Relationship between TATA-Binding Protein and Herpes Simplex Virus Type 1 ICP4 DNA-Binding Sites in Complex Formation and Repression of Transcription

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Received 24 March 1995/Accepted 31 May 1995

The herpes simplex virus (HSV) regulatory protein, infected-cell polypeptide 4 (ICP4), represses the transcription of promoters that have binding sites for ICP4 located near the transcription start site. It has also been shown that ICP4 binds such promoter DNA cooperatively with the TATA-binding protein (TBP) and TFIIB to form a tripartite protein-DNA complex (C. Smith, P. Bates, R. Rivera-Gonzales, B. Gu, and N. A. DeLuca, *J. Virol.* 67:4676–4687, 1993). In this study, we analyzed the effects of position and orientation of the ICP4-binding site relative to the TATA box in the ICP4 promoter on transcriptional repression by ICP4 and on the ability of ICP4 to form tripartite complexes with TBP and TFIIB. The results of this parallel study provide a strong correlation between tripartite complex formation and repression. Both tripartite-complex formation and transcriptional repression were efficient when the ICP4-binding site was downstream of the TATA box, within a short distance and in proper orientation. In addition, both tripartite-complex formation and repression were partially sensitive to the stereoaxial positioning of the ICP4-binding site relative to the TATA box. As a preliminary characterization of the tripartite complex, circular permutation analysis was performed to assess the distortion of the proximal promoter region in the tripartite complex. As previously reported, both TBP and ICP4 independently could bend DNA and the relative magnitude by which each of these proteins bent DNA in the tripartite complex was preserved. The results of this study suggest that the formation of tripartite complexes on a promoter is part of the mechanism of repression and that simple blocking as a sole result of ICP4 binding is not sufficient for full repression.

Transcription of eukaryotic viral genes by RNA polymerase II is often modulated by virus-encoded transcription factors. Therefore, regulation of the cellular transcription machinery by viral gene products has served as a helpful paradigm for understanding eukaryotic transcription. Viral transcriptional regulators, like their cellular counterparts, can interact with both general transcription factors, such as the TATA-binding protein (TBP), TBP-associated factors, and TFIIB (4, 40, 59, 61), or gene-specific transcription factors, such as RB, p53, and oct-1 (7, 13, 18, 20, 54). Several cellular factors, such as RB (33, 52) and YY1 (50, 57), and viral transcription factors, such as simian virus 40 large T antigen (5, 46), are known to both positively and negatively regulate transcription. Herpes simplex virus (HSV) encodes a 175-kDa phosphoprotein (6), referred to as infected-cell polypeptide 4 (ICP4), which can positively or negatively regulate RNA polymerase II-dependent transcription of many HSV genes. Genetic and biochemical analyses revealed that ICP4, like many cellular transcriptional regulators (reviewed in reference 45), contains discrete domains responsible for DNA binding, nuclear localization, and transcriptional regulation (9, 49, 56) and that the domain requirements for activation are greater than those for repression (56).

ICP4 interacts with both DNA and transcription factors in the transcription initiation complex to exert its regulatory effects. ICP4 is a sequence-specific DNA-binding protein (15, 37), which binds a fairly degenerate consensus sequence (27,

44). The most recent consensus derived from a statistical analysis of different potential binding sites is RTCGTCNNYNY SG, where R is purine, Y is pyrimidine, S is C or G, and N is any base (11). In vitro and in vivo studies collectively suggest that the binding of ICP4 to specifically located sites is not required for activation and that nonspecific DNA contacts may suffice (21, 58). However, specifically located ICP4-binding sites are required for repression (22, 43, 53). The HSV genes that ICP4 negatively regulates include those for the ICP4 gene itself (8, 48), the promoter for the latency-associated transcript (1), and the promoter for L/ST (3, 64). All these promoters contain strong ICP4-binding sites located near the transcription initiation site (2, 3, 16, 37, 47, 64). Mutation of this binding site also causes alteration in the temporal expression of the promoter, shifting its kinetics of expression to an earlier time postinfection and altering its sensitivity to inhibitors of DNA synthesis (35, 51). Biochemical studies with purified ICP4, TBP, and TFIIB showed that these proteins cooperatively interact on the ICP4 promoter containing a TATA box and a functional ICP4-binding site (59). It was hypothesized that the formation of the TBP-TFIIB-ICP4-DNA complex is involved in the mechanisms of ICP4 function, including repression.

