Stabilized Binding of TBP to the TATA Box of Herpes Simplex Virus Type 1 Early (tk) and Late (gC) Promoters by TFIIA and ICP4[∇]

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We have recently shown that ICP4 has a differential requirement for the general transcription factor TFIIA in vitro (S. Zabierowski and N. DeLuca, *J. Virol.* 78:6162–6170, 2004). TFIIA was dispensable for ICP4 activation of a late promoter (gC) but was required for the efficient activation of an early promoter (tk). An intact INR element was required for proficient ICP4 activation of the late promoter in the absence of TFIIA. Because TFIIA is known to stabilize the binding of both TATA binding protein (TBP) and TFIID to the TATA box of core promoters and ICP4 has been shown to interact with TFIID, we tested the ability of ICP4 to stabilize the binding of either TBP or TFIID to the TATA box of representative early, late, and INR-mutated late promoters (tk, gC, and gC8, respectively). Utilizing DNase I footprinting analysis, we found that ICP4 was able to facilitate TFIIA stabilized binding of TBP to the TATA box of the early tk promoter. Using mutant ICP4 proteins, the ability to stabilize the binding of TBP to both the wild-type and the INR-mutated gC promoters was located in the amino-terminal region of ICP4. When TFIID was substituted for TBP, ICP4 could stabilize the binding of TFIID to the TATA box of the wild-type gC promoter. ICP4, however, could not effectively stabilize TFIID binding to the TATA box of the INR-mutated late promoter. The additional activities of TFIIA were required to stabilize the binding of TFIID to the INR-mutated late promoter. Collectively, these data suggest that TFIIA may be dispensable for ICP4 activation of the wild-type late promoter because ICP4 can substitute for TFIIA's ability to stabilize the binding of TFIID to the TATA box. In the absence of a functional INR, ICP4 can no longer stabilize TFIID binding to the TATA box of the late promoter and requires the additional activities of TFIIA. The stabilized binding of TFIID by TFIIA may in turn allow ICP4 to more efficiently activate transcription from non-INR containing promoters.

The expression of herpes simplex virus type 1 (HSV-1) genes relies on the functions of the host cellular transcription machinery (2, 10). Initiation of transcription is a major control point of viral as well as cellular gene expression and requires the proper assembly of the general transcription factors TFIIA, -B, -D, -E, -F, and -H and RNA polymerase II into preinitiation complexes (PICs) on core promoter elements (reviewed in reference 49). PIC formation begins with the binding of the general transcription factor TFIID, a multisubunit complex composed of the TATA binding protein (TBP) in association with 10 to 14 TBP-associated factors, known as TAFs (reviewed in reference 1). The TBP subunit of TFIID recognizes and binds to the TATA box present on core promoters. Although TBP is sufficient for basal transcription initiation, activated transcription requires the TBP-associated factors of TFIID (4). Many cellular and viral activators have been shown to interact with the general transcription machinery through interactions with TAFs (5, 25, 27). TAFs also play an important role in recognition of non-TATA box core promoter elements, such as the INR, that may be present in place of or in addition to a TATA box on core promoters (41, 42). TAF250, TAF150, and TBP of TFIID are sufficient for INR element recognition and function. TFIID binding to the TATA box and/or to the INR is stabilized by TFIIA and TFIIB. Whereas

TFIIB is essential for basal transcription directed by either TBP or TFIID, TFIIA is dispensable for TBP-directed basal transcription. Although basal transcription with TBP does not require the general transcription factor TFIIA, TFIIA can stabilize TBP's interaction with the TATA box (3, 9, 39, 46, 48). In the absence of TFIIA, however, activated transcription does not occur, suggesting that although it may be dispensable for basal transcription with TBP, TFIIA plays an important role in transcription initiation and activation with TFIID (11, 44, 47, 53, 63, 68). The formation of a TFIID-TFIIA-TFIIB-DNA complex allows the subsequent recruitment of the rest of the general transcription factors, TFIIE, -F, -H, and RNA polymerase II, that are recruited individually or as preformed complexes to the core promoter completing PIC formation and thus ready to initiate transcription.

