

ICP4, the Major Transcriptional Regulatory Protein of Herpes Simplex Virus Type 1, Forms a Tripartite Complex with TATA-Binding Protein and TFIIB

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The ICP4 protein of herpes simplex virus can either increase or decrease the rate of transcription mediated by RNA polymerase II, depending on the target promoter. The interplay of DNA-protein and protein-protein contacts determining ICP4 function has yet to be characterized, and consequently the molecular mechanism by which the protein acts remains unclear. ICP4 can transactivate minimal promoters containing only TATA homologies, and therefore it is reasonable to hypothesize that ICP4 works by influencing the TATA-dependent assembly of general transcription factors via specific protein-protein interactions. This study directly addresses this hypothesis by determining whether ICP4 affects the assembly of general transcription factors on templates bearing a TATA box and an ICP4-binding site. Using gel retardation and footprinting assays, we found that ICP4 forms a tripartite complex with TFIIB and either the TATA-binding protein (TBP) or TFIID. The formation of this complex was not the result of simple tripartite occupancy of the DNA but the consequence of protein-protein interactions. In the presence of all three proteins, the affinity of ICP4 and TBP for their respective binding sites was substantially increased. Using mutant derivatives of ICP4 and defective versions of promoters, we also demonstrated that the ability of ICP4 to regulate gene expression correlated with its ability to form a tripartite complex with TFIIB and TBP *in vitro*.

Viruses that infect eukaryotic cells often encode transcriptional regulatory proteins to ensure the appropriate expression of genes during the viral life cycle. With such notable examples as the E1A protein of adenovirus (2, 19), large T antigen of simian virus 40 (34), and the E2 protein of papillomavirus (53), these proteins act to repress and/or activate transcription by a plethora of mechanisms. Herpes simplex virus (HSV) is complex in that it encodes several proteins that directly modulate viral gene expression. These proteins are VP16 (α -Tif, Vmw65, ICP25) (4, 54), ICP0 (α 0, IE110) (61), ICP27 (α 27, IE63) (60, 63), and ICP4 (α 4, IE175) (6). The involvement of multiple regulatory proteins is most probably to help mediate the complex temporal cascade exhibited by HSV's 75 transcription units. This cascade unfolds in roughly three phases: immediate early (IE or α), early (E or β), and late (L or γ) (27). The transcription of the five IE genes of HSV occurs without prior viral protein synthesis and is stimulated by VP16, a component of the infecting virus (4, 54). The IE proteins are absolutely required for progression to the E and L stages of the cascade (28). One IE protein, ICP4, is singularly required for viral infection to proceed past the IE phase of transcription (13, 56, 70).

Through studies involving viral genetics, transient assays, and biochemical analysis, it has been established that ICP4 is essential for virus growth (13, 56) and functions as a transcriptional activator in some cases (8, 15, 21, 46, 57) while operating as a repressor in others (8, 47). ICP4 binds DNA nonspecifically (20), but it also shows a significant preference for the consensus ATCGTCNNNNYCGRC, where R

= purine, Y = pyrimidine, and N = any base (16). When this site overlaps the start site of mRNA synthesis, ICP4 can inhibit transcription when provided in *trans* (8, 48, 59). However, in cases in which ICP4 serves to induce a gene, deletion of sites for which ICP4 shows relatively high affinity does not affect activation by ICP4 (68). In fact, exhaustive studies have failed to reveal any evidence for the existence of ICP4-specific induction sequences (5, 14). In addition, it has been possible to isolate mutant ICP4 molecules that exhibit reduced affinity for DNA *in vitro* but which still retain the ability to stimulate gene expression in the context of the infected cell (32, 65). Despite these observations, a strong correlation exists between mutants of ICP4 that can no longer bind DNA and the ablation of its transactivation function (50, 66). Considered collectively, these observations suggest that at least in some cases, ICP4 may transactivate by binding the target template in a manner that is characterized by low affinity and high degeneracy with respect to sequence specificity. Thus, it seems that the nonspecific and specific DNA-binding capabilities of ICP4 coupled with the positioning of high- and low-affinity sites collectively determine ICP4's ultimate effect on the activity of a promoter.

Studies have shown that ICP4 can transactivate transcription of minimal promoters in which the only recognizable *cis*-acting element is a TATA homology (30, 41, 64). Independent of how DNA binding relates to ICP4 function, this finding suggests that ICP4 operates through the transcriptional machinery acting at the TATA box. This machinery consists of the general transcription factors (GTFs) TFIID, TFIIA, TFIIB, TFIIF, TFIIE, TFIIH, and TFIIV. These proteins assemble on the DNA template in a very defined and ordered fashion along with RNA polymerase II (Pol II)

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to initiate transcription. The first factors to assemble are TFIID, TFIIA, and TFIIB (3, 40). TFIID consists of many different proteins nucleated in a complex containing the TATA-binding protein (TBP), a 43-kDa protein that specifically binds TATA boxes (26, 33, 52). TFIIB consists of a single protein (33 kDa) that binds to TFIID as well as TBP to form a DNA-protein complex termed DB (22, 52). While its exact molecular nature is still being defined, it is known that TFIIA participates in the formation of DA and DAB complexes (40).

The available evidence indicates that if indeed ICP4 interacts with the general transcription machinery, it probably does so directly or indirectly via TFIID. ICP4 can operationally substitute for the cellular transactivator Sp1, as evidenced by the fact that transcription driven by a promoter whose Sp1 sites are selectively deleted is reduced by only a factor of 2 to 3 in the presence of ICP4, compared with more than 30 in its absence (30). This indicates that ICP4 and Sp1 may act through a common pathway. Since Sp1 ultimately exerts its effects through TFIID (55), it is reasonable to suggest that ICP4 does so as well. In addition, it was also found that the ICP4 inducibility of a given promoter is inversely proportional to the promoter's natural affinity for TBP, at least in the cases tested (31). This finding suggests that ICP4 may work by recruiting TFIID to the preinitiation complex. Supporting this hypothesis are findings from *in vitro* transcription experiments using the pseudorabies IE protein, an analog of HSV ICP4. In these experiments, the inductive effects of the pseudorabies protein are eliminated when the DNA templates are presaturated with TFIID (1).

In this study, we directly assess the effect of ICP4 on the very first steps in the assembly of an active transcription complex. The experiments examine the interplay between ICP4, TBP (or TFIID), TFIIB, and DNA with and without the corresponding *cis*-acting binding sites for ICP4 and TBP. Specifically, we determine whether ICP4 can interact *in vitro* with TBP and TFIIB. In addition, the functional significance of these interactions is evaluated by using mutant derivatives of ICP4 that are deleted in functionally important regions of the molecule. It is found that ICP4 forms a tripartite complex with TBP and TFIIB when an appropriate DNA substrate is used and that this complex is the result of cooperative protein-protein interactions. The ability of ICP4 to bind to DNA is necessary but not sufficient for the formation of the tripartite complex, and ICP4's ability to form this complex correlates with its ability to activate transcription.