The requirement for an ICP4-binding site near the transcription start site for repression and the observation that ICP4 forms tripartite complexes with TBP and TFIIB on a repressed promoter led us to investigate whether ICP4 and the basal initiation complex require specific alignment for the observed regulation from such promoters and whether the extent of tripartite complex formation correlates with repression. To address this hypothesis, we altered the stereoaxial positioning, spacing, and orientation of the ICP4-binding site relative to the TATA box in the ICP4 promoter. The promoter constructs

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FIG. 1. Wild-type and mutant ICP4 promoter constructs. (A) Schematic diagram of plasmid p4. The thicker and thinner lines represent plasmid and viral DNA, respectively. The Sp1-binding site, TATA box, and ICP4-binding site are indicated by boxes. The transcription start site and the direction of transcription are shown by the arrow. The site of oligonucleotide insertion was the *Ava*I site. The location of the -40 primer used for primer extension is also shown. The primer was labeled with 32 P at the 5' end is indicated by an asterisk. The numbers (-108 and $+27$) are the nucleotide positions relative to the transcription start site. Abbreviations of the relevant restriction sites: A, *Ava*I; B, *Bam*HI; E, *Eco*RI. (B) DNA sequences of the different promoter constructs from the TATA box to the ICP4-binding site. The TATA box and the ICP4-binding site are shown in boldface letters. The transcription start site is indicated by an arrow.

were used as probes for DNA-binding studies with purified ICP4, TBP, and TFIIB and as templates for in vitro transcription studies. The results demonstrate that each of the three parameters spacing, phasing, and orientation affect both tripartite-complex formation and repression of transcription in vitro, to different degrees. Moreover, the degree of cooperative DNA binding by ICP4, TBP, and TFIIB correlated with the extent of repression, supporting a role for both DNA-protein and protein-protein interactions in ICP4-mediated repression.

MATERIALS AND METHODS

DNA templates. The plasmid p4, which contains a fragment of the ICP4 promoter from -108 to $+27$ relative to the transcription start site was used to construct all the ICP4 promoter templates. The ICP4 promoter fragment in p4 contains a strong ICP4-binding site centered at $+2$, a TATA box centered at -25 , and an Sp1-binding site centered at -80 . This fragment is sufficient for Sp1-responsive transcription of ICP4 promoter from the correct initiation site (22, 29). Insertion mutants of the ICP4 promoter were generated by digestion of p4 by *Ava*I (Fig. 1A) followed by insertion of synthetic DNA fragments of various lengths. Oligonucleotides $5'$ -CCGACGTGC3' and $5'$ -TCGGGACGCG3' were phosphorylated with T4 polynucleotide kinase. Upon hybridization, the double-stranded oligonucleotide provides *Ava*I-compatible ends and can multimerize. After ligation of the double-stranded oligonucleotides with DNA containing *Ava*I-compatible ends, the *Ava*I site in the left end (of the top strand) is not preserved. Excess molar concentration of the double-stranded oligonucleotides were ligated with *Ava*I-digested p4 to generate p18, p127, p136, p145, and p154, which contain two, three, four, five, and six copies of the double-stranded oligonucleotides, respectively. Plasmids p17 and p14 were fortuitous recombination products, generated presumably from plasmids with longer inserts. Plasmids p14, p122, and p140 are generated by digesting p4, p118, and p136, respectively, with *Ava*I, filling in the ends with Klenow enzyme in the presence of deoxyribonucleotide triphosphates, and then self-ligating with T4 DNA ligase. Plasmid p110 was generated by inserting a single copy of double-stranded oligonucleotide generated upon hybridization of $5'$ -CCGACGCGTC3' and $5'$ -TCGGGACGCG3' in the *Ava*I site of p4. The sequences of the promoter insertion mutants are shown in Fig. 1B. Plasmid p4R is identical to p4 except that the ICP4-binding site of ICP4 promoter ($5'$ -ATCGTCCACAGG3') was reversed by replacing DNA sequence from the *Ava*I site to the *Bam*HI site by the hybridization product of two 45-mer oligonucleotides.

Plasmid pBEND4 was constructed for use in the circular permutation analysis by inserting an 81-bp *Nae*I-*Sal*I fragment from plasmid p4 into the *Xba*I and *Sal*I sites of plasmid pBEND2 (31). The *Nae*I-*Sal*I fragment of p4 contains the TATA box and ICP4-binding site of the ICP4 promoter. The *Xba*I end of pBEND2 was filled in with Klenow enzyme prior to *Sal*I digestion. All the plasmid constructs

were sequenced to confirm the identity of the constructs by the dideoxy-chain termination method.

