The TATA Motif Specifies the Differential Activation of Minimal Promoters by Varicella Zoster Virus Immediate-early Regulatory Protein IE62*

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The immediate-early IE62 protein of varicella zoster virus is an acidic transcriptional activator capable of up-regulating many viral and cellular promoters with varying efficiencies. We demonstrate that, in the context of a minimal promoter, a TATA element is both sufficient and essential for IE62-mediated transcriptional activation. Differential levels of activation by IE62 in this context were conferred by a panel of naturally occurring sequence variations within the TATA motif itself. TATA motif-specific, differential induction was not obtained when the IE62 acidic activation domain was targeted as a GAL4 fusion protein to the same panel. The prototype acidic transactivator, VP16 of herpes simplex virus, failed to discriminate between these different TATA motifs when they were placed into an appropriate responsive promoter context. Nonetheless, a chimeric IE62 polypeptide substituted with the VP16 activation domain retained the ability to differentially modulate minimal promoters with various TATA motifs. Taken together with its binding to TATA box-binding protein (TBP) and transcription factor IIB in vitro, we suggest that IE62 has the unusual ability to achieve differential levels of transcriptional activation through different TATA motifs, which may be accomplished either directly or indirectly by recognizing conformational variations in DNA-bound TBP, TBP-transcription factor II A/B, or TBP-TATA-associated factor complexes.

Varicella zoster virus (VZV) causes chickenpox as a primary infection, and shingles as a reactivated infection. The lytic cycle of this virus begins with the expression of its major immediate-early protein, IE62, a DNA-binding trans-regulatory protein containing 1310 amino acid residues with a predicted molecular mass of 140 kDa (1). IE62 is a potent transactivator that stimulates transcription not only of target VZV genes, but also a variety of heterologous viral and cellular genes (2, 3). Although there is no definitive genetic proof as yet that IE62 is essential for VZV replication, its ability to regulate the expression of VZV genes of all three putative kinetic classes (immediate-early, early, and late) as well as its sequence homology and functional similarity to the essential ICP4 transactivator of herpes simplex virus (HSV) argue that it serves a critical role in the VZV replicative cycle. Both VZV IE62 and HSV-1 ICP4 bind to specific ATCGTC DNA sequences at the cap site of their own promoters, and these interactions are thought to be involved primarily in negative transcriptional autoregulation. However, the mechanism by which these proteins up-regulate the transcription of responsive viral promoters has not yet been well defined.

Accumulating evidence indicates that transcriptional activators, in general, enhance transcription by influencing or stabilizing functional interactions among the general transcription factors including TATA box-binding protein (TBP), TFIIA, and TFIIB, that are essential for the formation of a functional pre-initiation complex (reviewed in Refs. 4–12). In addition, the in vitro function of transcriptional activators requires a number of co-factors that are not essential for basal transcription in minimal reconstituted systems. Many of these co-factors are physically associated with the TBP polypeptide (TATA-associated factors or TAFs) and constitute the holotFIID fraction along with TBP. Certain transactivators have been shown to interact specifically with TBP and one or more TAFs, and these interactions appear to function as molecular adapters that bridge between the transactivator and the general transcription initiation machinery and serve to recruit TBP and enhance a rate-limiting step in transcription (reviewed in Refs. 5, 8, and 10).

An intriguing aspect of IE62 function is its capacity to activate in trans a diverse array of promoters that lack any apparent conserved motifs in their promoter and upstream regions (2, 3, 13). In addition, the magnitude of responsiveness to IE62 varies widely from promoter to promoter. Curiously, in both HSV and VZV, most of the immediate-early and early class promoters tend to have classical close-to-consensus TATA motifs, whereas late class promoters typically have highly divergent, non-consensus (but still AT-rich) TATA-like motifs. Both types of promoters respond to IE62 and ICP4, although in HSV other accessory viral factors clearly contribute to both maximal and appropriate temporal responses of late promoters.

Previous deletion analysis of the VZV IE62 promoter revealed that a minimal reporter construct possessing the TATA box with only 15 base pairs (bp) of adjacent upstream sequence retains responsiveness to IE62-mediated induction but not to its HSV homolog ICP4 (13). Because this upstream sequence lacks consensus recognition motifs for binding to IE62 or any other known transcription factors, we have focused our atten-

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‡ The abbreviations used are: VZV, varicella zoster virus; HSV, herpes simplex virus; EBV, Epstein-Barr virus; CMV, cytomegalovirus; HCMV, human cytomegalovirus; TBP, TATA box-binding protein; TF, transcription factor; TAF, TATA-associated factor; IFN, interferon; aa, amino acid(s); bp, base pair(s); nt, nucleotide(s); GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; PIPES, 1,4-piperazinediethanesulfonic acid; Inr, initiator; MIE, major immediate-early.