MATERIALS AND METHODS

Cells, viruses, and proteins. (i) **ICP4, purified from HSV-infected Vero cells.** Wild-type ICP4 was obtained from 10^8 Vero cells infected with HSV-1 (strain KOS). The truncated mutant, n208 ($\Delta 774$ –1298), was obtained from n208-infected Vero cells (10). Mutant X25 ($\Delta 30$ –274, $\Delta 774$ –1298) was obtained following infection of X25 cells with KOS. X25 cells harbor the gene expressing the X25 mutant. Since this gene retains the IE promoter/regulatory sequences, it can be induced to high levels by VP16 supplied by viral infection. X25 cells, their induction upon infection, and the subsequent purification of X25 homodimers were previously described (67). The viruses expressing the nd3-8 ($\Delta 30$ –142, $\Delta 774$ –1298), nd8-10 ($\Delta 142$ –210, $\Delta 774$ –1298), and d8-10 ($\Delta 142$ –210) ICP4 mutant proteins were constructed as described previously (10, 66), using the previously characterized plasmids pndi3-i8, pndi8-i10, and pdi8-i10, respectively (66). General proce-

dures for infection, harvesting of the cells, nuclear lysis, extraction, and subsequent chromatography to obtain purified ICP4 were as previously described (32, 65, 67).

(ii) **Human TBP.** The TBP expression plasmid pETHIID and *Escherichia coli* BL21 were generous gifts of Arnold Berk and have been described previously (33). Two liters of pETHIID-transformed BL21 cells was grown in $2\times$ YT medium (16 g Bacto tryptone, 10 g Bacto-yeast extract, and 15 g NaCl per liter) plus 0.4% glucose in the presence of 50 μ g of ampicillin per ml at 37°C until the A_{600} reached approximately 0.8. Isopropylthio- β -D-galactoside (IPTG) was added to a final concentration of 0.5 mM, and the cells were grown for an additional 3 h at 30°C. Lysis of the cells and fractionation of the extracts over DEAE-Sephacel and heparin-Sepharose were performed essentially as described previously (30, 31). Fractions were assayed by gel shift with a 29-bp double-stranded oligonucleotide encoding the adenovirus E1B TATA box as described previously (33) and by immunoreactivity with a mouse anti-TFIID antibody (generously provided by Robert Roeder, Rockefeller University). Relevant fractions were pooled and applied to a fast protein liquid chromatography (FPLC) Mono Q column equilibrated with buffer D (33) plus 0.1 M KCl at a rate of 1 ml/min. The TBP immunoreactive material present in the flowthrough was applied to an FPLC Mono S column equilibrated with buffer D plus 0.1 M KCl at a rate of 1 ml/min. Bound proteins were eluted with a continuous 0.1 to 1.0 M KCl gradient. Immunoreactive fractions were frozen in liquid nitrogen and stored at -80°C .

(iii) **Recombinant TFIIB (rTFIIB).** The TFIIB expression plasmid phiIIB was obtained from Dan Reinberg and has been described previously (22). The procedures for bacterial expression, lysis, and purification of TFIIB through the phosphocellulose column were as previously described (22). The TFIIB eluting from the phosphocellulose column at 0.5 M KCl was further fractionated on an FPLC Superose 12 column.

(iv) **GTFs.** Eighty liters of HeLa cells grown in suspension was used to make nuclear extracts by the method of Dignam et al. (12). The fractionation procedure was similar to that of Reinberg and Roeder (58) and Samuels et al. (62). The nuclear extracts were first fractionated through a phosphocellulose column to make 0.1 M KCl flowthrough (A), 0.3 M KCl (B), 0.5 M KCl (C), and 1 M KCl (D) fractions. Subsequently, all fractions were adjusted to 0.1 M KCl prior to their use in transcription or further fractionation. TFIIA activity in fraction A was eluted from a DEAE-Sephacel column with 0.3 M KCl buffer (AB). From fraction C, TFIIB, TFIIE/F, and RNA Pol II activities were eluted from the DEAE-Sephacel column with 0.1 M KCl (fraction CA), 0.25 M KCl (fraction CB), and 1 M KCl (fraction CC), respectively. Fraction D, containing TFIID activity, was loaded on a DEAE-Sephacel column, and TFIID was eluted with 0.25 M KCl buffer (fraction DB). The activities and relative requirements of the fractionated GTFs were assessed by performing transcription reactions using the adenovirus major later promoter (AdMLP) linked to the G-less cassette. *In vitro* transcription reaction mixtures contained 0.2 mg of template, 1 μ l of fraction AB (TFIIA), 4 μ l of fraction CB (TFIIE/F), 2 μ l of fraction CC (Pol II), 4 μ l of fraction DB (TFIID), 0.017 μ g of rTFIIB, 15 U of RNase T₁, 15 U of RNasin, 7 mM MgCl₂, 60 μ M ATP and CTP, 25 μ M UTP, 0.1 mM *O*-methyl GTP, and 5 μ Ci of [α -³²P]UTP. The transcription reaction mixtures were incubated at 30°C for 1 h; after phenol and chloroform extraction, the RNA transcripts were separated from free nucleotides through a