Proteins. HeLa cell nuclear extract was prepared by the method of Dignam (12). Recombinant human TBP (rTBP) was purified from extract prepared from *Escherichia coli* BL21 carrying plasmid pETHIID (30) as described previously (26). A fast protein liquid chromatography (FPLC) Mono S fraction of TBP was used throughout this study. Recombinant human TFIIB (rTFIIB) was purified from extract derived from *E. coli* carrying plasmid pFIIB (23) as modified by Smith et al. (59). An FPLC Superose 12 column fraction was used for this study. ICP4 was purified from HSV-1 (strain KOS)-infected Vero cell nuclear extract as previously described (27, 55).

In vitro transcription and primer extension. Supercoiled DNA template (26.6 μ g/ml) was incubated for 80 min at 30°C with nuclear extract (50 μ g of protein) in a buffer containing 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 60 mM KCl, 12% glycerol, 8.3 mM MgCl_2 , 0.6 mM each ATP, CTP, GTP, and UTP, 0.3 mM dithiothreitol, and 12 U of RNasin (Promega). Where indicated, 200 ng of ICP4 was added to the transcription mixture. The order of addition was (i) nuclear extract, (ii) ICP4, and (iii) DNA template suspended in reaction buffer. The reaction was stopped by the addition of stop buffer (0.25 M sodium acetate [pH 5.2], 15 mM EDTA), and the transcribed RNA was purified by two phenol extractions followed by ethanol precipitation. The purified transcripts were subjected to primer extension as previously described (25). The -40 sequencing primer for the pUC plasmids (New England Biolabs, Beverly, Mass.) was used for primer extension. Primers were labeled by phosphorylation with T4 polynucleotide kinase in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Primer extension products were resolved on 5% polyacrylamide-7.0 M urea sequencing gels. Following electrophoresis, the gels were fixed in 10% methanol-10% acetic acid solution and then dried. The dried gels were used for autoradiography and were also scanned for quantitative analyses with a radioanalytic imaging system (AMBIS Inc., San Diego, Calif.).

Electrophoretic mobility shift assays. The *Eco*RI-*Bam*HI fragments of p4, p4R, and p14 through p154 were used as probes for gel shift analysis. For pBEND4, the plasmid was digested with *Mlu*I, *Nhe*I, *Spe*I, *Dra*I, *Sma*I, or *Ssp*I and the fragments containing the direct repeat sequences of pBEND2 and the ICP4 promoter insert were used as the probes. In all cases, the DNA fragments were dephosphorylated with calf intestine alkaline phosphatase and then phosphorylated with T4 polynucleotide kinase in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The labeled DNA fragments were isolated from 5% polyacrylamide gels and were further purified with Elutip-D minicolumns (Schleicher & Schuell, Keene, N.H.) and Sephadex G-50 spin columns.

DNA-binding reactions and the subsequent gel shift analysis were performed as previously described (59), except that the KCl concentration of the binding buffer was 80 mM and the DNA-protein complexes were resolved with 3.5 to 4.0% native polyacrylamide gels containing $0.5\times$ TBE buffer (45 mM Tris base, 45 mM borate, 1 mM EDTA [pH 8.0]). The amounts of proteins used in the binding reaction were 50 ng of TBP, 100 ng of rTFIIB, and 50 ng of ICP4. The

gels were dried and exposed to Kodak X-Omat AR films and also scanned for quantitative analyses with the AMBIS radioanalytic imaging system.

Circular permutation analysis. The group of DNA probes generated from plasmid pBEND4 were incubated with ICP4, or TBP and TFIIB, or all three proteins, and the complexes were electrophoretically resolved, as described above. The mobilities of the bound and free probes were measured from the autoradiograms of the dried gels. The relative mobilities of the complexes (mobility of bound/free probes) were calculated and were plotted against the fractional distance. Fractional distance is the length in base pairs from the center of DNA-binding site to the 5' end of the top strand of the probe, divided by the length of the probe. The resulting data points were fitted by linear least-squares analysis to the equation of a parabola, $y = ax^2 + bx + c$, and the bend angle was determined for the TBP-TFIIB-DNA and ICP4-DNA complexes from the first- and second-degree parameters [$a = 2c(1 + \cos \theta)$ and $-b = 2c(1 + \cos \theta)$] of the equation, as previously described (17, 38). The angle of deflection of DNA from linearity (α) was calculated from the relationship $\alpha = 180^\circ - \theta$.

