is accumulating on the DNA sequence elements and protein-DNA interactions that regulate immediate-early genes (4, 8, 10, 24, 27, 36, 37, 40, 52, 53), but this type of information is not available for genes of the early and late classes.

The results of transfection experiments (15, 19, 26, 47, 48, 50) are consistent with results obtained with viral mutants that implicated ICP4 as a regulatory factor in establishing the orderly synthesis of viral mRNAs (9, 12, 62, 63). It is not clear whether the mechanism of action of ICP4 involves direct recognition of specific nucleotide sequences in viral DNA or whether the mechanism is indirect and is mediated through cellular proteins. It is possible that a protein with the size and complexity of ICP4 could regulate gene expression by more than one mechanism. If direct or indirect binding of ICP4 to DNA is required for it to function, then it should be possible to distinguish between the generalized DNA-binding properties of the protein (23, 28) and binding to specific nucleotide sequences in viral genes by identifying the sequences and showing that they have a role in modulating RNA synthesis.

Evidence is available that ICP4 interacts with sequences near the start of mRNA synthesis in several HSV genes, and from DNase protection experiments Faber and Wilcox have proposed a core sequence of ATCGTC for ICP4-binding sites in DNA (20, 21). Our earlier experiments on the regulation of the HSV glycoprotein D (gD) gene located ICP4-binding sites in three DNA fragments near the gD mRNA start site; using an in vitro transcription assay, we demonstrated that ICP4 stimulated the initiation of gD mRNA synthesis approximately fivefold (2).

The presence and organization of the three binding sites for ICP4 in the region of the gD promoter and the observation that ICP4 can increase gD promoter activity in vitro pose the question of whether the binding sites are involved in the effect on transcription. In this report, interactions between ICP4 and DNA fragments from the gD gene are documented by using gel mobility shift assays and the nucleotide sequences responsible for the interaction identified by DNase footprinting. We also provide functional evidence that three ICP4-binding sequences in the gD gene,

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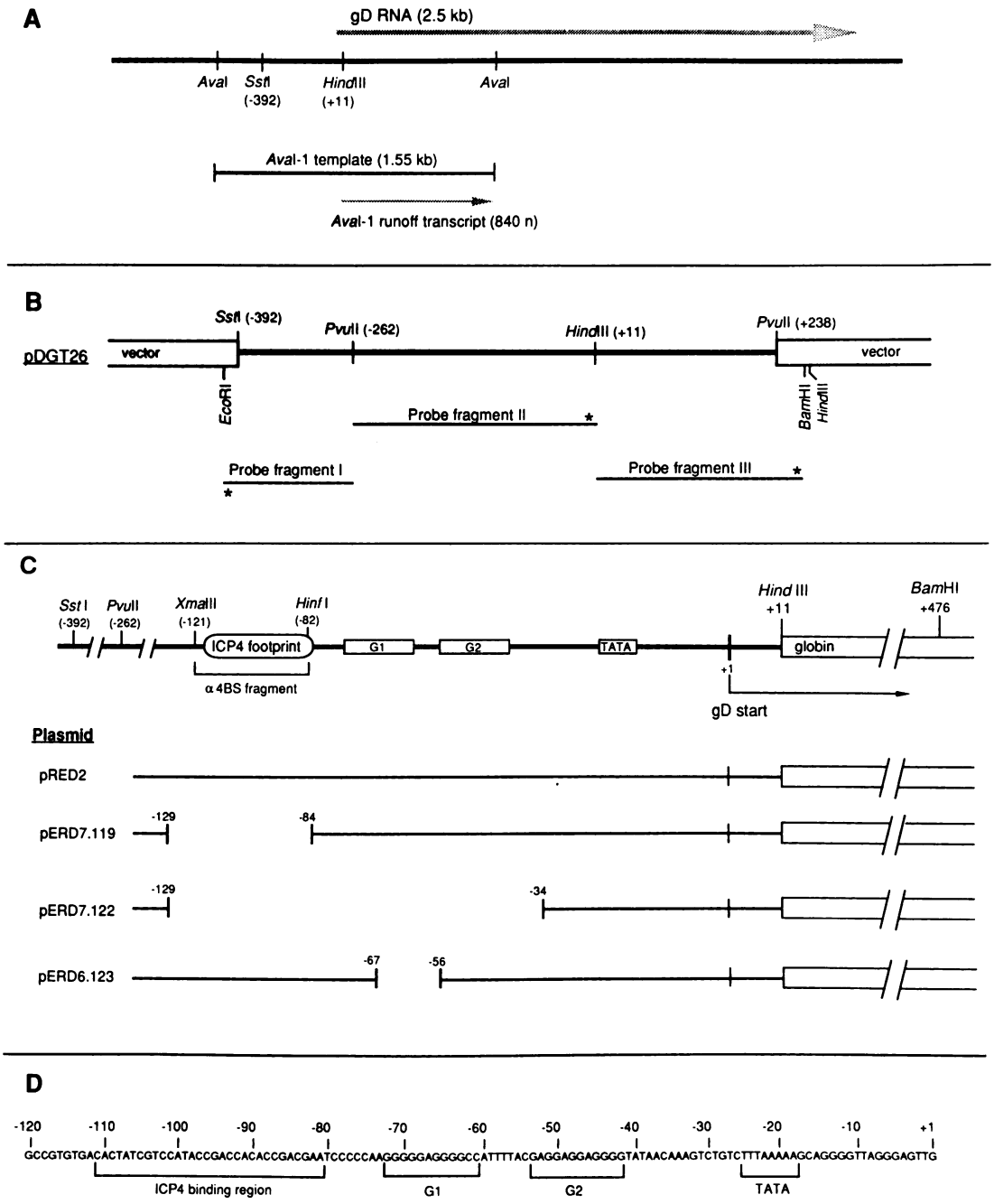

FIG. 1. Organization of the gD gene. (A) *Ava*I subfragment used for in vitro transcription experiments. (B) Illustration of the DNA probe fragments obtained for gel mobility shift assays and DNase footprinting. The *Sst*I-*Pvu*II subfragment was cloned into a Bluescribe vector, and the resulting clone was designated pDGT26. (C) gD internal deletion constructs. (D) Sequence of the gD promoter region (HSV-1 strain 17). The ICP4-binding region is that first reported by Faber and Wilcox (20).

two 5′ to the mRNA start and a third within the coding region, contribute to the stimulation of gD mRNA synthesis by ICP4 in vitro.