Transcriptional activators physically promote the formation of transcription preinitiation complexes by facilitating the recruitment of one or more general transcription factors to target promoters. The major HSV transcriptional regulatory protein, ICP4, has been shown to interact with components of the general transcription machinery to either activate or repress transcription (6, 29–31, 45, 61). One of the first gene products produced during infection, the 175-kDa protein ICP4 (55), localizes as a 350-kDa homodimer (51) in discrete foci in the nuclei of infected cells, where both viral transcription and replication are thought to originate (21, 22). ICP4 functions as the major transcriptional activator of early and late genes (13, 20, 24, 26, 52), making it essential for lytic infection (12, 16, 42, 66).

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ICP4, as a transcriptional activator, contains many conserved regions that include a DNA-binding, a nuclear localization, and both N- and C-terminal transactivation regions (15, 54, 57). Although the DNA-binding domain is essential to its function as an activator, the operationally defined DNA-binding domain alone is not sufficient for transcriptional induction, as demonstrated by the isolation of ICP4 deletion mutants that bind to ICP4 consensus sites but do not activate transcription. ICP4 does not require any single or collection of ICP4 specific binding sites, and no ICP4 sequence specific binding sites responsible for activation have been identified on early and late promoters (7, 17, 19, 60). ICP4 can bind DNA that has a fairly degenerate consensus sequence and has been shown to activate promoters *in vitro* without ICP4 sequence-specific DNA-binding sites (29).

During lytic infection viral gene expression proceeds from the expression of immediate-early to early to late genes (36). The expression of viral genes, which occurs in these three highly regulated phases, is mediated in part by the structural differences within the promoter architectures of each of the three classes of genes (65, 70). Since viral genes are transcribed by the cellular transcription machinery, the one element that most all HSV-1 promoters contain in common is a TATA box. Aside from a TATA box, however, the promoters of each of the three classes of genes are distinct, with a trending decrease in promoter complexity from immediate-early to early to late genes (reviewed in references 64 and 67). Immediate-early promoters, in addition to containing numerous cellular *cis*-activating sequences upstream of the TATA box, are the only promoters that contain viral specific activating sequences. Early promoters lack any viral specific sequences but still retain binding sites for cellular specific factors, such as Sp1 and CTF. Late promoters significantly differ from IE and E promoters in that they lack any influential upstream *cis*-acting sequences that are binding sites for either cellular or viral specific factors (23, 33, 35, 37, 40). For true late promoters, the sequences downstream from the TATA box are important for late gene regulation (29, 32, 33, 37, 62). The INR element, identified on many late promoters, is one such region downstream from the TATA box. INR elements, which overlap the initiation start site, are common to many cellular core promoters and can promote transcription initiation in the absence of a TATA box or can synergize the effects of a TATA box (reviewed in reference 59). These elements are specifically recognized by components of the general transcription factor TFIID (41, 42). The INR in addition to a TATA box has been shown to be crucial for ICP4 activation of late promoters and, unlike early promoters, ICP4 can activate transcription from these promoters with a relatively simple set of general transcription factors (29). Mutations in the INR element diminish the ability of ICP4 to activate transcription from these promoters (43).

Because early and late promoters are structurally distinct and yet still activated by ICP4, the cellular requirements for the activation of early and late genes by ICP4 may be different. Indeed, in an *in vitro*-reconstituted transcription system, ICP4 cannot activate transcription of a representative early promoter with a relatively simplified set of general transcription factors. Other cellular factors are required for the activation of early genes. ICP4, on the other hand, can efficiently activate

transcription of a representative late promoter with a reasonably simple set of general transcription factors dependent on the presence of a functional INR (29). In addition, we have recently reported that there is a differential requirement for the general transcription factor TFIIA for ICP4 activation of early versus late genes *in vitro* (69). TFIIA is required for efficient ICP4 activation of the early tk promoter. Surprisingly, however, TFIIA is dispensable for ICP4 activation of the late gC promoter. Dispensability of TFIIA for ICP4 activation of the late promoter requires an intact INR. In the absence of a functional INR, the additional activities of TFIIA allow ICP4 to overcome the necessity for an INR element. Moreover, the decreased expression of all three subunits of TFIIA during infection and its dispensability for late gene activation by ICP4 suggests that functions of ICP4 can substitute for the activities specified by TFIIA in a promoter-dependent manner. In an effort to delineate the basis for TFIIA's dispensability for ICP4 activation of late genes, the binding properties of ICP4, TFIIA, and TBP or TFIID on the tk, gC, and INR-mutated gC promoters were analyzed by DNase I footprinting analysis.