RESULTS

The focus of this study was to explore the relationship between the cooperative association of ICP4, TBP, and TFIIB on promoters containing both an ICP4-binding site and a TATA box and repression by ICP4. The formation of this complex was previously observed and was hypothesized to be involved in ICP4 regulatory functions, including repression (59). As a test of this hypothesis, we varied the position and orientation of the two binding sites relative to each other and conducted a quantitative comparison of the ability of ICP4 to participate in tripartite complexes and its ability to repress transcription.

Effect of relative TATA box and ICP4-binding-site position on tripartite complex formation and repression. Plasmids pi4, pi7, pi10, pi14, pi18, pi22, pi27, pi40, pi45, and pi54, which contain the ICP4 promoter sequence from -108 to +27 relative to the transcription start site (Fig. 1A) and 4 to 54 bp of DNA inserted between the TATA box and the ICP4-binding site (Fig. 1B), were used as templates for in vitro transcription in the presence and absence of ICP4. The inserted sequences (Fig. 1B) do not have any significant match to an ICP4-binding site according to the predictive model proposed by DiDonato et al. (11). Plasmid p4 was included in each transcription reaction mixture in equimolar amounts to the test template to serve as an internal control for minor sample losses incurred in the isolation of the transcribed RNA and the extended primer and for slight variations in template concentration. The transcripts were visualized as primer extension products resolved in sequencing gels. The primer extension products generated from each set of reactions are shown in Fig. 2A. Transcription was initiated in vitro from the wild-type ICP4 promoter from the authentic initiation site. The ICP4-binding site in the ICP4 promoter overlaps with the transcription start site. Therefore, insertion of linker DNA increased the distance between the TATA box and the ICP4-binding site, moving the original start site further downstream. As expected, transcription from all the insertion mutant templates started about 25 bp downstream from the TATA box. Thus, the length of the extension products increased by the length of the inserted sequences. Transcription from the p4 template was higher than from the remaining insertion templates, probably because of the alteration in the natural start site. However, repeated experiments demonstrated that transcription from all the insertion templates was nearly the same in the absence of ICP4. In the presence of ICP4, transcription from the different templates was affected differently. Repression by ICP4 was greatest on the natural p4 template. This template was repressed 5- to 10-fold. The absolute level of repression seen for individual insertion templates varied approximately twofold from experiment to experiment, as did the absolute level of repression of the p4 internal control. The global twofold variation was seen from experiment to experiment and may reflect slight differ-

FIG. 2. The effect of phasing and spacing of the TATA box and the ICP4-binding site on repression and tripartite-complex formation. (A) Effect on repression. Equimolar amounts of the indicated insertion mutant and the p4 template (as the internal control) were used in in vitro transcription reactions. The bottom bands represent transcription initiated from p4 template. Transcripts initiated from the insertion mutants have lower mobility because of the inserted sequences of variable lengths. The presence or absence of ICP4 in the reaction mixtures is indicated by + and -, respectively. (B) Effect on tripartite-complex formation. Labeled DNA probes generated from p4 and pi4 through pi54 were incubated with TBP, TFIIB, and ICP4 for 40 min at 30°C. The reaction mixtures were resolved on 3.5% polyacrylamide gels. The protein-DNA bands corresponding to the TBP-TFIIB-DNA complex, the ICP4-DNA complex, and the tripartite complex are indicated by DB, ICP4 and TPC, respectively.

ences in the relative concentrations of transcription factors and ICP4. Small variation within an experiment was evident from repeat experiments. This was controlled for by normalization to the internal control. Figure 2A shows that ICP4 noticeably repressed transcription from templates pi4 through pi22. However, repression was not seen with templates pi27 through pi54.

To observe the effect of the insertion on the ability of ICP4 to form tripartite complexes, labeled DNA probes generated from the ICP4 promoter (plasmid p4) and the insertion mutants (plasmids pi4 through pi54) were incubated with ICP4, TBP, and TFIIB and the resulting protein-DNA complexes were resolved by gel shift analysis. Control gel shift experiments showed that ICP4 alone or TBP-TFIIB specifically formed stable protein-DNA complexes with each of the DNA probes and that ICP4 bound equally well to all the probes in the absence of ICP4 (data not shown). In the simultaneous presence of all three proteins, three different complexes, namely, the TBP-TFIIB-DNA, the ICP4-DNA complex, and the ICP4-TBP-TFIIB-DNA complex, were formed with the DNA probes (Fig. 2B). The tripartite complex was predominant on the p4 probe but was also readily detectable with templates pi4 through pi22 (Fig. 2B). The formation of this