MATERIALS AND METHODS

Organization of the gD gene. The gD gene of HSV type 1 (HSV-1) is located in the *Bam*HI-J fragment of the viral genome (3, 38). The gD gene was subcloned as a *Sma*I fragment (pJB3) of HSV-1 (KOS), and with this subclone a restriction enzyme map was generated (3, 31). The 5′ end of the gD mRNA was located near the *Hin*dIII site (31, 64). The 1.5-kilobase DNA fragment produced by *Ava*I digestion (*Ava*I-1) was shown to serve as a template for in vitro transcription and by an immunoaffinity assay to bind ICP4 (Fig. 1A) (2, 31). To facilitate the study of ICP4 binding, an *Sst*I-*Pvu*II subfragment of *Ava*I-1 was subcloned into the *Sst*I-*Sma*I-cut Bluescribe vector (Stratagene, San Diego,

Calif.). The resulting plasmid, pDGT26, allowed efficient labeling of the three DNA fragments that bind ICP4 (Fig. 1B).

The segment within the *Pvu*II-*Hin*dIII fragment bounded by *Xma*III and *Hin*fI contains the reported nucleotide sequence protected by ICP4 from DNase digestion (Fig. 1C) (20). The details of subcloning the α4BS fragment have been published (60). In brief, digestion of plasmid pRED2 with *Xma*III and *Hin*fI gave a DNA fragment containing the ICP4-binding site; this fragment was isolated on a polyacrylamide gel. The 5′ ends of the fragment were filled in with deoxyribonucleoside triphosphates by using Klenow polymerase, *Xho*I linkers were attached, and the resulting 47-base-pair (bp) fragment was ligated into *Xho*I-cut pACYC177 (5). The recombinant plasmid pα4BS, containing the putative ICP4-binding site, was used as a source of competitor DNA to test the specificity of ICP4-DNA interactions. To obtain a competitor DNA fragment that contains the gD promoter sequence but lacks the α4BS sequence, plasmid pERD7.119 (Fig. 1C) was digested with *Xho*I and *Hin*dIII, and the resulting 94-bp fragment was isolated on a polyacrylamide gel. Restriction enzymes for cloning and construction of DNA probes were purchased from Bethesda Research Laboratories, Inc., and New England BioLabs, Inc. The techniques for constructing recombinant DNA molecules and transforming bacteria either were described in our earlier publication (3) or were standard procedures (41).

Mutant constructs. Plasmids containing mutations within the gD promoter have been described elsewhere (16). The 5′ region of the gD gene was joined to the body of the rabbit β-globin gene at the *Hin*dIII site, and recombinant methodology was used to produce defined deletions in the gD promoter segment of the hybrid construct. *Xho*I linkers were inserted at the junction points of the deletions. Several of these deletion mutants are shown in Fig. 1C.

In vitro transcription templates. DNA fragments for use as templates in the in vitro transcription assay were cut from the gD gene (pJB3) or the gD-globin hybrid constructs (16, 31, 51). For the hybrid constructs, the enzyme *Sst*I or *Pvu*II was used to cut the plasmid in the 5′ end of the gD promoter, and *Bam*HI was used to cut in the 3′ globin segment of the DNA (Fig. 1C). Fragments were purified on low-melting-point agarose as previously described (2). The concentration of DNA fragments was determined by visual comparison of ethidium bromide-stained gels containing the template fragments and several dilutions of standard DNA solution.

Runoff bands from in vitro transcriptions were located by autoradiography and excised. Background values were obtained by counting gel segments adjacent to the transcript band and were subtracted for each reaction.

Labeling of DNA fragments. DNA fragments were generated by digesting pDGT26 (Fig. 1B) or gD-globin constructs (Fig. 1C) with restriction enzymes. DNA probes were labeled at the 3′ end with [α-^{32}P]dATP (3,000 Ci/mmol) and the Klenow fragment of DNA polymerase. The other three unlabeled deoxyribonucleoside triphosphates were present during the incubation; unincorporated nucleotides were subsequently removed by Sephadex chromatography. A secondary cut was made in the DNA with an appropriate restriction enzyme, and the radioactive fragments were separated by electrophoresis on a polyacrylamide gel. After elution from the gel, DNA probes were used either directly or after concentration by ethanol precipitation. The specific activities of the probes were in the range of 10^4 to 10^5 cpm/ng. The restriction enzymes used to obtain each probe are described in the legends to the figures. Quantitation of radioactivity was either by Cerenkov counting or by placing excised gel bands in scintillation fluid and measuring the ^{32}P present in a scintillation counter.

Cells and virus. The procedures for growth of cultured cells, preparation of virus stocks, and plaque assay of virus have been previously described (57, 59). The virus, HSV-1 (KOS), was grown and assayed on a continuous line of rabbit skin cells. To prepare nuclear extracts of infected cells, HeLa cells were infected at a multiplicity of infection of 10 to 20 PFU/cell, and the nuclei were isolated after 8 h.

Nuclear proteins. Nuclear extracts were prepared from HeLa cells by the method of Dignam et al. (11). Suspension cells were used to obtain extracts for in vitro transcription, and monolayer cultures from infected or uninfected cells were used to prepare nuclear extracts for binding studies (51). Purified preparations of ICP4 (fraction VIII) were prepared as described by Faber and Wilcox (20) and, before use, were dialyzed against the *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid–glycerol buffer D of Dignam et al. (11). ICP4 (fraction IX) was purified from ICP4 (fraction VIII) by affinity chromatography on immobilized plasmid DNA containing multiple copies of the specific ICP4-binding sequence (35). ICP4 (fraction VIII) was approximately 5% pure, and ICP4 (fraction IX) was essentially homogeneous (35).

Monoclonal anti-ICP4 antibody. The monoclonal anti-ICP4 antibody H944, kindly provided by L. Pereira as an ascites fluid, was diluted with phosphate-buffered saline to give a protein concentration of 2 mg/ml.

Assays of DNA-protein interactions. The interaction between DNA fragments and proteins present in nuclear extracts or purified ICP4 (fraction VIII) was detected with a gel mobility shift assay. The procedure has been previously described (45, 58). In the case of the competition experiments, various amounts of the α4BS fragment containing the ICP4-binding site or other competitor DNA were added to reaction mixtures before the addition of nuclear extract or the ICP4 preparation.