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tion on the TATA element itself as a potential key factor in mediating the transcriptional effects of IE62. In this report, we present evidence to suggest that a novel TATA motif-dependent mechanism is both essential and sufficient for IE62-mediated transcriptional activation.

EXPERIMENTAL PROCEDURES

Target Reporter Gene Plasmids Containing Panels of Different TATA Motifs—Plasmids p62CATΔ258 and p62CATΔ45 have been described previously (13). In p62NTATA and p62NTATACAP, either 5 or 3 bp of the TATA or the Inr, respectively, were deleted by site-specific mutagenesis. In p62TATACAP the putative Inr element was changed from TCTCAG to TCTAAG. To create a TATA only synthetic promoter (pTATAX5CAT), a 44-bp oligonucleotide consisting of five tandem copies of the β-casein TATA sequence TATATATA was inserted at the XbaI site upstream of the CAT coding region in the plasmid pCAT-B (Basic, Promega Corp.). A parallel control plasmid (p2TATAX5CAT) contained a 44-bp oligonucleotide consisting of five tandem copies of the mutant sequence TATCGATC. In p62E1bTATA, a single copy 12-bp (TAATATAGGAGA) synthetic oligonucleotide bearing the adenosine E1b gene TATA motif was inserted immediately upstream of the CAT gene (15).

For analysis of the responsiveness of diverse TATA elements to IE62, a panel of target constructs was created. To do so, the TATA element in the p62NTATA target plasmid was modified by site-specific mutagenesis to resemble the TATA motifs of several viral and cellular genes. For example, p(T)ATTAAA bears the ATTAAA motif of the VZV thymidine kinase (TK) gene; pT/ATTAAATT bears the ATTAAAATT motif of the VZV glycoprotein C gene; pTATATAAA bears the TATATAAA motif of the adenosine A3.01 (pG-TATTTAAATT, pG-TATATATAA, and pG-TATATAA. However, this distance was reduced by 2 bp for TATATATAA mutant and by 3 bp for TATTAAA mutant.

A second set of TATA motif constructs was created, by cloning a 100-bp oligonucleotide cassette, containing five tandem GAL4-binding sites 28 bp upstream of the modified TATA boxes in the α-INF minimal promoter panel described above to yield the following set of GAL4-responsive reporter genes, pG5-TATTAAA, pG5-TATTTAAA, pG5-TATATAA, pG5-TATTAAAATT, pG5-TATATAAA, and pG5-TATATAA. In ICPO-CATΔ(9-2), the promoter elements, including several Oct-I-1AATGARAT type, VP16 response motifs extending from -800 to +120 within the HSV immediate-early ICP0 gene, drive the expression of CAT reporter gene (17). A third TATA motif panel was created based on this construct. Specifically, the native TATA element (GGGGTATATAAGTTTTAAGG) in ICPO-CAT was replaced by site-specific mutagenesis with a variety of TATA boxes, to generate ICPO-CAT/TATA with the TATA motif deleted, ICPO-CATM/TATTAAA (bearing the VZV IE62 TATA motif), ICPO-CATM/TATATAA (bearing the HCMV MIE TATA), and ICPO-CATM/TATATAAA (bearing the consensus MFP and HSP90 TATA).

Further, a fourth TATA motif panel was created by cloning a 23-bp oligonucleotide cassette bearing the Oct-I-1AATGARAT motif (GTCATGCTATATAGATTGTTTTAAAGG) immediately upstream of the modified TATA elements in the α-INF minimal promoter to yield a set of VP16-responsive, minimal reporter plasmids designated pVP-TATTAAA, pVP-TATTTAAA, pVP-TATATAAA, and pVP-TATATAA.

Effector Genes and CAT Assays—The pCMV62 plasmid expresses the VZV IE62 protein (3) from the HCMV MIE promoter. The pRL5 plasmid, which expresses both the immediate-early proteins IE1 and IE2 of HCMV, has been described previously (18). Plasmid p62GAL (9–120) expresses the chimeric protein GAL4-IE62 containing the activation domain of IE62 (aa 9–120) fused to the GAL4 DNA binding domain (aa 1–147) and has also been described previously (19). The VP16 plasmid (pGH12) expresses the intact HSV-1 VP16 effector protein from its cognate promoter (17). The plasmid pGH114 has been described earlier (13) and expresses ICP4 from the simian cytomegalovirus immediate-early promoter. The pPRV180 plasmid expresses the VZV180 of pseudorabies virus and was a gift from P. Sheldrick. The EIA expression plasmid (20) was a gift from G. Nabel. The plasmid pCMV82/16D is a derivative of pCMV62 and contains the coding region of HSV VP16 expression plasmid, an orientation (domain (dsDNA) fragment from pVP16 (CLONTECH), cloned in-frame 5’ to the IE62 coding segment extending from codon 96 to 1310. Effector and target reporter gene plasmid DNAs were electroporated into A3.01 cells (a human T lymphocytic cell line). The CAT activity was determined as described previously (3), quantitated with a PhosphorImager scanner (Molecular Dynamics), and expressed as percentage of conversion of [14C]chloramphenicol to its acetylated derivatives.