FIG. 3. Graphical representation of the phasing and spacing effects on repression and tripartite-complex formation. The data for this representation were derived from two independent experiments for repression and tripartite-complex formation, including that in Fig. 2. For repression, the ratio of the number of counts obtained in the presence of ICP4 to the number obtained in the absence of ICP4 for each insertion template was determined. This ratio was normalized to the ratio obtained for the internal control (p4 template) and is represented as the relative repression for the different promoter constructs. For complex formation, the fraction of DNA-bound ICP4 in the tripartite complex was calculated by dividing the number of counts in the area corresponding to the tripartite-complex band by the total number of counts present in the areas corresponding to the tripartite complex and the ICP4-DNA complex.

complex was greatly reduced with the rest of the templates (pi27 through pi54); however, trace amounts of complex could be detected upon prolonged exposure of the film. The apparent reduced binding of ICP4 in probes that form tripartite complexes poorly may result from the lack to cooperativity between ICP4 and TBP binding. This would also reduce the amount of ICP4 complex migrating at the position of the ICP4-only complex, since a proportion of the tripartite complex would be expected to dissociate in the process of electrophoresis. Also, consistent with previous findings (59), DNase I footprinting of isolated tripartite complexes formed on the p4 and pi7 templates revealed specific protection of both the TATA box and the ICP4-binding site (data not shown).

To determine whether the degree of repression from the different promoter constructs was related to the degree of tripartite-complex formation, the data derived from the gel shift and *in vitro* transcription experiments were quantified and compared graphically. The degree of ICP4-mediated repression from each template was normalized relative to the repression of the wild-type promoter. The data from duplicate experiments were averaged and plotted against the number of base pairs of DNA inserted between the TATA box and the ICP4-binding site (Fig. 3). The fraction of total ICP4-bound probe present in the tripartite complex was also determined for each of the templates. Again, the data from duplicate experiments were averaged and also plotted against the number of base pairs of DNA inserted between the TATA box and the ICP4-binding site (Fig. 3). It is evident from Fig. 3 that both tripartite-complex formation and ICP4-mediated transcriptional repression dropped in a similar fashion as the distance between the TATA box and the ICP4-binding site was increased. Second, in templates with TATA boxes and ICP4-binding sites separated by less than 40 bp of DNA, both ICP4-

mediated repression and, to a lesser extent, tripartite-complex formation were noticeably affected by the stereoaxial positioning of the two binding sites. Thus, tripartite-complex formation and ICP4-mediated repression were reduced more for the template pi4 than for pi10. This trend was evident but less pronounced for templates pi14 through pi27. Since the TATA box and the ICP4-binding site are separated by about 2.5 helical turns of DNA in the ICP4 promoter (10), the two binding sites are separated by about 3, 4, and 5 helical turns in the templates pi4, pi14, and pi27, respectively, and by approximately 3.5 and 4.5 helical turns in the templates pi10 and pi22, respectively. When the TATA box and the ICP4-binding site was separated by longer distances (50 bp or longer), tripartite-complex formation and transcription repression by ICP4 were not seen. The data in Fig. 3 demonstrate that both repression and tripartite-complex formation are concomitantly reduced when the ICP4-binding site and the TATA box are moved further apart, strongly suggesting that ICP4-mediated repression is related to the degree of tripartite-complex formation on the ICP4 promoter. To a lesser degree, the data suggest that a separation of an odd number of half-helical turns of DNA is preferred for both repression and tripartite-complex formation.

To investigate whether the orientation of the ICP4-binding site in the ICP4 promoter is functionally important in tripartite-complex formation and repression, we constructed a mutant promoter with the ICP4-binding site reversed in orientation relative to the TATA box (Fig. 4A). Both promoter constructs were used as templates for *in vitro* transcription. The transcripts generated from each template in the absence and in the presence of ICP4 are shown in Fig. 4B. In the absence of ICP4, transcription from p4R was less than that from p4 (about fourfold), probably because of the alteration of the native start site region. In the presence of ICP4, transcription of the less active p4R template was reduced approximately two-fold relative to that seen in the absence of ICP4, while transcription of the more active p4 template was barely detectable and was reduced by greater than 10-fold. Therefore, the magnitude of the repression was at least fivefold greater with the p4 template than with the p4R template.