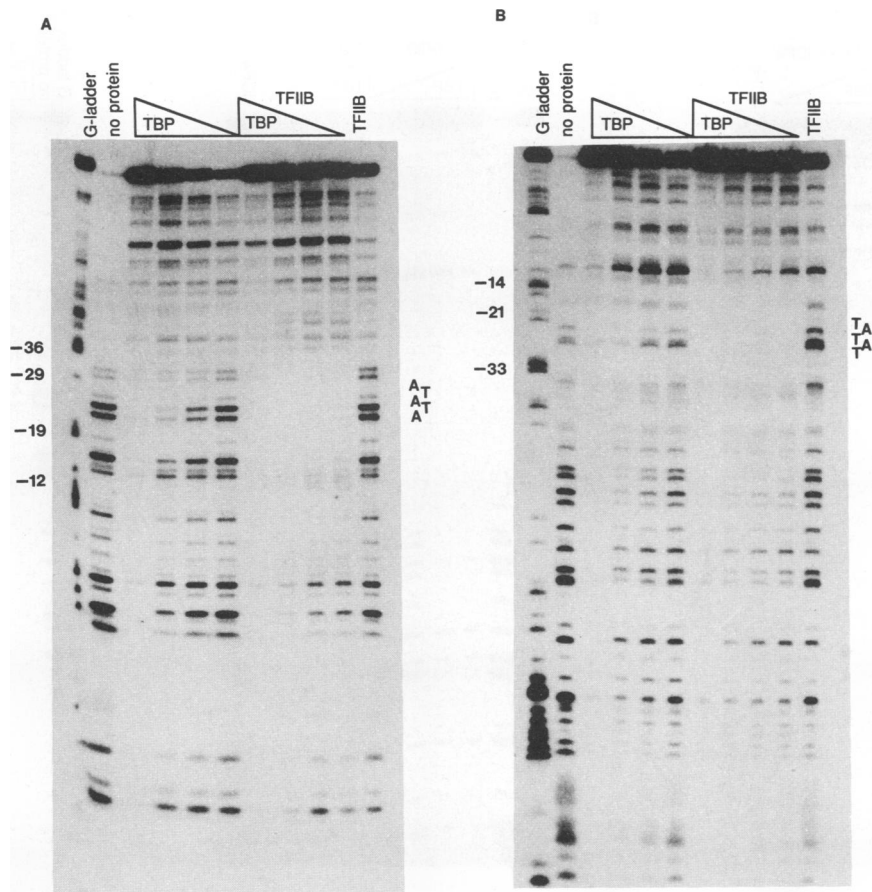


FIG. 2. Evidence that rTFIIB helps rTBP bind the TATA box. (A) The 130-bp ICP4 probe labeled on the coding strand was incubated with the indicated mixture of proteins, treated with DNase I, and then run on a sequencing gel. Different amounts of rTBP (400, 200, 100, and 50 ng) were reacted with the probe alone or with the probe in combination with a constant amount of rTFIIB (0.3 μ g), as indicated. As controls, the probe was incubated with buffer alone (no protein) or with rTFIIB alone (rightmost lane). For sizing, a G ladder of the probe was included (leftmost lane). The position of the TATA box is indicated; numbers refer to positions relative to the start site of transcription. (B) The same experiment performed with a probe labeled on the noncoding strand.

with the indicated proteins, treated with enough DNase I to introduce on average a single nick per template, and then run on a denaturing polyacrylamide gel. rTFIIB alone did not give a footprint. In contrast, rTBP alone resulted in weak TATA-specific protection at intermediate protein concentrations. The simultaneous use of rTBP and rTFIIB resulted in strong TATA protection at concentrations of rTBP which when used alone did not give a footprint. It appears that rTFIIB lowered rTBP's dissociation constant for DNA by a factor of 10. In addition, inclusion of rTFIIB broadened the footprint of rTBP. These results indicate that rTFIIB significantly aids the DNA-binding potential of rTBP, presumably through the formation of a DB complex having enhanced affinity for the TATA homology.

ICP4 facilitates the specific DNA binding of TBP-containing complexes. ICP4 was then assayed for its effect on the ability of TBP and TFIIB to protect the TATA box as measured by DNase I footprinting. In Fig. 3A, rTFIIB and rTBP were reacted with the 130-bp probe labeled on the coding strand in the presence or absence of ICP4. The amount of ICP4 used in this experiment is sufficient to give only a very weak footprint. Larger amounts of ICP4 result in a unique footprint extending from about -10 to $+15$ with respect to the start of transcription (data not shown). This stretch contains

a strong site for specific binding by ICP4 (17, 35, 45). The inclusion of ICP4 enhanced the DB footprint, as evidenced by the fact that at low concentrations of rTBP and rTFIIB (concentrations at which hardly any TATA protection was detected in the absence of ICP4), a very strong footprint was obtained in the presence of ICP4. The enhancement was approximately fivefold. The effect was reciprocal; i.e., the ICP4 footprint was enhanced by the presence of TBP and TFIIB, such that the region between -10 to $+15$ was clearly protected. To verify these results, DNase I footprinting was conducted on the noncoding strand (Fig. 3B). Again, the protection of the TATA box complex was greatly facilitated by ICP4. To assess whether these observations extend to a more physiologically relevant situation, human TFIID from HeLa cells was substituted for rTBP in this experiment. As shown in Fig. 3C, the enhanced protection of the TATA box in the presence of ICP4 also occurred when human TFIID was used in place of rTBP. The demonstration of the same effect with the human TFIID fraction, which has many other proteins tightly associated with TBP, indicates that the molecular events described above may occur under the conditions present in human cells, at least with respect to TFIID.

ICP4, TFIIB, and TBP form a tripartite complex on DNA.