RNase Protection Assay—In RNase protection assays, 40 μg of total cellular RNA extracted from transfected A3.01 T lymphocytic cells were hybridized with an antisense RNA probe corresponding to CAT gene for 12 h at 56 °C in 40 mM PIPES (pH 6.7), 500 mM NaCl, 1 mM EDTA, and 80% formamide. As internal controls, antisense RNA probes corresponding to the CD4 cell surface marker and two housekeeping genes, L32 ribosomal gene and glyceraldehyde-3-phosphate dehydrogenase, were also included. Samples were then treated with RNase A (40 μg/ml) and RNase T1 (2 μg/ml) for 45 min at 30 °C. The reactions were terminated by adding 50 μg of protease K and 10 μl of 10% SDS and incubated at 75 °C for 15 min. After phenol-chloroform extraction and ethanol precipitation, the samples were resolved on 6% denaturing polyacrylamide gel and subjected to autoradiography as well as phosphorimage analysis.

In Vitro Protein Matrix Affinity Binding Assays—Three overlapping segments (aa 9–410, 234–734, and 734–1310) spanning nearly the entire length of the 1310-aa IE62 protein, were cloned into an in vitro transcription/translation vector (21) to generate portions of the IE62 polypeptide in a rabbit reticuloocyte lysate system. Agarose beads coupled to purified intact 33-kDa human TFIIB synthesized in Escherichia coli were purchased from Santa Cruz Biotechnology (catalog SC4001AC). TBP-N (N-terminal) and TBP-C (C-terminal) GST-fusion proteins were expressed in E. coli and purified by incubating the bacterial supernatants with glutathione-Sepharose beads as described previously (2). Purified proteins were performed on polyacrylamide DNA-immobilized beads. Agarose beads were boiled in sample buffer and subjected to SDS-PAGE, followed by visualization of proteins with Coomassie Blue stain. Equal amounts (25 μg) of bead-bound proteins were incubated in vitro translated, 35S-labeled polypeptides representing segments of the IE62 protein. The binding buffer contained 140 mM NaCl, 50 mM Tris-HCl, 0.5% Triton X-100, 2 mM diithiothreitol, and 100 μg/ml ethidium bromide. Ten μg of poly[dG-dC]/poly[dG-dC] was added to the protein DNA-immobilized beads. After extensive washing to remove the unbound material, the beads were suspended in protein sample buffer, boiled for 5 min, and resolved by SDS-PAGE.

Electrophoretic Mobility Shift Assay (EMSA)—Electrophoretic mobility shift assays with purified recombinant human TATA-binding protein (VP16) synthesized in E. coli (Promega TPHD, catalog no. E9081) in ICPO-CATΔ(9-2) (20), the promoter elements, including several Oct-I-1AATGARAT type, VP16 response motifs extending from -800 to +120 within the HSV immediate-early ICP0 gene, drive the expression of CAT reporter gene (17). A third TATA motif panel was created based on this construct. Specifically, the native TATA element (GGGGTATATAAGTTTTAAGG) in ICPO-CAT was replaced by site-specific mutagenesis with a variety of TATA boxes, to generate ICPO-CAT/TATA with the TATA motif deleted, ICPO-CATM/TATTAAA (bearing the VZV IE62 TATA motif), ICPO-CATM/TATATAA (bearing the HCMV MIE TATA), and ICPO-CATM/TATATAAA (bearing the consensus MFP and HSP90 TATA).

Further, a fourth TATA motif panel was created by cloning a 23-bp oligonucleotide cassette bearing the Oct-I-1AATGARAT motif (GTCATGCTATATAGATTGTTTTAAAGG) immediately upstream of the modified TATA elements in the α-INF minimal promoter to yield a set of VP16-responsive, minimal reporter plasmids designated pVP-TATTAAA, pVP-TATTTAAA, pVP-TATATAAA, and pVP-TATATAA.

RESULTS

The TATA Element Is Essential for IE62-mediated Transcriptional Activation—The immediate-early protein encoded by the open reading frame 62 constitutes the major transcriptional regulatory protein of VZV. However, the mechanic
aspects of its transcriptional activity remain poorly understood, although its HSV counterpart ICP4 has been extensively studied. Our previous studies indicated important differences between these two related regulatory proteins of alphaherpesviruses. For example, unlike ICP4 which represses its cognate promoter activity, IE62 augments the transcription from its own promoter as well as that of the ICP4 promoter. In addition, despite ICP4's repressive effects on its own promoter, it enhanced the transcription from the IE62 promoter. However, the upstream cis-elements required for the induction of IE62 promoter by IE62 and ICP4 differed (13). Confirming our previous observations, as shown in Fig. 1 (panel A), the IE62 promoter with 258-bp upstream sequences with reference to its transcription start site (p62CATΔ558) responded positively when cotransfected with either IE62 or ICP4 (120- or 25-fold, respectively). In contrast, an IE62 promoter version having only 15-bp upstream sequences together with its TATA box (p62CATΔ45) responded only to IE62 (36-fold).