To investigate the ability of ICP4 to form tripartite complexes on these promoter constructs, labeled DNA fragments encompassing the ICP4-binding site, TATA box, and the transcription start site from p4 and p4R were incubated with ICP4 and/or TBP and the resulting complexes were resolved by electrophoresis in native polyacrylamide gels (Fig. 4C). ICP4 formed complexes with both the probes with nearly identical avidity. TBP also recognized the TATA boxes of both promoters and formed stable DNA-protein complexes in the presence of TFIIB. Tripartite-complex formation was clearly prevalent with the probe p4, which contains the ICP4-binding site in its natural orientation. However, tripartite-complex formation was significantly reduced with the p4R probe relative to the p4 probe. The fraction of ICP4-bound DNA in the tripartite complexes formed on the p4 and p4R probes was 91 and 39%, respectively. Data from two independent experiments indicated that tripartite-complex formation was approximately threefold greater with the ICP4-binding site in the natural orientation. Again, the data provide a correlation between the ability of ICP4 to form tripartite complexes and its ability to repress transcription.

Distortion of the promoter in the tripartite complexes. Both ICP4 (14) and TBP (24, 32, 34, 38) induce bends upon binding to DNA. Whether the bends induced by ICP4 and TBP are retained in the tripartite complexes was investigated, since distortion of the proximal promoter may be involved in some

FIG. 4. Effect of the orientation of the ICP4-binding site on repression and tripartite-complex formation. (A) DNA sequences between the TATA box and the ICP4-binding site of templates p4 and p4R. The TATA box and the ICP4-binding site are in boldface letters. The orientations of the TATA box and the ICP4-binding site are shown by the arrows. (B) The p4 and p4R templates were included in *in vitro* transcription reaction mixtures in the presence (+) and absence (–) of ICP4. The bands shown in the brackets indicate the primer extension products from transcripts initiated in the presence and in the absence of ICP4. (C) Effect of ICP4-binding-site orientation on tripartite-complex formation. Labeled DNA probes generated from p4 and p4R were incubated with ICP4 and/or TBP and TFIIB as indicated and then resolved in a 3.5% nondenaturing polyacrylamide gel. The protein-DNA bands corresponding to the DB complex, the ICP4-DNA complex, and the tripartite complex are indicated by DB, ICP4, and TPC, respectively.

aspect of ICP4-mediated repression. DNA probes with circularly permuted TATA boxes and ICP4-binding sites (Fig. 5A) were incubated with TBP and TFIIB and/or ICP4 and then resolved in native polyacrylamide gels. As expected, the mobility of DB and the ICP4-DNA complexes varied depending on the positions of TATA box and ICP4-binding site, respectively (Fig. 5B). The mobility of the tripartite complex also varied depending on the locations of the DNA-binding sites; however, the pattern produced in the gel shifts with the six different probes was not symmetrical (Fig. 5B). This is readily seen by comparing the pattern of the gel shifts produced by ICP4 and the tripartite complex in the same experiment (Fig. 5B, bottom).

Binding-site-dependent mobility variation of protein-DNA complexes is indicative of DNA bending (62). The relative mobility of the complexes versus fractional distance was plotted (Fig. 6) and analyzed as described in Materials and Methods. The analysis of the curve in Fig. 6A revealed that the DNA bend induced in the DB complex was approximately $87 \pm 5^\circ$. This is consistent with previous observations with TBP alone and indicates that the TBP-induced DNA bend is retained in the DB complex. Similar analysis of the curve in Fig. 6B yielded a bend angle of $62 \pm 2^\circ$ for the DNA in the ICP4-DNA complexes. Therefore, the obtained values for the human TBP-TFIIB and HSV-1 ICP4 are comparable to those previously reported for yeast and *Arabidopsis* TBP (32, 34, 38) and HSV-1 ICP4 (14), respectively. The relative mobility of the tripartite complexes plotted against the fractional distance of the ICP4 binding sites, TATA boxes, or points midway between the two sites is shown in Fig. 6C. It is clear that none of these treatments of the data resulted in a parabolic relationship, from which a bend angle can be correctly determined. This can be

reconciled by considering that the DNA in tripartite complex is bent by two different proteins, each to a different degree, at two different positions. If both proteins bent DNA to the same degree or if only one of the proteins bent DNA in the tripartite complex, the circular permutation analysis would result in a symmetrical pattern. In addition, the data imply that TBP is still bending DNA to a greater extent in the tripartite complex than in ICP4. The mobility of a protein-DNA complex is more strongly retarded when the bend occurs in the middle of the fragment. When the TATA box is more centrally located relative to the ICP4-binding site, the mobilities of the probes are retarded the most (Fig. 5B, probes M, N, and S). When the ICP4-binding site is more centrally located relative to the TATA box, the mobilities of the probes are retarded the least (Fig. 5B, probes A and P). Therefore, the lack of a parabolic relationship suggests that both ICP4 and TBP are bending DNA in the complex and the asymmetrical nature of the observed pattern of probe mobilities suggests that TBP is still bending the DNA in the tripartite complex more than is ICP4.