DNA-protein binding was also detected by a DNase I protection assay (footprinting) modified from the procedure described by Dynan (13). Binding reactions were carried out in a 50-μl volume containing about 3 ng of probe DNA labeled at a single 3′ end (15,000 cpm), 10 mM Tris hydrochloride (pH 7.6), 1 mM sodium EDTA, 0.1% Nonidet P-40, and 100 ng of poly(dI-dC). Reactions were initiated by the addition of 2 μl of purified ICP4 in buffer D (5 μg of ICP4 [fraction VIII] protein per reaction), and incubation was for 60 min at 20°C. Immediately before DNase treatment, 50 μl of a solution containing 5 mM $CaCl_2$–10 mM $MgCl_2$ was added to the binding reaction mixture. Typically 0.5 to 6 μl of a 5-μg/ml solution of DNase I (Sigma Chemical Co., St. Louis, Mo.) was added and allowed to digest the DNA for 60 s at room temperature (20 to 22°C). Digestion was terminated by the addition of 12 μl of 3 M ammonium acetate–0.25 M sodium EDTA. Samples were then digested for 30 min at 30°C with 20 μg of predigested pronase (Sigma) and extracted with phenol-$CHCl_3$. Carrier salmon sperm DNA was added to the aqueous phase, and the total DNA was precipitated with ethanol. DNA precipitates were dissolved in 50% formamide containing indicator dyes (0.15% xylene cyanol and 0.15% bromophenol blue), boiled for 2 min, placed on ice, and loaded onto an 8% polyacrylamide–urea sequencing gel.

G+A chemical cleavage reactions were performed on each probe and were used to identify band positions in the DNase-treated samples. In some cases, DNA standards were used as markers.

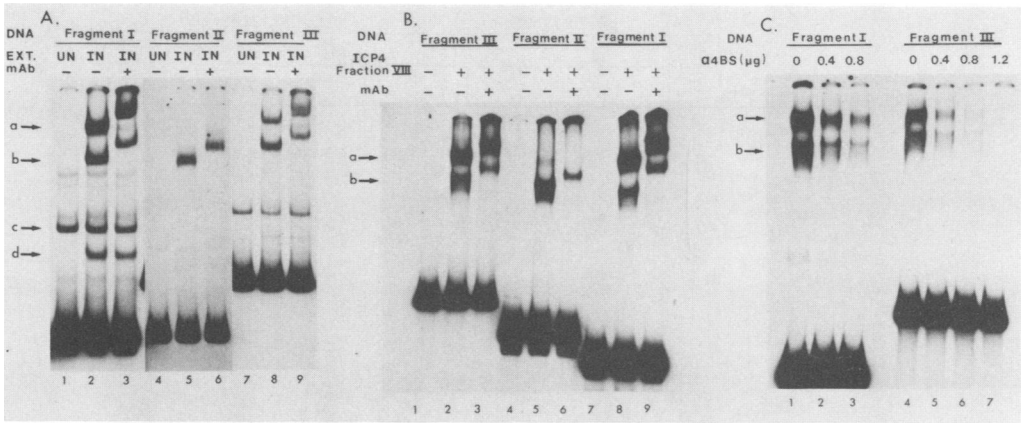

FIG. 2. Association of ICP4 with subfragments from the gD promoter-upstream region. (A) Protein(s) from cell extracts bound to DNA probes I, II, and III. Lanes 1, 4, and 7 show the incubation of uninfected cell extract with the probes. The complexes formed with infected extract are shown in lanes 2, 3, 5, 6, 8, and 9. In lanes 3, 6, and 9, monoclonal antibody against ICP4 was added after the incubation of extract with the probes. (B) Binding of ICP4 from fraction VIII to the probes. Lanes 2, 3, 5, 6, 8, and 9 contain 5 μg of fraction VIII. The addition of the anti-ICP4 monoclonal antibody is shown in lanes 3, 6, and 9. (C) Competition of the probe I and III complexes with unlabeled α4BS DNA. Competitor DNA was included in the reaction mixtures, as indicated, before the addition of fraction VIII. All gel mobility shift assays employed 4 μg of sonicated salmon sperm DNA as nonspecific competitor DNA.

RESULTS

Band shift assays of ICP4 binding to gD promoter DNA. An immunoaffinity assay with monotypic antibody against ICP4 has demonstrated ICP4 binding to three contiguous DNA fragments that span the gD promoter (2). This observation was confirmed and further defined by using the band shift assay to detect DNA-ICP4 complexes. From subclone pDGT26, which contains the 5′ region of the gD gene, three radioactive DNA fragments were obtained. The three fragments, *Eco*RI-*Pvu*II (fragment I), *Pvu*II-*Hin*dIII (fragment II), and *Hin*dIII-*Hin*dIII (fragment III) (Fig. 1B), when incubated with nuclear extracts from HSV-infected or uninfected cells, gave discrete radioactive bands with slower mobility than that of free DNA (Fig. 2A). However, the slowest-moving bands (a and b, or b alone) were only found with infected-cell extract (Fig. 2A, lanes 2, 5, and 8) and were shown to contain ICP4 by a further reduction in mobility when monoclonal antibody against ICP4 was added to the incubation mixture (Fig. 2A, lanes 3, 6, and 9). It should be noted that with fragment II a single ICP4-containing band was observed (Fig. 2A, lane 5, band b), whereas with fragments I and III two slow-moving bands (a and b) were found (Fig. 2A, lanes 2 and 8). Because anti-ICP4 antibody reduces the mobility of both bands a and b, we conclude that ICP4 is present in both DNA-protein complexes. Fragments I and III produced additional bands (c and d) after incubation with uninfected- or infected-cell nuclear extract. Band c was found with both extracts and represents cellular protein-DNA complexes. Band d, although restricted to the infected-cell extract (compare lanes 1 and 2), did not contain intact ICP4, since its mobility was not altered by anti-ICP4 antibody (compare lanes 2 and 3). However, we have not excluded the possibility that band d is generated by proteolytic degradation of band a or b. The specificity of DNA-protein binding was tested by using two unrelated DNAs as probes: a 113-bp DNA fragment from the vector segment of pDGT26 and a fragment from the early region of simian virus 40 including the 21-bp repeat sequences (14). With infected-cell extract the lower bands c and d were present with both DNAs, but the slower-moving complexes, bands a and b, containing ICP4 were not detected (data not shown). These results indicate that bands a and b in Fig. 2A are the result of interactions between proteins including ICP4 and specific nucleotide sequences in fragments I, II, and III. The nature of the complexes in bands c and d was not identified.

A repeat of the binding experiment with ICP4 (fraction VIII) confirmed that fragments I and III form two types of complexes (Fig. 2B, lanes 2 and 8) which on the basis of effects of anti-ICP4 antibody contain ICP4 (Fig. 2B, lanes 3 and 9). The faster-moving complexes, c and d, produced with unfractionated nuclear extracts (Fig. 2A) were not present when ICP4 (fraction VIII) was used, suggesting that the proteins responsible for these bands were removed by the chromatographic procedure used to purify ICP4 (fraction VIII).