Scrutiny of the IE62 promoter region in p62CATΔ45 revealed no recognition motifs for binding to IE62 or any other known transcription factors other than the TATA box. Considering that IE62 activates a wide array of viral and cellular promoters and the fact that the core promoter element-TATA box is a common motif present in all these responsive promoters, we focused on the TATA element as a potential mediator of the IE62 transcriptional activity.

To explore the contribution of the TATA element to IE62-mediated induction, a CAT reporter gene containing a 60-bp minimal promoter derived from the murine α4 IFN promoter elements (pIFN-TATA) (14) was electroporated into the human T-lymphocytic cell line A3.01 with or without a VZV IE62 effector plasmid. T lymphocytes have been implicated as important vehicles for the growth and spread of VZV in vivo (see Ref. 3 and references therein), and specific T cell tropism for VZV has been demonstrated (24). Our previous studies with VZV promoter targets were also carried out in these cells (3, 13, 19).

The minimal pIFN-TATA promoter target was induced by the cotransfected IE62 effector plasmid (Fig. 1, panel B), which expressed the intact IE62 protein under the control of the HCMV MIE promoter. Neither a control plasmid containing the HCMV MIE promoter region alone (data not shown), nor plasmids expressing the IE62 homologs ICP4 of HSV and IE180 of pseudorabies virus, the ICP0 protein of HSV, or the adenovirus E1A protein, resulted in any obvious increase in CAT activity when cotransfected with the target plasmids. The HCMV IE1/IE2, however, was able to activate pIFN-TATA significantly (Fig. 1, panel B).

Two synthetic minimal core promoters consisting, first, of a 12-bp TATATA element from the adenovirus E1B promoter in pE1B-TATA CAT (15) and, second, one containing three tandem copies of either a wild-type TATATA motif only or a 3TATA mutant motif inserted 5′ to the CAT reporter gene in pCAT-B vector (Promega Corp.) were also tested. Co-transfection with VZV IE62 gave 4-fold induction of pE1B-TATA CAT and 26-fold induction of pCAT-B, but gave no effect on either the pCAT-B or p3TATA CAT targets (data not shown).

All three minimal promoters used above lacked any recognized upstream elements other than the TATA boxes, but pIFNTATA does contain a putative pyrimidine-rich Inr element (CTCTTCAG), of a type that can substitute for TATA in some circumstances. Therefore, to assess the relative contributions of the TATA element and the Inr element in IE62-mediated transactivation, we performed transient expression assays with a set of minimal pIFNTATA derivatives in which both of these elements were individually mutated (Fig. 1, panels B and C). Deletion of 5 bp of the α-IFN TATA element itself (pIFNTATA) led to complete ablation of IE62 responsiveness (Fig. 1, panel B), while deletion or mutation of the Inr element...
To validate the differential activation of various TATA modified promoter constructs by IE62, it is crucial to assess whether the substitution of different TATA motifs influenced the basal promoter activity appreciably. Therefore, the basal activity of each minimal promoter construct examined in Fig. 2, was directly measured by a sensitive RNase protection assay following transfection of TATA modified promoter CAT plasmid DNA into A3.01 T cells. As evident from the data in Fig. 3, all minimal promoter versions were transcriptionally active, including the pIFN\textsuperscript{ATATA} (which still contains the core promoter IRr element to initiate transcription), resulting in appropriately sized protected fragment with the CAT probe (visible after 6-day exposure). Probes for housekeeping genes L32 ribosomal gene and glyceraldehyde-3-phosphate dehydrogenase gene as well as the abundantly expressed CD4 gene were included as controls for RNA integrity and quantitation standards. Upon quantitation of CAT transcripts from each promoter, the differences in intensity were less than 2-fold. For example, IE62 responsiveness (17-fold decrease) and substitution with the TATAAAA consensus sequence of the Ad2 MLP and HSP90 promoters conferred a minimal promoter is TATA motif-dependent. The parent TATA motif present in pIFN\textsuperscript{TATA} was modified to resemble a variety of consensus and non-consensus TATA motifs derived from several other viral and cellular genes, or VZV downstream target genes (see “Experimental Procedures”). Co-transfections into A3.01 cells and quantitation of CAT activity were done as described under “Experimental Procedures,” using 10\(^{10}\) or 10\(^{15}\) of the cell extract was used in the assay. The -fold inductions given for each target in the presence of IE62 represent the mean value of three independent experiments ± standard error: for pIFN\textsuperscript{TATA}, 15 ± 2.6; for pIFN\textsuperscript{TATATA}, 280 ± 32.2; for pIFN\textsuperscript{TATTATA}, 13 ± 1.9; for pIFN\textsuperscript{TATATA}, 54 ± 6; for pIFN\textsuperscript{TATATA}, 258 ± 30.9; for pIFN\textsuperscript{TATTATA}, 3 ± 0.6; and for pIFN\textsuperscript{TATATA}, 114 ± 20.2, respectively.