MATERIALS AND METHODS

Proteins. Purified recombinant TFIIA and TBP have been previously described (69). ICP4, n208, and X25 were purified from wild-type (KOS) and n208- and X25-producing virus-infected Vero cells, respectively, as previously described (6). TFIID was immunoaffinity purified from α 3 HeLa cells containing a hemagglutinin epitope-tagged TBP as previously described (6) and analyzed via silver stain analysis and Western blotting with antibodies directed against TAF250, TAF150, TAF55, and TBP. The relative amounts of TBP in immunoaffinity-purified TFIID were determined by Western blot analysis with an antibody directed against TBP and comparing the resulting signals to those from known amounts of purified rTBP. For all analyses, 2 ng of rTBP, 12 ng of rTFIIA, and 250 pg of TBP-equivalent TFIID were used unless otherwise noted.

DNase I footprinting analysis. The SgrAI-NheI fragments of the pgCLS1 and pgCLS8 plasmids (43) representing the wild-type gC and INR-mutated gC promoters, respectively, and the EcoRI-BglII fragment of the pLSWT plasmid (38) representing the tk promoter, were end labeled on the coding strand using polynucleotide kinase and [γ - ^{32} P]ATP. The labeled fragment was then purified and quantified. A total of 1 ng of an end-labeled probe (3×10^4 to 6×10^4 cpm/ng) and the indicated mixture of proteins were incubated for 30 min at 30°C in a 30- μ l reaction buffer consisting of 12.5 mM HEPES (pH 7.8), 60 mM KCl, 12.5% glycerol, 5 mM MgCl₂, 0.5 mg of bovine serum albumin/ml, 20 mM β -mercaptoethanol, and 0.006 μ g of poly(dG-gC)/ μ l. After incubation, 4 μ l of 10 $\times$ DNase I reaction buffer (50 mM CaCl₂, 100 mM MgCl₂) was added to each reaction, followed by incubation for an additional 10 min at 30°C. Then, 1 μ l of DNase I (0.125 U) was added to each reaction at room temperature for 1 min, and reactions were stopped by the addition of 40 μ l of stop buffer (0.2 M NaCl, 0.02 M EDTA, 1% [wt/vol] sodium dodecyl sulfate, 20 mg of tRNA/ml, 1 mg of proteinase K/ml). Reactions were then incubated at 37°C for 10 min after phenol and chloroform extraction and ethanol precipitation. Pellets were resuspended in 95% formamide and run out on denaturing 6% gels for gC promoter analysis or 8% denaturing gels for analysis of the tk promoter. Gels were dried and exposed to Amersham Hyperfilm.

RESULTS

ICP4, as well as TFIIA, stabilizes the binding of TBP to the TATA box of the wild-type late (gC) and INR-mutated late (gC8) promoters. TFIIA has been shown to stabilize the binding of TBP to the TATA box of cellular promoters (3, 9, 39, 46, 48). To determine whether ICP4 could also stabilize the binding of TBP to the TATA box, DNase I footprinting analysis was performed on the viral late gC promoter. Neither TBP nor TFIIA alone bound to the TATA box or any regions surrounding the TATA box (Fig. 1A, lanes 2 and 3). ICP4 alone at

FIG. 1. TBP-TATA interactions on the wild-type and INR-mutated late gC promoters by ICP4 and/or TFIIA. DNase I footprinting analysis was conducted with 2 ng of TBP, 12 ng of TFIIA, and either 5 or 10 ng of ICP4 on the wild-type (gC) (A) or the INR-mutated (gC8) (B) late promoters. The wild-type (gC) and the INR-mutated (gC8) late promoters were derived from the pgCLS1 and pgCLS8 plasmids, respectively. The gC8 promoter contains three mutations within the INR element that has been previously described (43).