As an additional test for the specificity of binding between ICP4 and fragments I and III, a competition experiment was performed with the α4BS DNA fragment previously shown to bind ICP4 (Fig. 1C) (60). Increasing the amount of the α4BS DNA in the binding reaction mixture reduced the amount of fragments I and III in the protein-DNA complexes a and b (Fig. 2C). Quantitation of the radioactivity in bands a and b shows that α4BS DNA is an equally effective competitor for both fragments. Adding 0.4 μg of α4BS DNA reduced the amounts of fragment I or fragment III in complex a and b to approximately 50% of the control values. The same results were obtained with ICP4 (fraction IX).

Using the gel mobility shift assay, we investigated the interaction between ICP4 and *Bst*NI-*Hin*dIII DNA fragments that span the gD gene promoter from nucleotides −166 to +11, including derivatives with defined deletions or additions (60). Although the wild-type promoter fragment from plasmid pRED2 yielded an intense band on the mobility shift gel, the equivalent *Bst*NI-*Hin*dIII fragment from plasmid pERD7.119, which lacks the region of the gD gene promoter from nucleotides −129 to −84, yielded no detectable band. Transfection experiments with deletion mutants have implicated a G-rich DNA sequence from nucleotides −56 to −67 as an important element in regulating transcription of the gD gene (16). Therefore, we tested the ability of ICP4 to bind to a *Pvu*II-*Hin*dIII fragment from plasmid

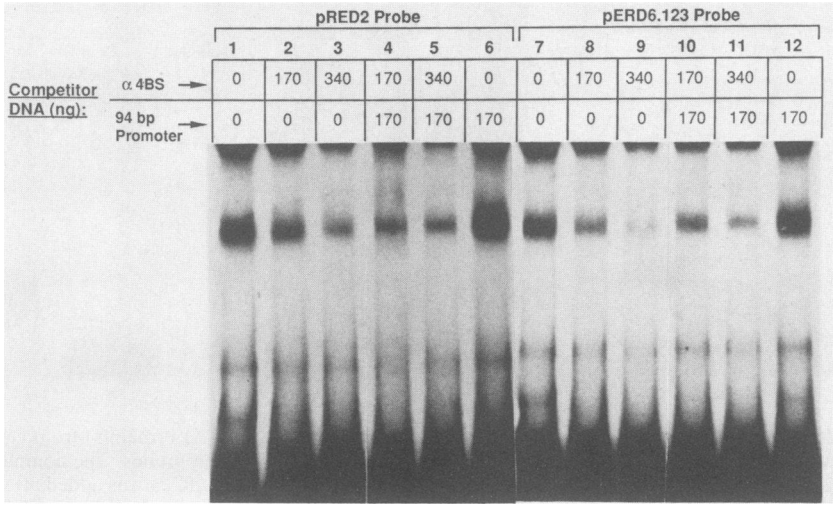

FIG. 3. Binding of ICP4 to gD probes that contain or lack part of the G1 region. Lanes show the complex formation of ICP4 from infected-cell extracts with a *Pvu*II (nucleotide −262)-*Hin*dIII (nucleotide +11) probe from pRED2 (lanes 1 through 6) and with the pERD6.123 probe (lanes 7 through 12). Competitor DNAs containing either the α4BS sequence (lanes 2 through 5 and 8 through 11) or the 94-bp promoter fragment (nucleotides −84 through +11) (lanes 4 through 6 and 10 through 12) were used as competitors as indicated.

pERD6.123, which lacks the G-rich region from nucleotides −56 to −67. This probe formed a DNA-protein complex with the same mobility as that of the wild-type promoter fragment (Fig. 3, lanes 1 and 7). These data, taken together with our earlier results, indicate that formation of a strong complex between ICP4 and the 5′ region of the gD promoter extending from nucleotides −166 to +11 requires nucleotides between positions −84 and −129 and that nucleotides −56 to −67 are not critical for ICP4 binding.

As a more sensitive approach to detect any influence the G-rich sequence could have on ICP4 binding, a gel mobility shift competition assay was carried out with radioactive DNA targets from pRED2 and pERD6.123. DNA fragments containing the ICP4-binding site (α4BS) or the 94-bp promoter region from nucleotides −84 to +11 were used as competitors. The retardation gel (Fig. 3) showed that the α4BS fragment was an effective competitor with both the pRED2 and pERD6.123 targets. Comparing the radioactivity present in lanes 3 and 9 of Fig. 3 showed that they were 35% of the control values in lanes 1 and 7, respectively. When added alone (lanes 6 and 12) or together with α4BS DNA (lanes 4, 5, 10, and 11), the 94-bp promoter fragment did not affect binding.

DNase protection experiments. To locate the nucleotide sequences in fragments I and III that complex with ICP4, DNase protection experiments analogous to those of Faber and Wilcox (20) were carried out. Fragment I contained a stretch of 19 nucleotides between positions −286 and −306 that were protected from digestion; this sequence has been designated site I (Fig. 4A). The 3′ boundary of site I was marked by a hypersensitive band at nucleotide −286. The DNA sequence of this region for HSV-1 (KOS) was determined (data not shown) and found to be very similar to the published sequence for HSV-1 strain 17 (43). The nucleotide sequence in site I (see Fig. 9) lacked significant homology with the consensus sequence (nucleotides −81 to −111) previously identified as an ICP4-binding site by DNase protection (Fig. 1D) (20). In view of this result and the presence of proteins other than ICP4 in fraction VIII, the DNase footprinting experiment was repeated with an apparently pure form of ICP4 (fraction IX). The footprints ob-