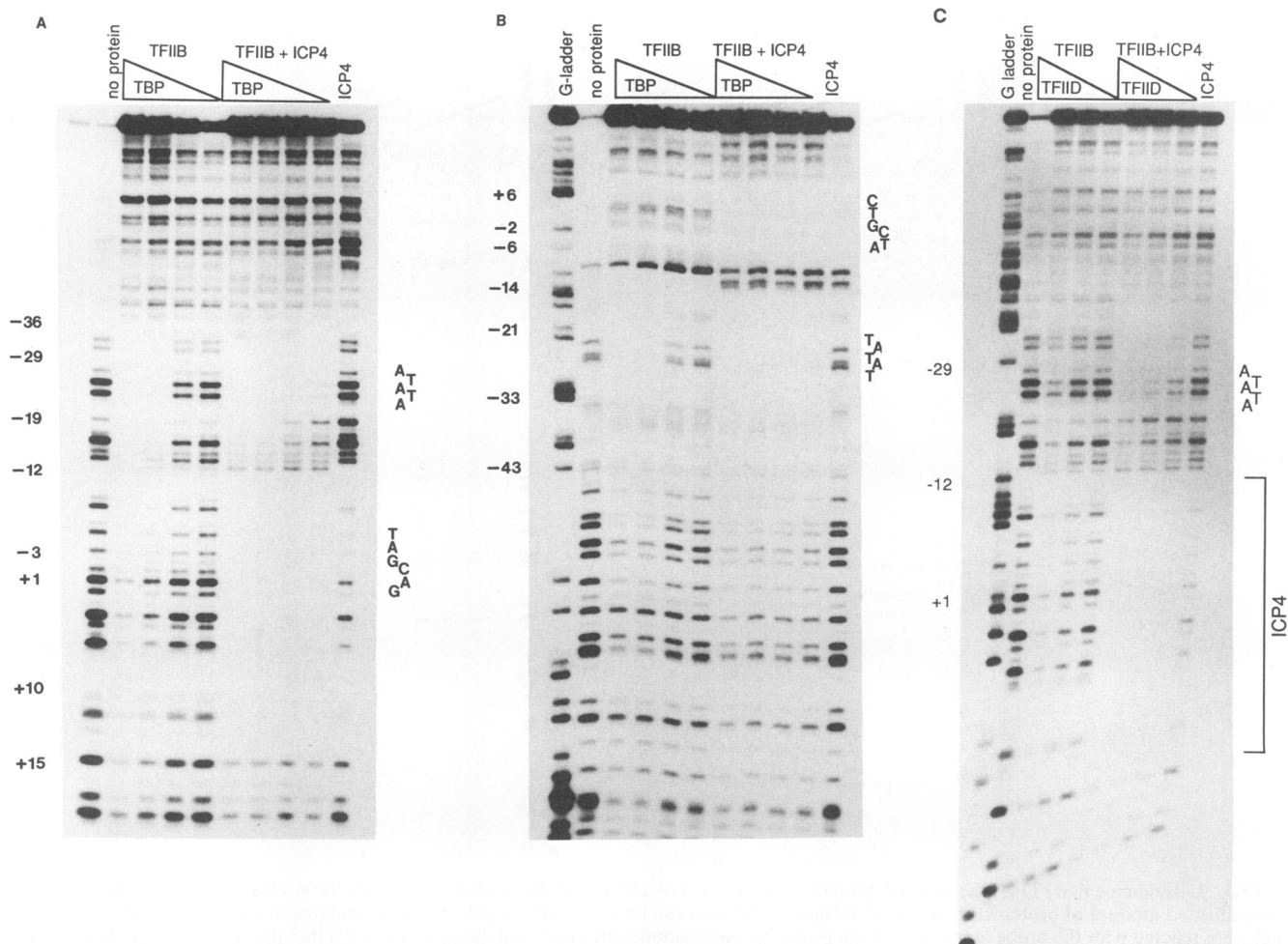


FIG. 3. Evidence that ICP4 helps DNA binding of the DB complex. (A) A DNase I protection assay was performed as for Fig. 2, using the 130-bp ICP4 probe labeled on the coding strand. Different amounts of rTBP (100, 50, 25, and 12.5 ng) and a constant amount of rTFIIB (0.3 μ g) were reacted with the probe alone or with the probe in combination with a constant amount of ICP4 (0.25 μ g), as indicated. As controls, the probe was incubated with buffer alone (no protein) or with ICP4 alone (rightmost lane). For sizing, a G ladder of the probe was included (leftmost lane). Positions of the TATA box and of the ICP4 binding site are indicated; numbers refer to positions relative to the start site of transcription. (B) The same experiment performed with probe labeled on the noncoding strand. (C) DNase I protection assay using HeLa TFIID in place of rTBP. The experiment shown in panel A was performed, substituting various amounts of HeLa TFIID (DB fraction) for rTBP.

The simplest explanation of ICP4's effects is that ICP4 forms a complex with rTBP and rTFIIB and that cooperative protein-protein and protein-DNA interactions are involved in its assembly. To confirm the existence of this putative tripartite complex, we used a gel retardation assay. The assay involved reacting rTFIIB and rTBP, singly or in combination, in the presence of ICP4 and analyzing the products on native polyacrylamide gels to determine whether novel ICP4-containing complexes were formed. As probe, we again used the *EcoRI-BamHI* fragment spanning the ICP4 transcriptional start site. The simultaneous use of ICP4, rTBP, and rTFIIB resulted in a novel complex (DB4 in Fig. 4) exhibiting a very low electrophoretic mobility, consistent with the supposition that it represents a tripartite complex containing ICP4, rTFIIB, and rTBP. At the concentrations used, neither rTBP nor rTFIIB alone supershifted the ICP4-DNA complex. However, at very high protein concentrations, rTBP and rTFIIB alone were capable of altering the mobility of the ICP4-DNA complex (data

not shown). At the relatively low concentrations used in this study, the simultaneous addition of rTFIIB, rTBP, and ICP4 was necessary to generate a supershifted complex. Densitometric analysis of the data in Fig. 4 confirms the cooperativity in DNA binding seen in the previous DNase I protection assays. When used alone, ICP4 bound 9% of the available probe (Table 1). When rTBP and rTFIIB were used together, 29% of the probe participated in DB complexes. When all three proteins were present, 15% of the probe was taken up in tripartite complexes. If ICP4, rTBP, and rTFIIB did not engage in cooperative interactions, then one would expect the latter value to be less than 9%, considering the fact that the probe was used in excess. Stated differently, if the binding of ICP4 and DB were independent events, then the proportion of the probe bound by both components would be the product of the proportions bound by the components when used alone, i.e., 3%. The fact that the observed value was 15% (five times the value expected of simple co-occupancy) reaffirms the conclusion that cooper-

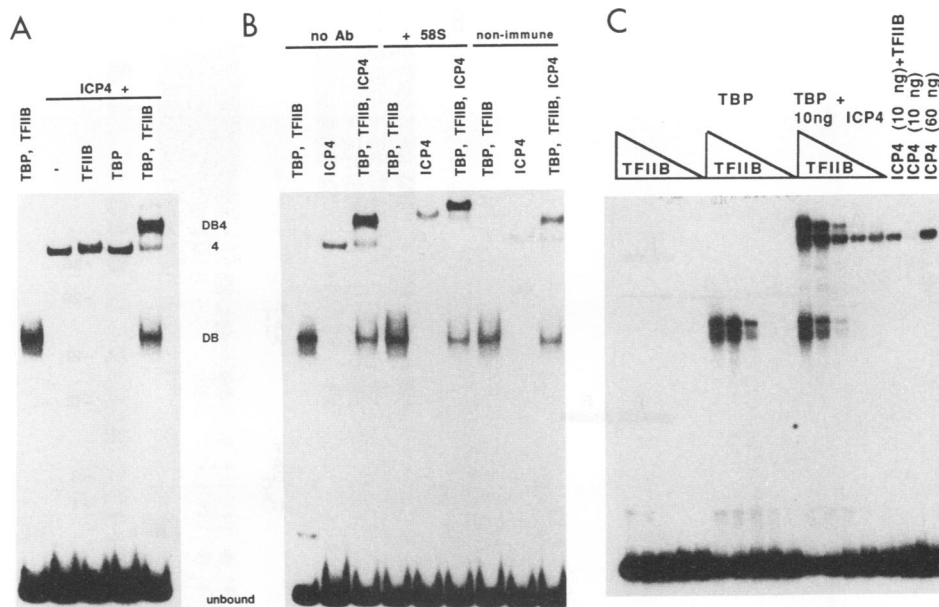


FIG. 4. Formation of a novel complex upon the simultaneous addition of TBP, TFIIB, and ICP4. (A) ICP4 (60 ng), rTFIIB (0.5 μ g), and rTBP (50 ng) were used in the indicated combinations. (B) ICP4, rTFIIB, and rTBP were used with the indicated antibodies (Ab). The 58S antibody reacts with ICP4 near the carboxyl terminus (10). (C) Constant amounts of rTBP (60 ng) and ICP4 (10 ng) were titrated against various amounts of rTFIIB (0.5, 0.25, 0.125, 0.063, and 0.032 μ g) in a standard gel shift assay. At low relative concentrations of ICP4, the inclusion of TFIIB in the binding reaction reproducibly enhanced the binding of ICP4; this is shown in the third lane from the right, in which 125 ng of TFIIB was added along with 10 ng of ICP4.

ative DNA binding is exhibited by ICP4, rTBP, and rTFIIB when used in combination.