| TATA motif | Gene | Sequence |
|------------|------|----------|
| TATTTAA    | α4 interferon | GGACTTTGCCCCTATTATAGAGAGATGTACAG |
| ΔTATA      | α4 interferon | GGACTTTGCCCCTAGAGAGATGTACACAGCA |
| TATATAA    | HCMV MIE    | GGACTTTGCCCCTATTATAGAGAGATGTACAG |
| TATATATAA  | β-Casein    | GGACTTTGCCCCTATTATAGAGAGATGTACAG |
| TATAAAA    | Ad2 MLP, HSP90 | GGACTTTGCCCCTATTATAGAGAGATGTACAG |
| (T)ATTTAA  | VZV IE62    | GGACTTTGCCCCTATTATAGAGAGATGTACAG |
| (T)ATTATAA | VZV IE62    | GGACTTTGCCCCTATTATAGAGAGATGTACAG |
| (T)ATTATAA | VZV gp C    | GGACTTTGCCCCTATTATAGAGAGATGTACAG |

**FIG. 2.** The magnitude of the IE62-mediated activation of a minimal promoter is TATA motif-dependent. The parent TATA motif present in pIFN\textsuperscript{TATA} was modified to resemble a variety of consensus and non-consensus TATA motifs derived from several other viral and cellular genes, or VZV downstream target genes (see “Experimental Procedures”). Co-transfections into A3.01 cells and quantitation of CAT activity were done as described under “Experimental Procedures,” using 10\(^{10}\) or 10\(^{15}\) of the cell extract was used in the assay. The -fold inductions given for each target in the presence of IE62 represent the mean value of three independent experiments ± standard error: for pIFN\textsuperscript{TATA}, 15 ± 2.6; for pIFN\textsuperscript{TATATA}, 280 ± 32.2; for pIFN\textsuperscript{TATTATA}, 13 ± 1.9; for pIFN\textsuperscript{TATATA}, 54 ± 6; for pIFN\textsuperscript{TATATA}, 258 ± 30.9; for pIFN\textsuperscript{TATTATA}, 3 ± 0.6; and for pIFN\textsuperscript{TATATA}, 114 ± 20.2, respectively.
Twenty micrograms of each plasmid DNA were transfected into A3.01 cells, and after 36 h cellular RNA was extracted. Forty micrograms of cellular RNA were hybridized with a 209-nt, 32P-labeled, antisense riboprobe corresponding to CAT gene. The expected size of the protected fragment is 180 nt. As internal controls labeled antisense riboprobes for CD4 (191 nt in length and when protected yields a 162-nt fragment), L32 ribosomal (141 nt in length and when protected yields a 113-nt fragment) and glyceraldehyde-3-phosphate dehydrogenase gene (124 nt in length and when protected yield a 96-nt fragment) were also included in the RNase protection assay. The protected RNA fragments were resolved on 6% sequencing gel and subjected to autoradiography analysis

In rats, the abundant expression of both IE62 and GC polypeptides despite their highly divergent TATA motifs during VZV infection very likely indicate their transcriptional competence. Taken together, these data suggest that within the limits of sensitivity of each assay employed the modifications of TATA elements assessed in this study do not appreciably alter the basal activity of the minimal promoter and the differential activation seen in the presence of IE62 with various TATA motifs is most likely to be a function of the VZV IE62 itself.