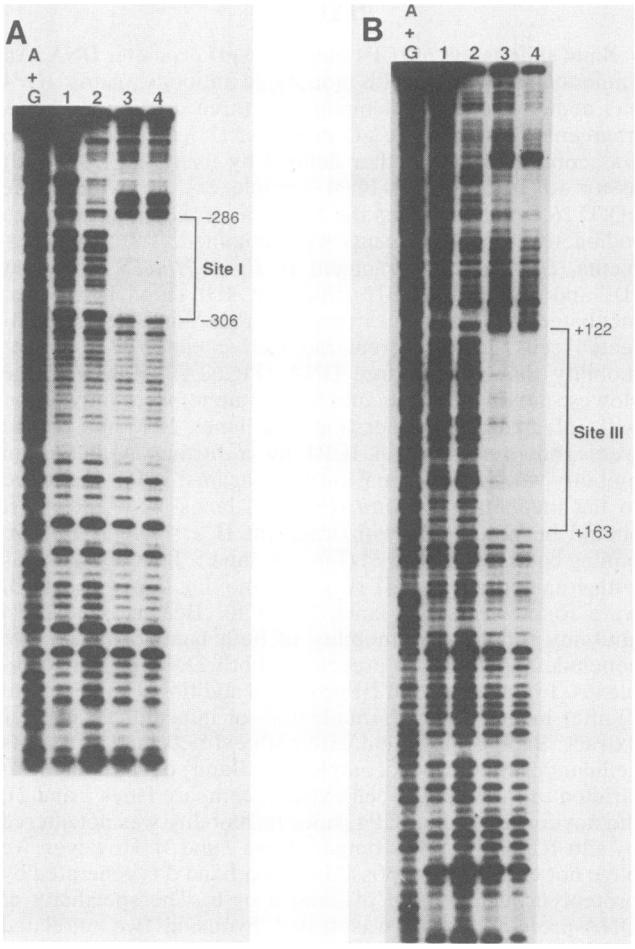

FIG. 4. DNase I footprint of ICP4 (fraction VIII) on the fragment I and fragment III probes. (A) Footprint of the *Eco*RI-*Pvu*II fragment from pDGT26. Lanes 1 and 2 show the ladder formed by DNase treatment alone. Lanes 3 and 4 contain ICP4 (fraction VIII). (B) Footprint of the *Hin*dIII-*Bam*HI fragment from pDGT26. Lanes 1 and 2 show the DNase ladder. Lanes 3 and 4 contain ICP4 (fraction VIII).

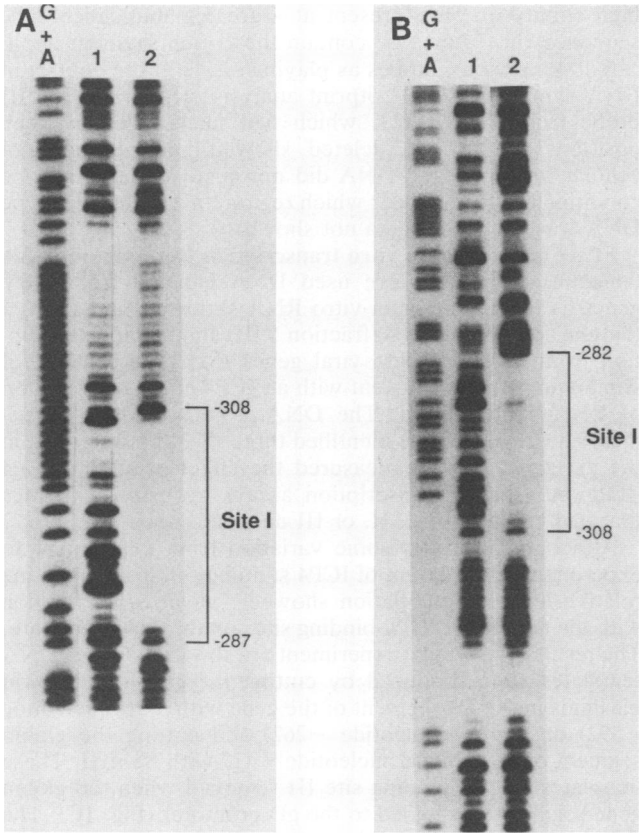

FIG. 5. DNase I footprint of ICP4 (fraction IX) on the fragment I probe. The *Sst*I-*Pvu*II subfragment from pDGT26 was subcloned into Bluescribe and labeled at either end by using restriction sites in the flanking polylinker. Footprints of the bottom (A) and top (B) strands with ICP4 (fraction IX).

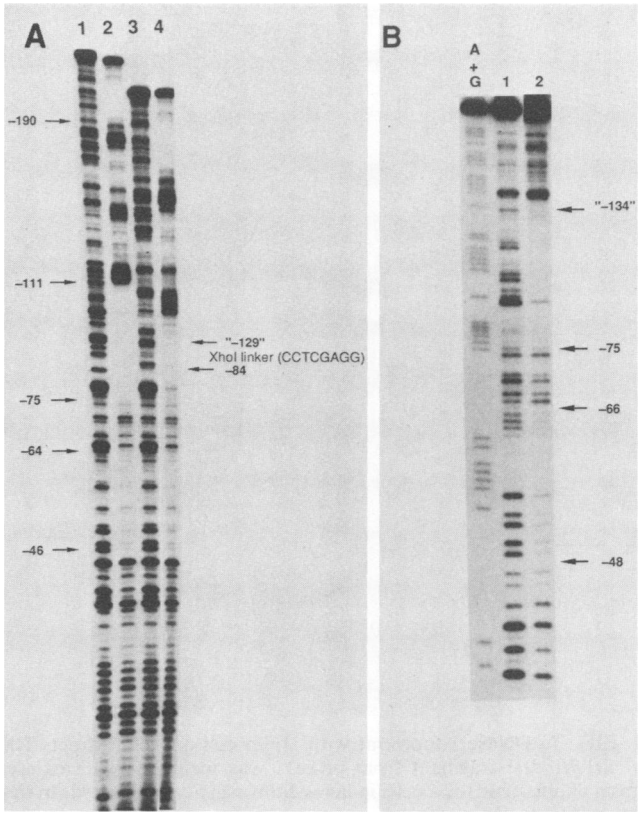

FIG. 6. DNase I footprint of ICP4 (fraction VIII) on the wild-type (pRED2) and pERD7.119 promoter fragments. (A) In lanes 1 and 2 the pRED2 *Pvu*II-*Hin*dIII probe was used, the top strand labeled with the Klenow fragment at the *Hin*dIII site, and in lanes 3 and 4 the pERD7.119 probe was used. In lanes 2 and 4 ICP4 (fraction VIII) was added. (B) Footprint of ICP4 (fraction VIII) on the *Bst*NI (nucleotide −166)-*Hin*dIII (nucleotide +11) pERD7.119 probe; the bottom strand labeled with T4 polynucleotide kinase at the *Hin*dIII site. Lane 1 shows the DNase I ladder with the probe; lane 2 shows the DNase I ladder in the presence of ICP4 (fraction VIII). Sequence coordinates in quotations indicate the nucleotide positions as they would occur in the wild-type gD sequence.

tained for each strand were in the same location, indicating that ICP4 binds directly to site I (Fig. 5).