In an effort to confirm the identities of the proteins participating in the DNA complexes identified in Fig. 4A, we performed antibody supershifting experiments. In Fig. 4B, a monoclonal antibody (58S) against ICP4 was used. As shown, the antibody supershifted only the two foremost retarded complexes, the two complexes putatively identified in Fig. 4A as being the ICP4-DNA complex and the ICP4-rTBP-rTFIIB-DNA complex. However, when polyclonal antibodies against rTBP and rTFIIB were used, no supershifting was observed; not even the DB complex was affected (data not shown). We assume that the antigenic epitopes of rTBP and rTFIIB are not available for antibody binding when the proteins are associated with DNA. This

assumption is not groundless, given similar observations reported previously (40).

The importance of TFIIB in the formation of the tripartite complex and most likely its participation was implied by the observation that in the absence of TFIIB, tripartite complexes did not form (Fig. 4A). Figure 4C shows that the concentration dependence of TFIIB for tripartite complex formation is similar to that for DB complex formation. This finding further highlights the importance of TFIIB for tripartite complex formation and also indicates that the tripartite complex can form at physiologically relevant concentrations of cofactors insofar as the *in vitro* assembly of the DB complex reflects a physiologically relevant phenomenon.

DNase I footprint analysis was conducted on the individual DNA-protein complexes in a further effort to identify the proteins and nucleotide sequences involved (Fig. 5). Preparative gel retardation reactions were performed with the protein mixtures indicated in Fig. 5A and the 130-bp probe labeled on the coding strand. The reactions were treated with DNase I, run on native TBE gels, and exposed to film. The bands marked A through F were cut out, and their DNA was eluted. The eluted DNA was then run on a denaturing polyacrylamide gel to determine which sequences were protected from DNase I cleavage. DNA from band A represents nonretarded probe and was susceptible to DNase I cleavage throughout its entire length. DNA from band B represents probe complexed with ICP4 alone. In this case, sequences extending from about -10 to at least +10 with respect to the transcriptional start site were protected. This stretch includes the sequence ATCGTC known to be involved in ICP4-DNA interactions (16). DNA from band D represents retarded probe resulting after reaction with ICP4 and rTBP. In this case, not only was the ICP4 footprint obtained, but also some very weak protection, covering the

TABLE 1. Complex-forming ability of TBP, TFIIB, and ICP4

Protein(s) in reaction ^a	Fraction of probe in complex ^b			
	DB	ICP4	DB4	Unbound
TBP, TFIIB	29			65
Wild-type ICP4		9.0		82
TFIIB, ICP4		10		84
TBP, ICP4		10		84
TBP, TFIIB, ICP4	15	3.0	15	58

^a Purified proteins added in each reaction to generate the data in Fig. 4.

^b The appropriate lane in Fig. 4 was scanned with a densitometer, and the value shown represents the percentage of the total measured intensity present in each complex. DB, the complex labeled DB in Fig. 4, having the mobility of that generated by the simultaneous addition of rTBP and rTFIIB; ICP4, the complex having the mobility of that generated by the sole addition of the purified ICP4 peptide; DB4, the novel complex appearing only after the simultaneous addition of the ICP4 peptide, rTBP, and rTFIIB; unbound, the band having the same mobility as that generated by the probe run alone on the gel.

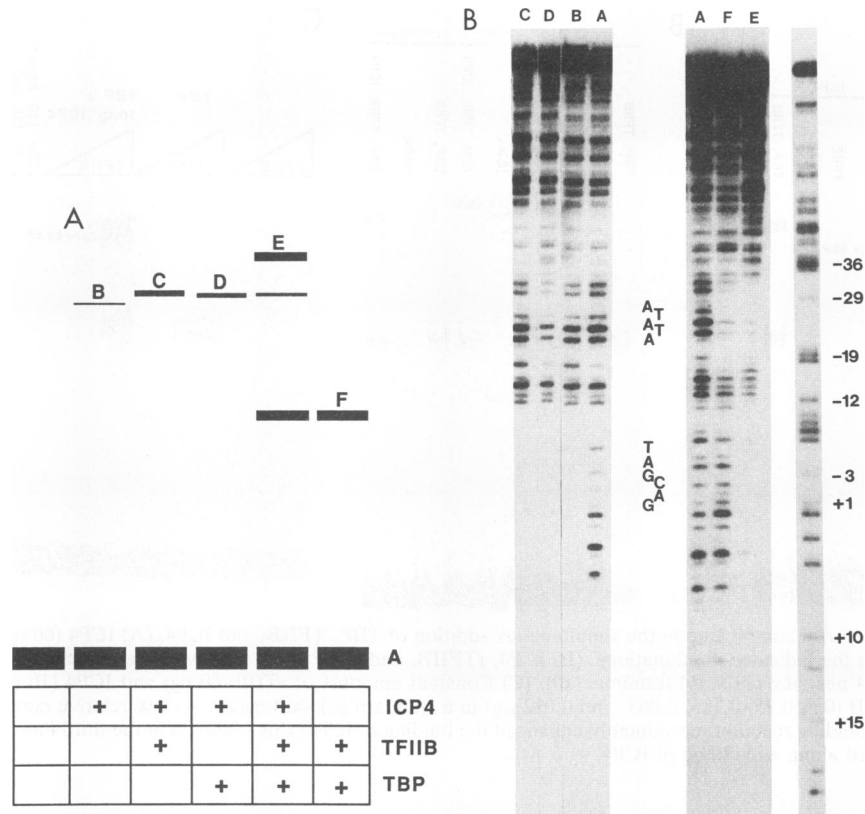


FIG. 5. DNase I footprinting of the complexes isolated by gel shift. DNase I footprinting of the complexes isolated by gel shift was performed as described in Materials and Methods. (A) Representation of the preparative gels run to isolate the indicated protein-DNA complexes. DNA from these complexes was run on the 8% sequencing gel shown in panel B. On the extreme right is shown the G ladder used to size the DNase I cleavage products; numbers are relative to the transcriptional start site. Also indicated are the TATA homology and the sequence ATCGTC, which has been shown to be important for specific DNA binding by the ICP4 protein.

area extending from about -29 to -18 , an area that contains the TATA box, was observed. This result indicates a low level of co-occupancy of the DNA template by rTBP and ICP4. DNA from band C represents retarded probe resulting after reaction with ICP4 and rTFIIB. In this case, the cleavage pattern was nearly identical to the pattern observed with ICP4 alone. DNA from band F represents the retarded probe resulting from adding rTBP and rTFIIB. The resulting DNase I profile demonstrated that sequences around -36 to -18 were protected in the complex. This stretch contains the TATA homology. DNA from band E represents the supershifted probe resulting from reaction with ICP4, rTBP, and rTFIIB altogether. In this case, both the TATA box and the ICP4 binding site were strongly protected, consistent with the supposition that the supershifted complex seen in Fig. 4A represents a high-order complex containing ICP4, rTBP, and probably rTFIIB.