**TATA Motif-dependent, Differential Induction Is Not Conferred by the Activation Domain of IE62**—We next sought to determine whether TATA motif-dependent, differential induction is conferred by the activation domain of IE62 itself or by some other segment of the IE62 protein. This is of special relevance in the light of recent identification of a coactivator protein (PC4) that mediates acidic activator transcription and contains SEAC motifs in common with VZV IE62 and many other viral transactivators (28, 29). The strong acidic activation domain of IE62 is localized to the negatively charged N-terminal region (aa 9–120) of the 1310-amino acid protein (19, 30). Previously, we have shown that the IE62 activation domain (aa 9–120) when expressed as a fusion protein with the yeast GAL4 DNA-binding domain (GAL4-IE62) efficiently activates promoters with upstream GAL4 binding motifs but not promoters without such sites (19). In addition, the deletion of this single activation domain of IE62 results in inactivation of transcriptional activity, although the deletant molecule is still capable of competitively interfering with the transcriptional activity of the native IE62 (19). To assess whether the activation domain itself is responsible for both transcriptional activation as well as sensing the differences in the TATA motif leading to differential responsiveness, five tandem GAL4-binding sites were placed upstream of the TATA box in each of the panel of TATA substituted promoters described earlier in Fig. 2. The insertion of GAL4 binding sites neither affected the basal activities of the promoters nor their ability to respond differentially to native IE62 when cotransfected (data not shown). When this panel was cotransfected with the GAL4-IE62 chimeric plasmid, all promoters responded vigorously (Fig. 4). However, the levels of activation varied by less than 2-fold upon substitution of the consensus TATA motif with a variety of other non-consensus TATA motifs, which in the context of minimal promoters lacking the GAL4-binding sites led to nearly 100-fold TATA-dependent differences in activation by the full-length IE62 protein. Although these findings suggest that the activation domain is not sufficient to impart differential induction under these circumstances, one could argue that the tethering of activation domain via a GAL4 bridge may not represent natural conformation or recruitment of IE62. However, if a region other than the activation domain of IE62 is responsible for the TATA motif-dependent, differential activation of promoters, then, theoretically, if the modular activation domain of IE62 is replaced with a heterologous activation domain, the chimeric protein might still be able to display TATA motif-dependent differential effects mimicking a more natural setting if such a molecule is transcriptionally functional. In selecting an appropriate heterologous activation domain, the preponderance of acidic residues, as well as the comparable sizes and transcriptional strengths of the modular domains (19), favored VP16, the prototype of acidic transcriptional activators (31). However, it was crucial that the substituted activation domain be free of any inherent TATA motif-dependent effects. Although HSV VP16 remains one of the most extensively studied viral transcriptional activators, the influence of the TATA motif, if any, for VP16-mediated transcriptional activity has not been described. We therefore first examined whether the TATA motif influences VP16-mediated transcriptional activity.

The promoter for the HSV immediate-early gene encoding the ICP0 protein is efficiently activated by VP16. VP16 responsiveness is mediated primarily by an interaction with the cellular Oct-1 protein and subsequent binding to multiple upstream Oct-1/TATAAGARAT DNA elements in the ICP0 promoter (32, 33). As shown in Fig. 5 (panel A), VP16-mediated activation of the ICP0 promoter was changed minimally (range: 11–15-fold) by substitution of its TATAAG element with several of the TATAAGARAT element examined earlier in Fig. 2. Nonetheless, it still remains possible that the apparent insignificance of the TATA element for the VP16-induced activation of ICP0 promoter resulted from compensatory effects of other transcription factors such as Sp-1 that binds to the upstream elements in this complex promoter. To address this issue, we devised a strategy to direct VP16 to the minimal promoter panel used in Fig. 2. A 23-bp synthetic Oct-1/TATAAGARAT element was inserted immediately upstream of the TATA box. As shown in Fig. 5 (panel B), when this panel was cotransfected with a VP16 expression plasmid, once again there was no differential TATA motif-dependent induction of the target plasmids. Equally important was the fact that in the context of a minimal promoter, or for that matter in the context of a natural target promoter (ICP0 promoter), VP16 was capable of inducing transcription in the absence of a TATA box.

Having confirmed that VP16 is devoid of any inherent TATA motif-dependent effects, a chimeric IE62 with VP16 activation domain substituting the cognate activation domain of IE62 was constructed (IE62/16AD). When IE62/16AD was co-expressed with the minimal promoter panel examined in Fig. 2, with modified TATA elements, the induction of the minimal promoters were differentially modulated as was seen with the native
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Fig. 4. The N-terminal acidic activation domain of IE62 is not sufficient to mediate TATA motif-dependent, differential modulation. A 100-bp oligonucleotide cassette containing five tandem GAL4 binding sites (68) was placed upstream of the TATA box in the same panel of target reporter genes used earlier in Fig. 2. The chimeric protein GAL4-IE62 expressed from pB2GAL9-120) contains the activation domain of IE62 (aa 9–120) fused to the DNA binding domain (aa 1–147) of yeast GAL4 protein. Because of the sensitivity of the targets to the GAL4 chimeric effector, the amounts of the target and effector DNAs used in co-transfections were reduced to 100 ng each.

IE62 (compare Fig. 2 versus Fig. 6). These results taken together with the lack of TATA specificity obtained with VP16 (Fig. 5), and also by the HCMV IE1/IE2 proteins (see Fig. 1, panel B), supports the notion that TATA motif-dependent, differential induction is an inherent property of IE62 and not a general property of viral transactivators and a region other than the activation domain of IE62 dictates this unique phenomenon.