For fragment III a stretch of 40 nucleotides from positions +122 to +163 was protected from DNase digestion by ICP4 (fraction VIII) (Fig. 4B). The protected region, designated site III, contained a sequence very similar to that previously identified as responsible for ICP4 binding in other HSV genes (20, 21). A hypersensitive site at nucleotide +122 marked the upstream boundary.

In vitro transcription from a mutant gD promoter that lacked site II (pERD7.119) was slightly stimulated by ICP4 (fraction VIII) (see Fig. 8). Although the band shift assay failed to detect a complex between ICP4 (fraction VIII) and the pERD7.119 fragment in previous studies (60), a DNase footprint with this fragment and ICP4 (fraction VIII) revealed protection of a region including the *Xho*I linker inserted at the site of the deletion that extended from nucleotides −129 to −84 (Fig. 6A, lane 4; Fig. 6B, lane 2). By using the wild-type promoter probe from pRED2, a footprint was obtained between nucleotides −75 and −111 (Fig. 6A, lane 2), similar to previous reports (20). Binding to the pERD7.119 probe was weak, since increasing the nonspecific competitor poly(dI-dC) from 100 ng to 1 μg eliminated the footprint with this probe but not with the pRED2 probe (data not shown). For both the pRED2 and pERD7.119 probes, the footprints showed some protection from nucleotides −46 to −64 (Fig. 6A, lanes 2 and 4; Fig. 6B, lane 2) and in the region around nucleotide −190 (Fig. 6A, lanes 2 and 4). These are highly G-rich regions of the DNA, and the interpretation of these results is presented in the Discussion.

As mentioned above, in vivo measurements of RNA synthesis from mutant forms of the gD promoter implicated the G-rich sequence at nucleotides −68 to −46 as important for basal levels of transcription (16). We utilized the DNase protection assay to detect interaction between cellular factors and this region. Nuclear extract from uninfected cells protected sequences between nucleotides −33 and −75 (Fig. 7). This segment of DNA could be divided into two regions ("a" and "b") on the basis of the amount of nuclear extract required to obtain protection. At high concentrations of nuclear extract (Fig. 7, lane 2), protection extended over the whole region, but as the amount of nuclear extract in the assay was decreased, the extent of protection was limited to region "a" (nucleotides −48 to −75) (Fig. 7, lanes 3, 4, and 5). It appears that uninfected-cell nuclear extract contains one or more factors that bind to DNA upstream from the TATA box. The protein(s) that bound to region "b," which was nearer the TATA box, bound weakly or were present at low abundance. Protein(s) that bound to region "a" had a

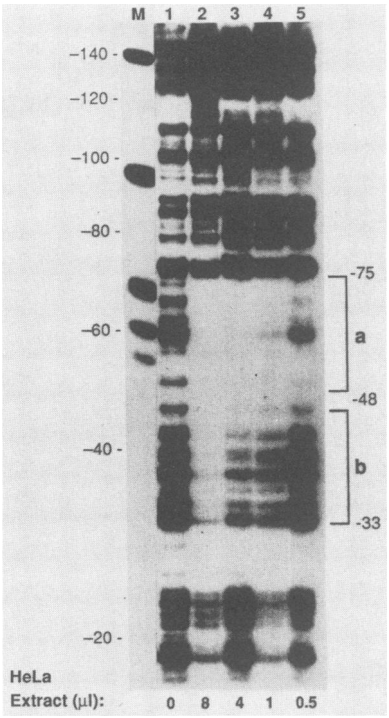

FIG. 7. DNase I footprint with HeLa cell nuclear extract. The *Pvu*II-*Hin*dIII fragment from pRED2 was incubated with extract from uninfected HeLa cells in lanes 2 through 5, as indicated. In this case poly(dI-dC) was used as a nonspecific competitor.

high affinity or were present at a greater abundance. The sequences in region "a" contain the G-rich segment implicated by previous studies as playing a role in the control of RNA synthesis (16). Footprint analysis of a *Pvu*II-*Hin*dIII probe from pERD6.123, which had nucleotides between positions −56 and −67 deleted, showed that the binding of cellular factors to the DNA did not occur under the same conditions as those under which region "a" of the wild-type DNA was protected (data not shown).

Effect of ICP4 on in vitro transcription from mutant DNA templates. Previously we used DNA isolated from HSV genes as templates for in vitro RNA synthesis and showed that the addition of ICP4 (fraction VIII) stimulated transcription from early and late viral genes (51). The kinetics of stimulation were consistent with an ICP4 effect on initiation of RNA synthesis (2). The DNA-protein binding experiments described above identified three ICP4-binding sites in the gD gene, and we measured the effect of adding ICP4 (fraction VIII) to transcription assays by using templates that had binding site I, II, or III deleted.

Although there was some variation from experiment to experiment in the extent of ICP4 stimulation, in all cases the relative level of stimulation showed a positive correlation with the number of ICP4-binding sites on the DNA template. The results of a typical experiment are shown in Fig. 8. DNA templates were produced by cutting the gD-globin hybrid plasmids in the gD segment of the gene with *Sst*I (nucleotide −392) or *Pvu*II (nucleotide −262) and cutting the globin segment of the gene at nucleotide +477 with *Bam*HI. These templates all had binding site III removed when the globin gene segment was joined to the gD promoter (Fig. 1C). The templates cut in the gD segment of the gene with *Sst*I