concentrations of 10 ng but not 5 ng, bound to regions surrounding, but not directly at the TATA box since DNase I-sensitive sites were present at the TATA box (Fig. 1A, lanes 4 and 5). This is probably due to saturation of nonspecific binding by ICP4. TFIIA, as previously observed for cellular promoters, stabilized the binding of TBP to the TATA box of the gC promoter, evidenced by DNase I protection of the TATA region (Fig. 1A, lane 6). ICP4 also stabilized the binding of TBP to the TATA box in the absence of TFIIA, suggesting that ICP4 alone is sufficient to stabilize TBP binding to the TATA box (Fig. 1A, lanes 7 and 8). These results suggest that ICP4 can substitute for TFIIA in promoting the stabilized binding of TBP to the TATA box of the gC promoter.

Mutations in the INR significantly reduce the ability of ICP4 to activate transcription (29, 43). The INR-mutated late gC (gC8) promoter is essentially composed of only a TATA box since three nucleotides have been mutated within the INR element (43), rendering this promoter less responsive to ICP4 activation in the absence of TFIIA (69). When this promoter was substituted for the wild-type gC promoter, TFIIA stabilized the binding of TBP to the TATA box of this promoter (Fig. 1B, lane 18). Similar to what we observed for the wild-type promoter, ICP4, like TFIIA, was also able to stabilize the binding of TBP to the TATA box (Fig. 1B, lanes 19 and 20). This suggests that ICP4 can substitute for TFIIA in stabilizing the binding of TBP to the TATA box of the late promoter regardless of the presence or absence of a functional INR element.

To determine the lowest concentration of ICP4 able to stabilize the binding of TBP to the TATA box of either the wild-type or the INR-mutated late promoter, varying concentrations of ICP4 were used in the presence or absence of TBP. ICP4 at 2.5 ng retained the capacity to efficiently stabilize the binding of TBP to the TATA box of both promoters (Fig. 2B and data not shown). The ability of ICP4 at 2.5 ng to stabilize TBP binding to the TATA box was comparable to TFIIA's ability to stabilize the binding of TBP. These results also further support that ICP4 does not require the presence of a functional INR to stabilize the binding of TBP to the TATA box of the late promoter.

Regions of ICP4 required for the stabilized binding of TBP to the TATA box. ICP4 is a large structurally complex molecule that contains discrete domains conserved across many ICP4 homologs of alphaherpesviruses. Two domains (Fig. 2A) that have been shown to contribute to the ability of ICP4 to activate transcription are located N- and C-terminal to the DNA-binding domain (57, 58). n208 is a 774-amino-acid derivative of ICP4 that lacks the C-terminal transactivation domain (15). This polypeptide still retains a number of physical and functional properties characteristic of the intact molecule such as the N-terminal transactivation domain, the DNA-binding and dimerization domain, and the nuclear localization signal (57). Despite the presence of these functional domains, n208 is defective for viral growth due to a block at the stage subsequent to viral early gene expression (15).

Because n208 retains the ability to bind DNA, autoregulate, and activate a number of viral genes (14, 15), the regions still present in this molecule may contribute to the ability of ICP4 to stabilize the binding of TBP to the TATA box. When substituted for full-length ICP4, n208, like TFIIA, was able to efficiently stabilize the binding of TBP to the TATA box, at concentrations as low as 1 ng (Fig. 2C). These results suggest that the N-terminal transactivation domain of ICP4 may be sufficient to stabilize binding of TBP to the TATA box. In addition, no differences were evident in the ability of n208 to stabilize TBP binding to either the wild-type or the INR-mutated late promoter (data not shown), suggesting again that the INR is not required for ICP4 to direct or facilitate binding of TBP to the TATA box.