DISCUSSION

Biochemical and genetic studies have demonstrated that ICP4-binding sites located near the start of transcription are required for repression by ICP4 (22, 43, 53). In the case of the ICP4 promoter, there is an ICP4-binding site approximately 23 bp downstream of the TATA box. It has also been shown that ICP4 will form a multiprotein complex (tripartite complex) on this promoter in the presence of TBP and TFIIB (59). The assembly of this complex is cooperative since its formation is favored over the formation of the TBP-TFIIB-DNA or ICP4-DNA complexes. This report provides a quantitative correla-

FIG. 5. DNA bound to the TBP-TFIIB, ICP4, and tripartite complexes is distorted. (A) The set of DNA probes generated from plasmid pBEND4 for the circular permutation analysis. The locations of the TATA box and the ICP4-binding site (ICP4 BS) are shown in each of the probes by a square and a rectangle, respectively. The restriction endonuclease sites used to generate the probes are also indicated (A, *SmaI*; D, *DraI*; M, *MluI*; N, *NheI*; P, *SspI*; S, *SpeI*). (B) Circular permutation analysis of the DB, ICP4-DNA, and tripartite complexes. The probes shown above were individually used for gel shift analysis following incubation with TBP and TFIIB (top), ICP4 (middle), and all three proteins (bottom). Shown are the relevant complexes seen in the gel shift. The letters above the lanes represent the restriction endonuclease used to generate the probes used in the indicated gel track.

tion between the ability of ICP4 to form this complex and its ability to repress transcription of its own promoter. These studies also address the requirement for the proper orientation and phasing of the ICP4-binding site relative to the TATA box for both repression and complex formation. The approach taken to measure repression by ICP4 was the use of a reconstituted *in vitro* repression assay. We chose this approach over inserting the promoter constructs into the virus because it has previously been shown that repression by ICP4 can alter the kinetics or maximal time of expression during viral infection (35, 51) and because the *in vitro* system is more amenable to analysis of multiple mutant constructs.

The findings of the functional studies demonstrated the following: (i) increasing the spacing between the ICP4-binding site and the TATA box results in a decrease in the ability of ICP4 to repress transcription and a proportional decrease in the ability of ICP4 to participate in tripartite complexes; (ii) there is a small degree of helical preference for both repression and complex formation when the distance between the ICP4-binding site and the TATA box is relatively small; and (iii) reversing the orientation of the ICP4 binding site results in a decrease in the ability of ICP4 to repress transcription and form tripartite complexes. These observations provide a strong

FIG. 6. Curves of relative mobility versus fractional distance. (A) For the DB complex, the relative mobility was plotted as a function of the fractional distance of the TATA box. The data points were fitted by linear least-squares analysis to the equation $y = 1.43x^2 - 1.17x + 0.68$ ($r = 0.99$). A DNA bend angle of $87 \pm 5^\circ$ for the DB complex was calculated from the first- and second-order terms of the equation from two independent experiments, as described in Materials and Methods. (B) For the ICP4-DNA complex, the relative mobility was plotted as function of the fractional distance of the ICP4-binding site. The data points were fitted by linear least-squares analysis to the equation $y = 0.31x^2 - 0.33x + 0.30$, ($r = 0.99$). The calculated bend angle for ICP4 was $62 \pm 2^\circ$. (C) For the tripartite complex, the relative mobility was plotted as a function of the fractional distance of the ICP4-binding site, the TATA box, and a point between the ICP4-binding site (ICP4 BS) and the TATA box.

mechanistic connection between the ability of ICP4 to repress transcription and the participation of ICP4 in complexes with TBP and TFIIB.

The observation that the axial phasing of the ICP4-binding site and the TATA box is not as important as absolute distance may reflect some degree of flexibility inherent in the system. Precedent for this consideration is provided from studies examining the activation of the adenovirus E1B promoter by Sp1 (63). In that study, it was found that the spacing between the TATA box and the Sp1-binding site was more important than phasing for activation. However, some degree of optimal phasing was observed when the distance between the Sp1 site and

the TATA box was short. This situation is similar to what we observed for repression by ICP4. How suboptimal spacing is partially compensated for remains to be determined. ICP4 is a very large and elongated dimer molecule, and it would not be surprising if it were multivalent or if regions of the molecule were flexible. McGeoch et al. (41) noted that the regions of ICP4, which are conserved between HSV and varicella-zoster virus, are sometimes separated by "spacer regions" predicted to form a random-coil structure. We have noted that some of these regions greatly contribute to the Stokes radius of the molecule (unpublished observations). Perhaps these regions are also flexible to some extent. This hypothesis is supported by the observation that the effect of phasing was most clearly seen within one helical turn of the natural spacing between the two sites. In this situation, the ability to compensate for suboptimal phasing would be more limited by spatial constraints.