ICP4 DNA binding is not sufficient to form the tripartite complex. A set of ICP4 mutant proteins purified from mutant virus-infected cells was assayed for the ability to form tripartite complexes (Fig. 6A). The primary structures of these proteins are shown relative to that of the wild-type protein in Fig. 6B. All five mutant proteins contain the DNA-binding domain (49, 66), bind DNA (66), and yield wild-type footprints at least when assayed on the ICP4 promoter (66; unpublished data). Densitometry data from Fig. 6A are shown in Table 2. For wild-type ICP4, n208, and nd3-8, the proportion of the probe bound in tripartite com-

plexes was significantly greater than the product of the proportions bound by DB and the ICP4 proteins when assayed individually. As previously discussed, this finding is indicative of cooperative binding. In contrast, for X25, nd8-10, and d8-10, the proportions of the probe bound in novel complexes were substantially reduced and approximately equal to the products of the proportions bound by DB and the mutant proteins alone. As previously discussed, this finding indicates simple co-occupancy of the probe resulting from independent binding events. With respect to mapping the region of ICP4 that is involved in the interaction with the DB complex, the following reasoning points to the region extending from amino acids 142 to 210. The failure of X25 to interact cooperatively implicates the region from 30 to 274. The wild-type ability of nd3-8 eliminates amino acids 30 to 142 from consideration while implicating amino acids 142 to 274. The failure of nd8-10 and d8-10 discounts amino acids 210 to 274 as being necessary. Therefore, the residues residing between 142 to 210 contributes to the ability of ICP4 to cooperatively interact with the DB complex.

ICP4 forms a tripartite complex with rTBP and rTFIIB on the TK promoter, an inducible template. In the previous gel retardation experiments, the *Bam*HI-*Eco*RI fragment spanning the ICP4 transcriptional start site was used as a probe. This fragment contains an ICP4 binding site overlapping the region where transcription initiates. The binding of ICP4 to this site is involved in the repression of transcription. We wanted to determine whether ICP4 could form a tripartite

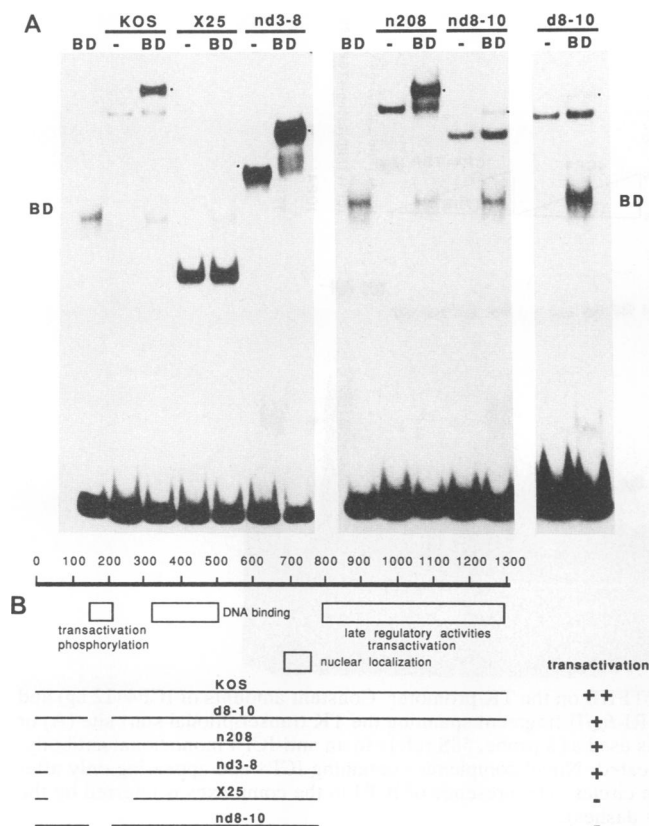


FIG. 6. (A) Evidence that DNA binding of ICP4 is not sufficient to form the tripartite complex. About 12 ng of purified ICP4, n208, X25, nd3-8, d8-10, or nd8-10 was used in conjunction with the indicated proteins in a standard gel shift assay using the 130-bp *Bam*HI-*Eco*RI fragment spanning the ICP4 transcriptional start site. For rTBP, 60 ng was used; for rTFIIB, 250 ng was used. Novel complexes are indicated by dots. The intensity of this complex was used to obtain the value for the DB4 complex in Table 2. (B) Diagram of ICP4 and the mutant proteins along with their associated activities (9, 10, 66). The domains of ICP4 are those described previously (10, 49, 50, 66).

complex with use of a template whose transcription is induced by ICP4. The *Eco*RI-*Bgl*II fragment spanning the transcriptional start site of the inducible thymidine kinase (TK) promoter was chosen. This fragment, spanning -75 to +54 with respect to the start of transcription, contains a TATA box, an Sp1 site, and a low-affinity ICP4 binding site located approximately 40 bp downstream of the transcriptional start site (30). In Fig. 7A, ICP4 alone retards the probe and the amount of complex 4 is increased by the addition of TFIIB. The simultaneous addition of ICP4, TBP, and TFIIB resulted in a complex of even lower mobility than complex 4 (marked with open circles). Note that the anti-ICP4 monoclonal antibody 58S further retarded the novel complex formed in the presence of rTFIIB, rTBP, and ICP4 (marked with a dash and open circle). The experiment in Fig. 7B recapitulates the one in Fig. 7A except that a linker-scanning mutant of the probe was used. This mutant, designated LS -29/-18, harbors a defective TATA box (43). As shown, this mutation reduced the ability of rTFIIB and rTBP to form the rTFIIB-rTBP complex, as expected. In addition, it abolished the ability of rTBP and rTFIIB to cause a supershifting of the ICP4-DNA complex. This result underscores

TABLE 2. Complex-forming ability of mutant ICP4 proteins

Protein(s) in reaction ^a	Fraction of probe in complex ^b			
	DB	ICP4	DB4	Unbound
TBP, TFIIB	19			76
Wild-type ICP4		4.2		90
TBP, TFIIB, ICP4	8.9	5.3	18	67
n208		8.8		82
TBP, TFIIB, n208	2.9	8.9	19	69
nd3-8		35		61
TBP, TFIIB, nd3-8	3.0	18	47	34
X25		34		65
TBP, TFIIB, X25	4.3	34	1.8	53
nd8-10		6.8		87
TBP, TFIIB, nd8-10	12	11	1.7	74
d8-10		1.0		93
TBP, TFIIB, d8-10	4.9	2.0	0.3	86

^a Purified proteins added in each reaction to generate the data in Fig. 6A. The ICP4 peptides are diagrammed in Fig. 6B.

^b Each lane in Fig. 6A was scanned with a densitometer, and the value shown represents the percentage of the total measured intensity present in each complex. DB, the complex labeled DB in Fig. 4 and BD in Fig. 6A, having the mobility of that generated by the simultaneous addition of rTBP and rTFIIB; ICP4, the complex having the mobility of that generated by the sole addition of the purified ICP4 peptide; DB4, the novel complex appearing only after the simultaneous addition of the ICP4 peptide, rTBP, and rTFIIB. unbound, the band having the same mobility as that generated by the probe run alone on the gel.

the point that the observed supershifting is a phenomenon requiring the direct participation of rTBP. In summary, these experiments suggest that tripartite complexes can form on templates that are either inducible or repressed by ICP4 and that this ability does not require the immediate juxtaposition of the TATA box and the ICP4 binding site.