X25, diagrammed in Fig. 2A, is similar to n208 in that it lacks the C-terminal transactivation domain (residues 775 through 1298) but, in addition, also lacks the N-terminal transactivation domain (residues 30 through 274). The removal of both regions results in a mutant protein that still retains sequences sufficient for site-specific DNA binding and multimerization and yet lacks the ability to activate transcription (56). To further test whether the N-terminal transactivation domain was important for stabilized binding of TBP, X25 was substituted for ICP4. X25 at various concentrations was unable to stabilize the binding of TBP to the TATA box (Fig. 2D). This confirms that regions specified within the N-terminal transactivation domain of ICP4 are necessary for stabilizing the binding of TBP to the TATA box. These data also show that the DNA-binding activity of ICP4 is not sufficient to facilitate TBP binding.

ICP4 stabilizes the binding of TFIIID to the TATA box of the wild-type but not the INR-mutated late promoter. ICP4 alone is sufficient to stabilize the binding of TBP to the TATA box of

FIG. 2. Comparison of ICP4 derivatives to stabilize TBP-TATA interactions on the wild-type late gC promoter. (A) Domain map of ICP4 and properties of mutants relative to wild-type ICP4. The important regions of ICP4 are indicated by boxes, with their amino acid location indicated on the scale directly above the boxes. The primary structure of each of the mutant molecules relative to the wild-type (wt) protein is indicated. Transactivation in vivo was determined by transient-transfection assays and/or from the transcription rates of selected viral genes in the context of viral infection. DNA binding was determined by electrophoretic mobility shift assay. This summary was derived from several previous studies. The abilities of ICP4 and ICP4 derivatives to stabilize TBP-TATA interactions on the wild-type late gC promoter were determined by using 2 ng of TBP with either wild-type ICP4, varying from 1 to 10 ng (B); the ICP4 C-terminal domain mutant (n208), varying from 1 to 20 ng (C); or the ICP4 C- and N-terminal domain mutant (X25), varying from 1 to 20 ng (D), and was compared to TFIIA-stabilized TBP-TATA interactions.

both the wild-type and the INR-mutated late promoter (Fig. 1). However, in the context of cells, TBP is usually found associated in multisubunit complexes such as TFIID. To determine whether ICP4 was also able to stabilize the binding of TFIID to the TATA box of the wild-type late promoter, TFIID was substituted for TBP. The individual proteins TBP, TFIIA, TFIID, and X25 at 10 ng were unable to bind to the gC promoter alone (Fig. 3, lanes 3 to 6). TFIIA stabilized the binding of either TFIID or TBP to the gC TATA box (Fig. 3, lanes 9 and 10). Full-length ICP4, observed to stabilize the binding of TBP, also appeared to stabilize the binding of TFIID to the TATA box of the gC promoter (Fig. 3, lanes 12, 13, 15, and 16). X25, unable to stabilize the binding of TBP to the TATA box (Fig. 2D), also did not promote TFIID-TATA interactions (Fig. 3, lanes 11 and 14). ICP4-stabilized binding of TFIID appeared similar to the ICP4-stabilized binding of TBP (Fig. 3, compare lanes 15 and 16 with lanes 12 and 13), and this was comparable to the TFIIA-stabilized binding of TFIID and TBP (Fig. 3, compare lanes 12, 13, 15, and 16 with

lanes 9 and 10). These results suggest that ICP4 alone can substitute for TFIIA in the ability to stabilize TFIID binding to the wild-type gC promoter.

Figures 1B and 2B suggest that ICP4 stabilizes the binding of TBP to the TATA box in the absence of a functional INR. However, ICP4's ability to stabilize the binding of TFIID to the TATA box may differ and may require the presence of a functional INR element. To determine whether an intact INR element was required for the ability of ICP4 to stabilize the binding of TFIID to the TATA box, the INR-mutated late gC8 promoter was analyzed (Fig. 19). Similar to the wild-type promoter, neither TBP, TFIIA, affinity-purified TFIID, nor the ICP4 derivative X25 alone was able to bind to the INR-mutated late promoter (Fig. 4A, lane 5, and Fig. 4B, lane 6). ICP4 alone at both concentrations of 10 and 5 ng affected the footprint of the gC8 promoter (Fig. 4A, lanes 6 and 7). Due to this, a lower concentration of ICP4 at 2.5 ng that did not bind the gC8 promoter was also analyzed (Fig. 4B, lane 8). As observed with the wild-type late promoter, TFIIA stabilized the binding of