The finding that reversal of the ICP4-binding site relative to the TATA box also simultaneously decreased both repression and tripartite-complex formation again provides a mechanistic connection between the two phenomena. That some repression and tripartite-complex formation was seen with the reversed site may reflect the above considerations or the existence of a low-affinity ICP4-binding site due to the degree of degeneracy in the ICP4-binding site (11). However, the data clearly show that one orientation is greatly preferred for repression, suggesting that ICP4 predominantly represses transcription in a direction-dependent manner and not by just simply blocking transcription. The directional requirement for the ICP4-binding site in repression is also supported by previous findings that three promoters, which are repressed by ICP4, i.e., ICP4 itself (8, 48), latency-associated transcript (1), and L/ST (3, 64), all possess ICP4-binding sites oriented in the preferred orientation for tripartite complex formation.

The data in this study all demonstrate a correlation between the extent of tripartite-complex formation and repression. From this and the cooperative nature of complex formation, we infer that there are protein-protein contacts between ICP4 and the DB complex on DNA. It is possible that the distortion created by the binding of ICP4 to DNA is transmitted down the helix to the TATA box such that TBP binds with higher affinity. The binding of ICP4 to DNA has previously been shown to affect the susceptibility of a nearby TATA box to chemical modification (10). We have shown that DNA binding by ICP4 is necessary but not sufficient to form tripartite complexes (21a), and therefore we favor the notion that a region of ICP4 is necessary to contact the DB complex, leading to cooperative binding. Moreover, it has been previously demonstrated that the spacing between the TATA box and the ICP4-binding site in the ICP4 promoter is 2.5 helical turns (10). When the ICP4-binding site was moved farther away, we observed that the optimal spacing for complex formation and repression was an odd number of half-helical turns. Since TBP (32, 34, 39, 60) and ICP4 (11, 27) bind DNA in the minor and major grooves, respectively, a separation of an odd number of half-helical turns would align the binding sites on the same side of the helix, providing the best opportunity for protein-protein contacts between the two DNA-binding proteins.

ICP4 may negatively affect transcription by multiple mechanisms. Indeed, small negative effects that do not approach the effects seen with ICP4-binding sites located near cap sites have been seen with upstream ICP4-binding sites (43). Perhaps an upstream ICP4-binding site interferes weakly with the communication between upstream activators and the transcription complex. Our hypothesis for how ICP4 strongly represses transcription through the cap site mechanism is that it initially engages the cellular transcription machinery in structures sim-

ilar to the tripartite complexes we have described. While this implies a more complicated process than simply blocking via DNA binding, it does not rule out the possibility that overproduction of ICP4 leads to some repression by blocking. We have also noted that both ICP4 and TBP (TFIID in the cell) bend DNA, and these distortions are apparently conserved in the tripartite complexes. The distortion of the start site/TATA box region may lead to repression by not allowing other proteins such as activators to function. We have previously shown that ICP4 does not repress basal transcription but, rather, generally inhibits the function of activators (21a, 22). It is possible that the conformation of this complex does not allow activators to function by prohibiting the formation of higher-order transcription complexes. The cellular proteins NC1, NC2, and Dr 1 sequester TBP on the promoter in an unproductive complex by inhibiting the further formation of the basal transcription complex (28, 42). ICP4 would work similarly to inhibit activated transcription. The participation of ICP4 in the transcription complex may limit the access of coactivators to the complex, which are necessary for activator function. One such coactivator, p15 or PC4 (19, 36), has amino acid sequence similarity to a region of ICP4 which is important for the formation of tripartite complexes (59). p15 also forms tripartite complexes with TBP and TFIIB (36). It is possible that the formation of the ICP4-TBP-TFIIB complex precludes the association of p15. Alternatively, the participation of ICP4 in tripartite complexes may prohibit activators from functionally interacting with TBP-associated factors. These possibilities are currently being tested.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI27431.

We thank Michael Carrozza for valuable discussions and comments on the manuscript.

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