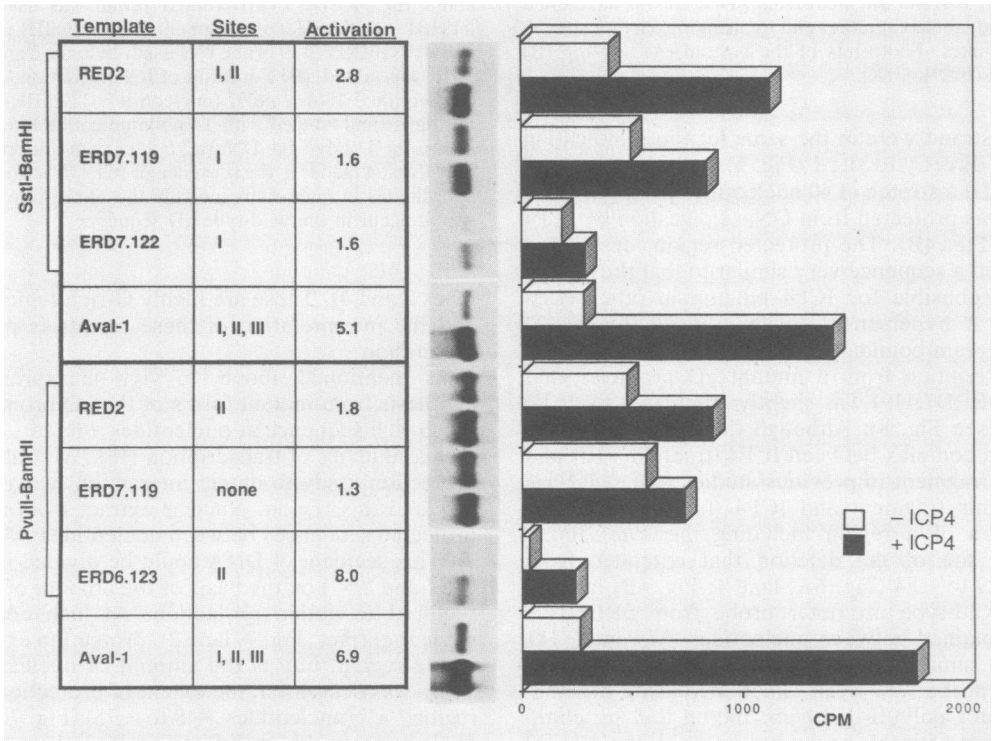

FIG. 8. In vitro transcription from gD deletion template DNAs. The *Sst*I-*Bam*HI fragment or *Pvu*II-*Bam*HI fragment from the DNA constructs was used as a template in the presence or absence of ICP4 (fraction VIII; 18 μg of protein per reaction). The two template sets were tested in independent experiments, each in comparison with the *Ava*I-1 control fragment. In vitro transcriptions were carried out as previously described (2) with Dignam extracts (11) from uninfected HeLa cells. The solid bars indicate basal transcription levels from the templates, and the shaded bars represent transcription in the presence of ICP4 (fraction VIII).

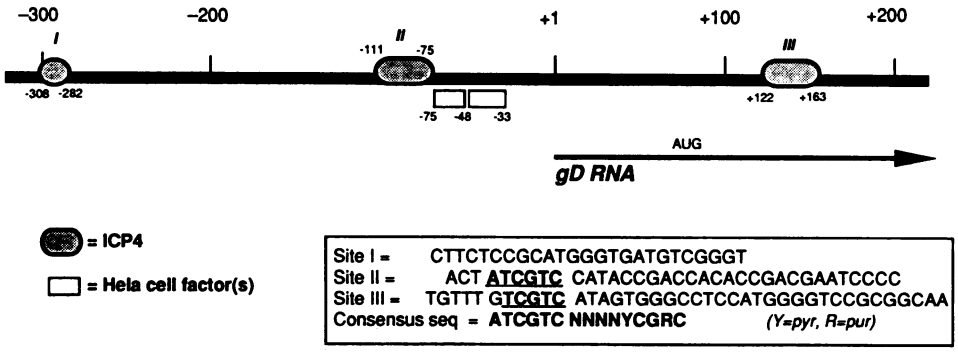

FIG. 9. Summary of DNA-protein interactions across the gD promoter-upstream region. The sequences shown for sites I and III are those of HSV-1 (KOS), determined by sequencing the regions. The site II sequence is from HSV-1 strain 17 (43). The consensus sequence for ICP4 binding is that proposed by Faber and Wilcox (20).

contained an intact ICP4-binding site I, whereas the templates cut with *Pvu*II lacked site I (Fig. 1C).

The series of plasmids shown in Fig. 1C contained deletions that covered ICP4-binding site II (pERD7.119), the G-rich region (pERD6.123), or both of these segments of the promoter (pERD7.122). Therefore, depending on whether *Sst*I or *Pvu*II was used to cut the plasmid, the DNA fragments obtained had zero, one, or two ICP4-binding sites. The levels of transcription obtained with these DNAs were compared with that obtained with the *Ava*I-1 fragment that contains the three ICP4 sites present in the wild-type gD gene (Fig. 1A). Inspection of runoff assays showed that DNA fragments from plasmids that lacked the G-rich segment from about nucleotides −60 to −73 had a reduced ability to serve as a template for RNA synthesis. As we reported previously (2), transcription from the *Ava*I-1 fragment was stimulated by ICP4 (fraction VIII), and the stimulation appeared higher with the *Sst*I-*Bam*HI templates than with the *Pvu*II-*Bam*HI templates from pRED2.

Quantitation of the radioactivity in the runoff transcripts by counting the bands excised from the gel confirmed the qualitative conclusions drawn from the autoradiographs. ICP4 (fraction VIII) produced the strongest stimulation when the *Ava*I-1 fragment was the template (five- to sevenfold), and the *Sst*I-*Bam*HI fragments with site I present gave a greater stimulation than did comparable *Pvu*II-*Bam*HI templates with site I removed. Templates derived from the deletion mutant pERD7.119, which showed only weak binding to site II (Fig. 6), were stimulated to a lesser degree than were comparable DNA fragments from pRED2 (Fig. 8).

The low level of transcription with templates lacking the G-rich sequences made quantitation difficult. However, the template from pERD6.123 was of special interest because it contained an intact ICP4-binding site II and a small deletion over the G1 box. In three experiments with this template the stimulation by ICP4 (fraction VIII) ranged from three- to eightfold. In our experiments, the extent of stimulation by ICP4 increased when the number of binding sites was increased from zero to three.

DISCUSSION

Experiments with mutant virus and transfections with isolated genes have shown that the protein ICP4 plays an important role in activating transcription of the early and late HSV genes (10, 12, 18, 49). Initial biochemical studies showed that ICP4 is a large phosphorylated protein that binds DNA (23, 28, 65). It was subsequently demonstrated that sequence-specific binding of purified ICP4 to DNA is stabilized by the presence of cellular proteins (P. Kattar-Cooley and K. W. Wilcox, personal communication). This observation raises important questions about the mechanism by which ICP4 regulates RNA synthesis. For example, (i) does ICP4 depend on interactions with cellular factors to establish specific binding to certain sites in DNA, and (ii) is DNA binding an essential component for the role of ICP4 in gene activation?

In this paper we present experimental evidence that there are three binding sites for ICP4 on the gD gene near the start site for mRNA synthesis. Two of these, sites II and III, contain a sequence similar to the core element, ATCGTC, identified by Faber and Wilcox as present in several DNA fragments that bind ICP4 (Fig. 9) (20, 21). DNase protection experiments showed that site I does not contain the core sequence (Fig. 9), and we conclude that ICP4 binds strongly to at least two distinctly different sequences in the gD gene, one that has previously been recognized (20) and a second that is novel. The site I sequence was not present in the DNA fragments studied by Michael et al. (44), and the possibility must be considered that ICP4 binds to a number of nucleotide sequences present in HSV and other viral genes. A search of a viral gene bank by using the IBI/Pustell sequence program has located sequences with homology to site I in other HSV genes and in cytomegalovirus, Epstein-Barr virus, and other animal virus genes.