FIG. 3. Analysis of the abilities of TFIIA, ICP4, and/or X25 to stabilize TFIID-TATA interactions on wild-type INR containing late gC promoter. To compare the ability of TFIIA and ICP4 to stabilize either TBP or the TBP multisubunit complex, TFIID's interaction with the TATA box on the wild-type late promoter was evaluated. DNase I footprinting was performed on the gC promoter with 2 ng of TBP, 250 pg of TBP-equivalent TFIID, 12 ng of TFIIA, 10 ng of the N- and C-terminal ICP4 mutant X25, and either 5 or 10 ng of wild-type ICP4.

either TBP or TFIID to the TATA box of the INR-mutated late promoter (Fig. 4A, lanes 8 and 9, and Fig. 4B, lanes 9 and 10). X25 again had no effect on the ability of either TBP or TFIID to bind the TATA box (Fig. 4A, lanes 10 and 13, and Fig. 4B, lanes 11 and 14). Although ICP4 could stabilize TBP binding to the TATA box of this INR-mutated promoter at concentrations ranging from 10 to 2.5 ng (Fig. 4A, lanes 11 and 12, and Fig. 4B, lanes 12 and 13), ICP4 was unable to efficiently stabilize the binding of TFIID to the TATA box of the INR-mutated promoter, at all concentrations (Fig. 4A, lanes 14 and 15, and Fig. 4B, lanes 15 and 16). TFIID in the presence of ICP4 appeared to be similar to ICP4 alone, indicating that ICP4 could not efficiently stabilize the binding of TFIID to the TATA box of an INR-deficient late promoter (Fig. 4B, compare lanes 15 and 16 with lanes 7 and 8). The additional activities of TFIIA were required to stabilize TFIID-TATA interactions (Fig. 4B, lanes 24 and 25). These results suggest that although ICP4 can stabilize the binding of TBP to a promoter consisting of only a TATA box, ICP4 alone is not sufficient to stabilize the binding of TFIID to a promoter con-

taining only a TATA box. Stabilized binding of TFIID to a promoter containing only a TATA box required the activities of TFIIA. Thus, in the absence of a functional INR element on a late promoter, ICP4 cannot substitute for TFIIA to stabilize TFIID binding to the TATA box.

ICP4 facilitates TFIIA in stabilizing the binding of TBP to the early promoter TATA box. Unlike the gC promoter, which contains a fairly strong TATA box, the tk promoter has a lower-affinity TATA consensus sequence (8). Whereas 3 ng of TBP alone could bind the gC TATA box (data not shown), the same concentration of TBP alone could not bind to the TATA box of the tk promoter. Increasing concentrations of TBP showed that 30 ng of TBP alone was required for half-occupancy of the tk TATA box (Fig. 5). TFIIA reduced this to 20 ng. The addition of ICP4 to TFIIA further reduced the amount of TBP required to achieve half-occupancy to 10 ng. Thus, higher concentrations of TBP are required to bind to the TATA box of the tk promoter, and TFIIA significantly reduced the amount required. ICP4 further reduced the amount of TBP required, probably by facilitating the ability of TFIIA to stabilize the binding of TBP to the TATA box.

DISCUSSION

In this study the binding properties of TFIIA, ICP4, and TBP/TFIID were examined on representative early, late, and INR-mutated late promoters. Our results indicate that ICP4 could substitute for TFIIA in stabilizing the binding of TBP to both the wild-type and the INR-mutated late promoters. For the early promoter, ICP4 facilitated the ability of TFIIA to stabilize the binding of TBP to the TATA box. When the TBP-containing complex TFIID was substituted for TBP, ICP4 was only able to stabilize TFIID binding to the TATA box of the wild-type late promoter and not TFIID binding to the INR-mutated late promoter. Stabilized binding of TFIID to this promoter required TFIIA. These results suggest that the efficient ICP4 activation of the wild-type gC promoter in the absence of TFIIA may be explained by the ability of ICP4 to substitute for TFIIA in stabilizing the binding of TFIID to the TATA box in the presence of a functional INR element. In the absence of a functional INR, ICP4's ability to activate transcription is severely compromised due to the inability of ICP4 to efficiently stabilize TFIID binding. TFIIA is required to stabilize TFIID-TATA box interactions on this INR-mutated promoter, alleviating the requirement for an intact INR, allowing ICP4 to more effectively activate transcription from this promoter. Thus, ICP4 can substitute for TFIIA in stabilizing the binding of TFIID to a late promoter containing a functional INR element but cannot substitute for TFIIA in stabilizing the binding of TFIID in the absence of a functional INR element.