A Region of IE62 That Interacts with TBP and TFIIIB in Vitro Maps Outside of the Activation Domain—Given the clear involvement of TATA element in IE62-mediated transcriptional activation, we assessed whether IE62 physically interacts with the essential components of the basal transcriptional machinery that assemble on the TATA element. The results of protein-protein affinity binding studies, shown in Fig. 7, indicate that it does interact with both TBP and TFIIIB. When various in vitro translated, 35S-labeled, VZV IE62 polypeptide segments were incubated with purified, bacterially expressed TBP or TFIIIB immobilized on beads, the IE62 protein bound to TFIIIB (lanes 2) and to the evolutionarily conserved C-terminal segment (lanes 4) of TBP (GST/TBP-C) but not to its N-terminal segment (lanes 3) (GST/TBP-N). The region of IE62 that interacted most strongly with the basal transcription factors mapped between aa 234 and 734, well downstream from the only defined activation domain, between aa 9 and 120. A weaker interaction also occurred with the aa 734–1310 region, suggesting that IE62 possesses multiple domains that contact basal transcription factors. This situation contrasts with the results for several other well defined viral transactivators such as HSV VP16, Ad2 E1A, and EBV Zta, which bind strongly to TFIID directly through their activation domains (34–37). The HCMV IE2, however, resembles VZV IE62 in that it binds to TBP strongly through a region that is independent of its acidic activator domain (22, 38).

Nonetheless, the activation domain is critical for transcriptional activity of the full-length, 1310-amino acid IE62 protein, since the deletion of 85 amino acids extending from codon 10 to 95 (IE62ΔAD) resulted in complete ablation of the transcriptional activity (Fig. 8). Moreover, consistent with the notion that high affinity binding to both TBP and TFIIIB are integral to transcriptional activity of IE62, an 80-amino acid deletion (aa 416–506) within this region (IE62Δ416–506) resulted in loss of transcriptional activity. The expression and intracellular distribution profiles of both mutants IE62ΔAD and IE62Δ416–506 were identical to that of full-length, wild-type IE62 (data not shown), and both mutants competitively inhibited the transcriptional activity of full-length IE62 as shown in Fig. 8. When 10 μg of IE62 plasmid was cotransfected with either 5 or 15 μg of IE62ΔAD, the induction of the reporter gene was reduced by 70% or 90%, respectively. Similarly, cotransfection of IE62 plasmid with 5 or 15 μg of IE62Δ416–506 resulted in 60% or 85% reduction in wild-type IE62-mediated inducibility, suggesting that the transcriptional-deficient mutants are still capable of efficiently sequestering cellular components involved in IE62-mediated transcription. Alternate possibility that the competitive inhibition may operate at the DNA binding step rather than at the level of transcription is unlikely since the target construct pIFNTATA lacks any IE62 binding ATCGTC sites.

TATA Motifs That Confer Widely Differing Responsiveness to IE62 Display Comparable Levels of Binding to TBP in Vitro—TFIID is a high molecular weight, multiprotein complex consisting of TBP, which specifically recognizes and binds to the TATA boxes of class II gene promoters (39, 40), and a number of associated factors (TAFs). While both consensus (TATAAA or TATAAAA) and non-consensus TATA elements bind TFIIID (41), it has been suggested that the strength of the interaction between TFIIID and the TATA element correlates with the basal promoter activity (42). Certain transcriptional activators such as Zta and ICP4 of EBV and HSV, respectively, induce transcription by enhancing or stabilizing TBP binding to the TATA box. Promoters with TATA elements that bind TBP suboptimally are usually responsive to these activators but not to the promoters with TATA elements that avidly bind TBP (35, 43). It should be noted, however, that the promoters that responded to IE62 most vigorously, in contrast, possessed apparently consensus or near consensus TATA motifs, for example TATATATA, TATATAAA, or TATATAT. Although only limited information exists in literature as to the binding affinities of TBP to various TATA motifs (41, 43), two of the motifs i.e., TATATATAA and TATATATATA, that conferred high activity in the presence of IE62 have previously been shown to display apparent dissociation constants (Kd) of 2 × 10−9 M, a value that is among the highest reported for any TATA motif (41). Since no information exists in the literature with regard to TBP binding efficiency to other TATA motifs evaluated in our study, we assessed the ability of each of the motifs to bind purified recombinant human TBP in an in vitro electrophoretic mobility shift assay to examine any possible link between TBP binding to a particular TATA motif and the level of its responsiveness to IE62. The synthetic oligonucleotides representing different TATA motifs used as probes in the EMSA are listed in Table I. An additional probe with TACAAA motif of adenovirus EIIa promoter, which was originally used by Huang et al. (42) to demonstrate minimal TBP binding by DNase foot-printing assay, was included in our TBP EMSA to validate our assay conditions. First, the amount of TBP added to the binding reaction was titrated (20, 8, and 4 ng of TBP) against a fixed amount of each probe (100 fmol) and the fraction of probe that
complexed with the added TBP was quantitated. As shown in Fig. 9 (panel A), at high concentrations of TBP (20 ng), all TATA motifs used in the present study displayed significant TBP-binding profiles, with a difference less than 3-fold between the highest (TATATAA) and the lowest (TATTTAA). A similar trend was evident at low TBP (4 ng) concentration as well. The apparent reduction in binding to TATTTAAA probe in Fig. 9 at low TBP concentrations, however, was not reproducible. In contrast, the probe with a deletion in the TATA motif (∆TATA) failed to elicit any TBP binding at all. Having failed to detect any gross differences in direct binding of TBP to our panel of TATA motifs, we sought to confirm this observation by a competition binding assay in which TBP binding to the consensus TATATAAA motif was competed with a 100-fold excess of unlabeled competitor sequence. Again, as seen in Fig. 9 (panel B), no significant differences were noted. Thus, no definitive correlation between the binding of TBP to specific motifs and the extent of IE62-mediated activation could be discerned. For example, the TTATTTAAA motif from the IE62 promoter and the TATTTAAAATT motif from the glycoprotein C promoter bound TBP strongly, yet the αIFN-CAT reporter genes containing these elements responded weakly to IE62 (13- and 3-fold, respectively). The fact that the most vigorous IE62-mediated activation was achieved with TATA motifs that display the highest affinities toward TBP, taken together with the observation that the motifs that were relatively refractory to IE62 induction still bound TBP efficiently, therefore, argue in the case of IE62, for a mechanism that is fundamentally different from what has been proposed for ICP4 and Zta transcriptional activators.