Both fragments I and III gave two distinct bands on mobility shift assays (Fig. 2). These bands could be the result of two distinct binding sites on each fragment, the association of different forms of ICP4 with a single site, or the presence of different cellular proteins with ICP4 in the complexes. We failed to located two binding sites by using DNA subfragments in the band shift assay or by DNase footprinting, and we found the same bands with highly purified ICP4 (fraction IX) (data not shown). We consider that the most plausible explanation for the two bands is different ratios of ICP4 to DNA in the complexes.

The correlation between the number of DNA-binding sites on the DNA template and the stimulation of in vitro transcription indicates that ICP4 binding contributes to the stimulation of gD mRNA synthesis. This result is consistent with in vivo experiments with recombinant promoter constructs containing three or five copies of the site II sequence placed in the site II location (60). We do not have direct evidence that sites I and III play a role in regulating gD mRNA synthesis, but it should be possible to test their role

by altering the nucleotide sequences in sites I and III as well as their relative positions in the gene. The normal spacing of sites I, II, and III at approximately 200-bp intervals would permit cooperative binding of ICP4 to DNA, and protein-protein interactions between ICP4 molecules on these sites could alter the structure of the DNA in the gD promoter (54).

In vitro transcription experiments have indicated that ICP4 facilitates the initiation of RNA synthesis from the gD gene (2). Initiation complex formation requires the presence of several transcription factors (56), and we may hypothesize that the mechanism of ICP4 action is mediated through one or more of these cellular transcription factors. By binding to DNA, ICP4 could stimulate factor binding or substitute for cellular factors and thereby increase the rate of complex formation. To explore this possibility, it is important to identify binding sites for cellular transcription factors on the gD gene and to determine whether the factors interact with ICP4. Examination of the nucleotide sequence 5′ to the mRNA start site reveals TATA boxes at nucleotides −20 and −40 and two G-rich regions at nucleotides −50 and −70 (Fig. 1D). Based on transfection experiments, the TATA box and G-rich regions are required for RNA synthesis (16), and our in vitro transcription assays confirm these results (Fig. 8). We have shown by DNase footprinting that the G-rich sequence at nucleotides −60 to −70 binds a cellular factor(s) (Fig. 7). We also observed protein(s) binding to G-rich DNA located between ICP4-binding sites I and II at approximately nucleotides −190 and −220 (Fig. 6), but we have no evidence that these DNA-protein interactions have functional importance.

At first sight the results described here are not entirely consistent with earlier observations on the DNA sequence requirements for regulated expression of the gD promoter in transfection experiments (15, 16). These studies showed (i) that fully efficient transactivation of the gD promoter required sequences from nucleotides −83 to +11 only, (ii) that within this region two G-rich sequences and the TATA box were important *cis*-acting signals, and (iii) that no sequences solely and specifically involved in transactivation by HSV-1 gene products could be detected. However, in these earlier studies infectious virus was used to activate the transfected gD promoter, thus supplying (in addition to ICP4) all of the viral gene products, including other transactivators such as ICP0 and ICP27. Second, it is not known whether other ICP4-binding sites in the tetracycline resistance gene in the vector (20) or even weak novel sites such as that at the *Xho*I linker in pERD7.119 (Fig. 6) could have contributed to the transactivation of the plasmid-borne gD promoter by ICP4. Such distant or weak sites may explain the slight transactivation in cotransfection experiments of gD promoters lacking consensus ICP4-binding sites (60).

For the gD gene we focused on ICP4-DNA binding and the role this interaction plays in controlling RNA synthesis. However, many if not most of the HSV promoters activated by ICP4 do not contain the core consensus sequence ATCGTC (19, 42, 43). To explain the activation of these genes, it has been proposed that ICP4 interacts directly with transcription factors to modify their binding properties (18). According to this proposal the nucleotide sequence involved in binding would reflect the properties of the transcription factor rather than ICP4. Although there is genetic evidence that cellular TATA box factors and certain TATA sequences are the targets for ICP4 activation, additional biochemical experiments are required to show whether ICP4 and the TATA box factors or other cellular transcription factors are physically associated (19). This mechanism of transactivation in which a viral protein interacts with a cellular factor has been shown to operate with the adenovirus protein E1A. E1A, which is not itself a DNA-binding protein, acts in conjunction with cellular factors to regulate transcription from viral and cellular genes (46). The analogy between E1A and ICP4 may be quite close, since the cellular factor ATF, which responds to E1A, binds to the nucleotide sequence (A/T)CGTC, which is homologous to the ICP4 core sequence ATCGTC (39). In addition, mutants in the E1A gene can be complemented by herpesvirus immediate-early gene products (22). Recently, the immediate-early gene product of pseudorabies virus was shown to interact with the cellular transcription factor TFIID (1). These data suggest that common elements are involved in transactivation by proteins from very different viruses.

Alternatively, the core consensus sequence ATCGTC may represent only one of a family of binding sites recognized by ICP4. Clearly, as exemplified by site I at nucleotide −300 in the gD region, there are other sequences to which ICP4 can bind. Such redundancy of recognition sequences would have a number of consequences, such as the presence of one or more ICP4-binding sites within promoter regions lacking the ATCGTC consensus and difficulty in predicting such sites on the basis of DNA sequence homology alone.

A protein the size of ICP4 may be expected to bind to DNA and to complex with proteins in a variety of ways, thereby stimulating or inhibiting RNA synthesis by several molecular mechanisms. Whether transcription from a particular gene is stimulated or inhibited by ICP4 probably depends on the nature of the cellular factors involved in initiation complex formation, the organization of the ICP4-binding sites on the gene, and how ICP4 affects the interactions between transcription factors, DNA, and RNA polymerase. Although there may be common features that separate HSV genes into early and late classes, it appears that each gene will have to be examined individually until a pattern is established.

ACKNOWLEDGMENTS

We thank Lenore Pereira for providing the anti-ICP4 monoclonal antibodies. We thank Betsy Litman for assistance with gel mobility shift experiments. Helen Bruggmann assisted with preparation of nuclear extracts and DNase footprinting.

This work was supported by Public Health Service research grants A121402-03 and AI 17246 from the National Institutes of Health and with funds from the Fonds National Suisse de la Recherche Scientifique. D.G.T. was supported by Public Health Service neurovirology training grant T-32 Ns07321-01 from the National Institutes of Health.

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