Regions of ICP4 required for the stabilized binding of TBP to the TATA box. The observation that ICP4 and n208 directly stabilize the binding of TBP to the TATA box of the gC promoter is not unexpected since both forms of ICP4 have been shown to interact with TBP in solution (6). The ability of n208, which lacks amino acids beyond 778, to both interact with TBP in solution and to stabilize the binding of TBP to the TATA box of the late promoter (Fig. 2C), suggests that the C-terminal transactivation domain is dispensable for TBP in-

FIG. 4. Footprinting analysis of TFIID-TATA interactions on the INR-mutated late gC8 promoter in the presence of TFIIA, ICP4, and/or X25. To compare the abilities of TFIIA and ICP4 to stabilize the binding of TFIID to the TATA box of the wild-type (gC) versus the INR-mutated (gC8) late promoters, footprinting analysis of the INR-mutated late promoter was conducted using the same concentrations of proteins as for the wild-type INR-containing gC promoter. Due to the ability of ICP4 to bind to regions of this promoter at either 10 or 5 ng (A), a lower concentration of ICP4 at 2.5 ng that did not bind to this promoter was also analyzed (B).

teraction and TATA box stabilization. The inability of X25 to stabilize TBP-TATA or TFIID-TATA interaction strongly suggests that regions specified within amino acids 30 to 274, which include the N-terminal transactivation domain, are necessary for the ability of ICP4 to stabilize TBP-TATA interactions and may be necessary for direct interaction with TBP. Moreover, this suggests that the DNA-binding domain alone, which is still intact in X25, is not sufficient to stabilize TBP binding to the TATA box.

Although both ICP4 and n208 are able to interact with TBP in solution (6) and can efficiently stabilize binding of TBP to the TATA box (Fig. 2B and C), neither one of these molecules is able to activate transcription with TBP (29). Activated transcription by ICP4 requires the TBP-associated factors of TFIID. ICP4's C-terminal domain has been shown to interact with TAF250 of TFIID to promote transcription PIC formation (6). Mutants that lack the C-terminal transactivation domain, such as n208 and X25, do not interact with TFIID in solution (6). The fact that n208 interacts with TBP in solution but not with TFIID implies that TBP is inaccessible in the context of TFIID in solution. Interestingly, however, n208, but

not X25, is still able to recruit and stabilize the binding of TFIID to an immobilized gC promoter template, suggesting that, in the presence of a TATA box, n208 can still stabilize TFIID binding (28). The results presented here support the idea that the N-terminal transactivation domain of ICP4 is required for this effect. n208 bound to promoter DNA may encourage a rate-limiting conformational change during weak TFIID-TATA interactions, exposing TBP and allowing for interactions with the N-terminal domain of n208 that in turn may serve to stabilize TFIID binding to the TATA box.

Activated transcription not only requires the TAFs of TFIID but also requires the general transcription factor TFIIA (11, 44, 47, 53, 63). Activators can interact directly with TFIIA to mediate stabilized interaction with TBP and the PIC. TFIIA, as well as TAFs, directly interact with and stabilize the binding of TBP within TFIID to the TATA box of core promoters (3, 9, 39, 48). Dynamic interplay exists between TFIIA and TAFs that regulate the binding of TFIID to promoter DNA. TFIIA mediates an activator-induced conformational change in the transcription factor TFIID that significantly alters the interaction of TAFs with promoter DNA (18, 53). The amino-termi-

FIG. 5. TBP-TATA interactions on the early tk promoter by TFIIA and ICP4. Due to the inability of 2 ng of TBP to stably bind to the TATA box of the early tk promoter by 12 ng of TFIIA (data not shown), TBP was titrated at higher concentrations to determine the amount necessary to bind to the tk promoter. A total of 12 ng of TFIIA was added alone or in combination with 10 ng of ICP4 to the TBP titration to determine the effect on TBP-TATA interaction.