DISCUSSION

The assembly of an active transcription complex on TATA-containing promoters begins with the binding of TFIID, TFIIB, and TFIIA. The data presented in this study show that ICP4 can participate in the formation of transcription complexes at this very early stage of assembly. Specifically, the simultaneous addition of ICP4, rTFIIB, and rTBP to a DNA template containing an ICP4 binding site and a TATA box resulted in a unique complex lower in electrophoretic mobility than the complexes obtained with any of the proteins used singly or in dual combinations. ICP4 resides in this unique complex, as evidenced by the fact that the complex exhibited a characteristic ICP4 footprint (Fig. 5) and by the fact that it was further shifted by an anti-ICP4 antibody (Fig. 4B and 7). rTBP resides in the complex, as evidenced by the fact that the complex protected the TATA box from DNase I cleavage (Fig. 5). The question of whether rTFIIB resides in the complex is not as straightforward because rTFIIB does not give telltale sequence-specific footprints. Nevertheless, rTFIIB is strongly implicated by the fact that in its absence the supershifted complex simply did not form (Fig. 4A). In addition, its presence facilitated the formation of the tripartite complex and the DB complex with the same concentration dependence. Consequently, we can conclude that ICP4, rTBP, and rTFIIB form tripartite complexes on suitable templates.

Both protein-protein and protein-DNA interactions mediate the formation of the novel complex. Two separate lines of evidence underscore the importance of protein-protein interactions. First, ICP4, rTBP, and rTFIIB help each other

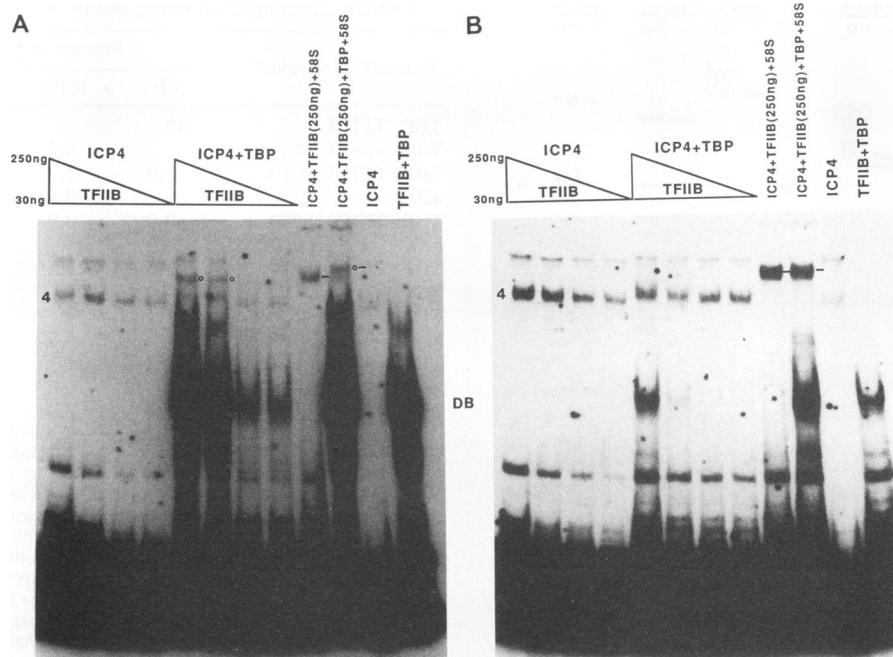


FIG. 7. Evidence that ICP4 forms a tripartite complex with rTBP and rTFIIB on the TK promoter. Constant amounts of ICP4 (12 ng) and rTBP (0.06 μ g) were titrated against various amounts of rTFIIB. The *EcoRI-BglII* fragment spanning the TK transcriptional start site (A) or the same fragment but bearing a mutated TATA box (LS -18/-29) (B) was used as a probe. 58S refers to an anti-ICP4 monoclonal antibody. The mobilities of the ICP4-alone (complex 4) and DB complexes are indicated. Novel complexes containing ICP4 and appearing only after the simultaneous addition of TBP, TFIIB, and ICP4 are indicated by open circles. The presence of ICP4 in the complexes is inferred by the ability to supershift a given complex by the addition of 58S (indicated by dashes).

bind DNA. As shown in Fig. 2, rTFIIB enhanced the DNA binding of rTBP by 10-fold. Figure 3 shows that ICP4 enhanced the DNA-binding potential of the rTFIIB-rTBP complex by fivefold, the effect being reciprocal. Consequently, the combination of ICP4, rTBP, and rTFIIB results in a 50-fold stimulation of specific DNA binding. This cooperativity can best be interpreted as being a consequence of protein-protein interactions that translate into lower rates of dissociation from the DNA template. Precedents for this sort of scenario are numerous, with such notable examples as the repressor protein of phage lambda and the large T antigen of simian virus 40 (7, 25). The other line of evidence implicating the critical importance of protein-protein interactions is the fact that specific ICP4 mutants failed to cooperatively form the tripartite complex despite their ability to bind DNA. This result indicates that efficient assembly of the complex is not a consequence of simple co-occupancy of the DNA template but requires that specific protein-protein contacts be made between the participating components. As demonstrated in Fig. 6, the ability of ICP4 to cooperatively form the tripartite complex maps between amino acids 142 and 274.

Evidence that specific protein-DNA interactions are involved in the assembly of the tripartite complex includes not only the footprint data but also the fact that a test DNA bearing a mutated TATA box fails to form the structure (Fig. 7). When the results of Fig. 4 and 7 are compared, it is evident that tripartite complexes form more efficiently on the fragment derived from the ICP4 promoter than on the fragment derived from the TK promoter. Two factors probably contribute to this difference. First, the binding site on the ICP4 promoter fragment exhibits a much higher affinity for ICP4 than does the site on the TK promoter fragment

(32). Second, the distance between the ICP4 and TBP binding sites is different for the two promoters. Therefore, tripartite complexes may form with varying degrees of efficiency, depending on the relative affinities of the TATA box and ICP4 binding sites for their respective proteins and on the degree of separation between the participating *cis*-acting sites. Additionally, if the ICP4 binding site in the ICP4 promoter is mutated such that ICP4 no longer binds to the residual sequence, tripartite complexes do not form (data not shown). Collectively, these data demonstrate the importance of the nucleating effect of DNA on the cooperative assembly of the tripartite complex. This is further underscored by the observations that in the absence of DNA, these proteins have a relatively low affinity for each other, at least in our hands (data not shown).