nal domain of TAF250 contains a negative regulatory element that inhibits TBP-TATA complex formation, demonstrating that at least one TAF can preclude the interaction of TBP with the TATA box. TFIIA, as a positive-acting general factor, has been shown to competitively derepress the inhibitory effect of TAF250 by destabilizing the interactions between TAF250 and TBP (18, 53). Thus, it is of interest that ICP4 interacts with TAF250 of TFIID and that it can substitute for TFIIA in stabilizing the binding of TFIID to the TATA box of an INR-containing promoter. ICP4 may function in a manner analogous to TFIIA in that it may alleviate the inhibitory effects of TAF250. This may in turn allow the N-terminal region of ICP4 to interact with TBP to stabilize its interaction with the TATA box in the context of TFIID.

Role of the INR in ICP4-stabilized binding of TFIID. Previous analysis of the promoter requirements for ICP4 activation of viral late genes has determined that the INR element, which is present on most true late promoters, is the only other element required in addition to the TATA box for efficient ICP4-activation, suggesting that late promoters are quite simple and unusual in nature since these are the only two promoter elements required for activation. Mutations within the INR element of a representative late promoter severely compromise the ability of ICP4 to activate transcription both in

vitro and in vivo (29, 43). Like cellular promoters, immediate-early and early promoters contain numerous *cis*-acting sequences for transactivators that work synergistically to recruit and stabilize the transcription machinery through interactions with different components of the general transcription factors. However, ICP4 alone can activate transcription with a relatively simple set of general transcription factors without the requirement of other cellular or viral specific activators in the presence of only a TATA box and a functional INR element (29). In addition, the general transcription factor TFIIA is not required for ICP4 activation of a late promoter containing an INR and a TATA box (69). This is quite intriguing since TFIIA has previously been shown to play an unusually important role in INR-mediated transcription (34, 50, 63). TFIIA has been shown to selectively enhance TFIID transcription from a promoter that contains both a TATA box and an INR (18). TFIID alone has been shown to have similar affinities for TATA and TATA-INR promoters. TFIIA strongly enhances TFIID binding to a TATA-INR promoter, while having little effect on binding to a TATA box only promoter. TFIIA-induced conformational changes are essential for the sequence-specific TFIID interaction with the INR. ICP4 may be functioning in a similar manner to TFIIA. Through protein-protein interactions and potential conformational changes that occur within both ICP4 and TFIID, ICP4 may induce interactions of TAFs with the INR in a manner similar to that of TFIIA.

For the tk gene, given that ICP4 cannot effectively stabilize the binding of TFIID to the TATA box of a non-INR containing promoter in the absence of TFIIA (Fig. 4) and that the TATA box of the tk promoter is much weaker than the gC TATA box requiring a higher concentration of TBP to bind to the tk TATA box (Fig. 5), it would be reasonable to assume that ICP4 alone would not be able to stabilize the binding of TFIID to the tk TATA box in the absence of TFIIA. This is also suggested by the requirement of TFIIA for efficient ICP4 activation of the tk promoter (69). Because ICP4 reduces the concentration of TBP required for TFIIA-stabilized TBP-TATA interactions on the tk promoter (Fig. 5), it is likely that ICP4 synergizes with the actions of TFIIA to stabilize TBP-TATA interactions on this promoter. In the context of TFIID, TFIIA or ICP4 may promote weak TFIID-TATA interactions that are strengthened by one another.

In the context of HSV infection, TFIIA is present early during infection and is required for activation of early genes. ICP4, in cooperation with other cellular activators that require TFIIA, works to recruit and promote stabilized binding of TFIID to the TATA box of the early promoters. As infection proceeds, TFIIA expression decreases, coincident with its dispensability for INR-containing late gene expression since ICP4 can substitute for TFIIA in stabilizing the binding of TFIID to the TATA box, as well as induce interactions of TAFs with the INR. The present study clarifies one of the many viral mechanisms that exist for the attenuation of early gene expression while allowing the subsequent expression of late gene expression.

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