It having been established that rTBP, rTFIIB, and ICP4 form a tripartite complex and that the complex's efficient assembly requires specific protein-protein and protein-DNA interactions, the question of functional relevance arises. The ability of ICP4 to form the tripartite complex maps between amino acids 142 and 274. Within this area is a serine-rich tract that is highly conserved among the ICP4 analogs of a variety of herpesviruses (42), has been genetically implicated as a site of phosphorylation (10), and is a target for cellular kinases A and C (unpublished data). It is conceivable that the phosphorylation state of this tract modulates ICP4's interaction with the DB complex. This possibility is currently being studied. This area has also been previously identified as being involved in transactivation (66). n208 and nd3-8 are capable of activating transcription and are able to form the complex, while X25 and nd8-10 are deficient for both transactivation and complex formation. Consequently,

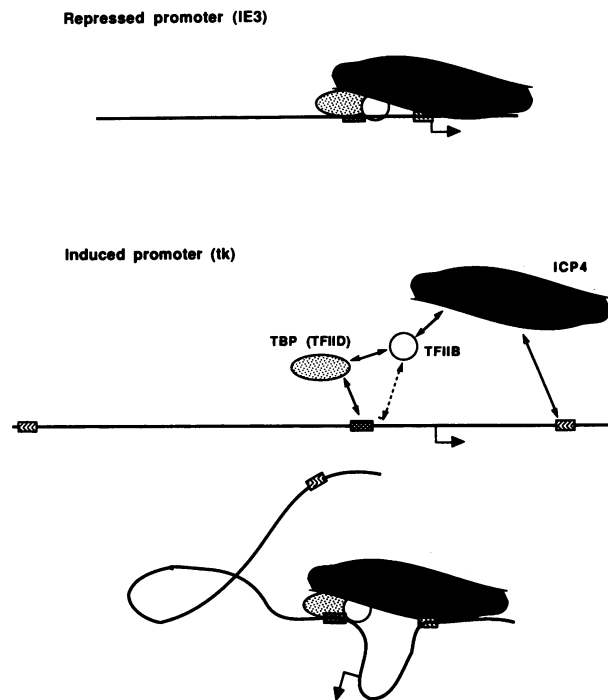


FIG. 8. Models of how ICP4 may repress and activate transcription. The arrows indicate interactions as identified either by DNase I footprinting assays or by cooperativity in DNA binding. The arrows beneath the templates indicate the start sites of transcription. The checkered boxes represent TATA boxes. The other boxes depict ICP4 binding sites. High-affinity ICP4 binding sites positioning ICP4 over the start of transcription may result in repression, whereas ICP4's potential interaction with many lower-affinity sites throughout the genome may result in transactivation by recruiting the GTFs without tightly binding to the start of transcription.

the data indicate that formation of the tripartite complex has implications for the events involved in ICP4 function.

However, interaction with the DB complex cannot be the sole mechanism by which ICP4 stimulates gene expression. As shown in Fig. 6, the d8-10 protein is unable to cooperatively interact with the TBP and TFIIB to form tripartite complexes. Despite this deficiency, proteins bearing the d8-10 and d8-10-like mutations (51) are one-half to one-third as active as wild-type protein in transient expression assays, and viruses expressing these mutant proteins are able to generate high enough levels of early and late gene products to grow at 2% of the level of the wild-type virus. Consequently, it is reasonable to suggest that ICP4 has multiple mechanisms by which it stimulates gene expression, one involving the tripartite complex and the region of ICP4 between amino acids 142 and 210 and the others possibly involving some yet to be identified activities or interactions mediated by the carboxyl-terminal 524 amino acids of ICP4.

Figure 8 is a cartoon summarizing the interactions implicated by the results of this study and their possible functional consequences. With respect to the question of how ICP4 stimulates transcription via the tripartite complex, it is important to note that the results reported here repeatedly underscore the fact that ICP4 increases the DNA affinity of the DB complex. This is most dramatically seen in Fig. 3. As shown, ICP4 increases the DNA affinity of the DB complex by fivefold. This is also true in the case of human TFIID (Fig. 3C). Consequently, the data suggest that ICP4 en-

hances gene expression by recruiting DB to the DNA template, a rate-limiting step in the assembly of the transcriptional preinitiation complex (39). Alternatively, it is conceivable that ICP4 transactivates by substituting, or by serving as a bridging agent, for one or more of the other cofactors among the myriad participating in the preinitiation complex. The data in this report do not address this latter possibility, but the interactions implied by these two hypotheses are not mutually exclusive and may both occur.

If indeed ICP4 acts as a nucleating agent by binding DNA and stabilizing the DNA association of the proteins which it contacts, then DNA binding is predicted to play a critical role in ICP4 functioning by this mechanism. However, in seeming contradiction to this, the specific ICP4 binding site mediating the formation of the tripartite complexes on the TK promoter shown in Fig. 7 is not necessary for ICP4 induction in the context of the viral chromosome (24). In fact, as previously mentioned, no single site or collection of sites that uniquely specify induction by ICP4 has been identified (68). However, the sequence requirement for specific ICP4 binding is known to be highly degenerate (11, 44), resulting in numerous binding sites of variable affinity for ICP4 scattered throughout the HSV genome. Therefore, the abundance of ICP4 binding sites may make the absence of any single ICP4 site of little consequence in the context of the entire viral chromosome. Alternatively, interactions with other proteins bound to the DNA may substitute for the effect of specific DNA binding seen in this study. In addition, viral genes may be transactivated by ICP4 by alternative mechanisms, such as implied by the activity and properties of d8-10.

An exception to this hypothesis is when a high-affinity site overlaps the start of transcription as in the case of the ICP4 promoter. Configured in this way, an ICP4 binding site results in repression when ICP4 is supplied *in trans*. Most of the data shown in this study are based on use of this promoter and show that ICP4 enhances the formation of the DB complex on DNA. This seeming paradox is reconciled upon considering that RNA Pol II and the preinitiation complex often assemble over the transcriptional start site of a promoter (3). Consequently, ICP4's occupancy of this site may preclude the further assembly of the transcription complex beyond DB formation or interfere with the activity of TBP-associated factors for upstream activating proteins such as Sp1 despite facilitating the formation of the preinitiation complex.

The data presented in this report clearly indicate that ICP4 interacts with the transcriptional preinitiation complex and that this interaction may have physiological significance for viral replication. This scenario is not unprecedented. The human cytomegalovirus 80-kDa IE protein interacts directly and specifically with the evolutionarily conserved carboxyl-terminal domain of rTBP (23). VP16, a transactivator of HSV that functions at a different stage in the viral transcriptional program than does ICP4, also interacts with rTBP (69). The same holds true for the Zta protein of Epstein-Barr virus and the E1a protein of human adenovirus (29, 37, 38). The latter finding is particularly interesting with respect to the present work because it has been shown that the ICP4 analog of pseudorabies virus complements E1a mutants of adenovirus for early gene expression, implying a similar pathway of action (18). Elucidating how ICP4 and these other viral transactivators interact with the host transcriptional apparatus should lead to a better understanding of the regulation of transcription and to new targets for antiviral chemotherapy.

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