**DISCUSSION**

The VZV IE62 transcriptional regulatory protein possesses an unusual, TATA motif-dependent mechanism by which it mediates activation of simple, minimal eukaryotic promoter targets. Large variations in the levels of activation of various promoters tested were evident according to which particular TATA sequence was present in them. The one other viral transactivator known to activate minimal promoters via a TATA-dependent mechanism is the adenovirus E1A protein. E1A, however, exhibits an absolute requirement for one specific motif, namely TATAA, for transactivation to occur (44, 45). Similarly, the muscle-specific enhancer of the myoglobin gene has been shown to function in the presence of its cognate TATAAAA motif, but not with the TATTTAT element from the SV40 promoter (46). The activity of cellular transcription factor ATF has also been shown to be sensitive to TATA motif changes (47).

The results presented here argue that VZV IE62 acts primarily by a mechanism that does not involve or require upstream targeting sites or factors and that it is unusually sensitive to the nature of the specific TATA motifs present. Such a mechanism differs significantly from the strong and relatively specific requirements for upstream targeting by other well studied viral and eukaryotic transactivators. For example, HSV VP16, Ad2 E1A, and EBV Zta interact in vitro with TBP and TFIIIB (and TAFs) through their activation domains, whereas VZV IE62 interacts with TBP and TFIIIB through a region(s) that is distinct from its activation domain. VP16 and E1A principally target upstream sites through indirect protein: protein interactions with Oct-1, and Rb:E2F or ATF-2, respectively, whereas Zta binds directly to upstream ZRE motifs and acts as a bridge between these sites and TFIID, resulting in TFIIA-dependent stabilization of TBP binding to weak TATA motifs such as GATAAA (55, 48). The HCMV IE1/IE2 transcription activators are also clearly different from IE62 in that they target promoters lacking TATA motifs. Both HCMV IE2 and HSV ICP4 also interact with TBP and TFIIIB in vitro through regions that are distinct from their activation domains (22, 49). Although ICP4 represses upstream activator-mediated transcription including that of Sp-1, presumably through formation of a tripartite complex consisting of TBP, TFIIIB, and ICP4 itself, the activation induced by ICP4 correlates with its interaction with TFIIID perhaps via TAF250 requiring the C terminus of ICP4 (49–51). IE62 also differs from ICP4 in possessing a powerful N-terminal activator domain that functions effectively in a GAL4 fusion protein in comparison to a weak, promoter-specific modular activation domain recently reported in ICP4 (19, 52). In addition, our previous work (13) showing
that the IE62 promoter positively responds to both ICP4 and IE62 indicates that the promoter elements required for activation by VZV IE62 and HSV ICP4 are different, a finding that was confirmed in the present study by the absence of activation of pIFNTATA by ICP4 (Fig. 1). While the TATA box is dispensable for ICP4-mediated activation of promoters, it does appear to influence the magnitude of activation such that it activates promoters with TATA boxes with low affinity for TBP more efficiently than those with high affinity for TBP (43). The related pseudorabies virus IE180 protein also has been shown to activate suboptimally utilized promoters and contains an N-terminal activator domain (53), but VZV IE62 differs from it in that the TATA motif alone is a sufficient cis-acting element to mediate transactivation by IE62, but not by IE180 (54).

Simon et al. (44) showed that, despite similar binding to TFIIID and in vitro functionality for the Ad MLP TATAAAA motif and the SV40 TATTATTAT motif, only the former responds to E1A transactivation. In this regard, VZV IE62 resembles E1A, except that a much broader pattern of TATA motifs are recognized (i.e. weak or non-responsive for TTTTTAA and TATTTAAAATT; moderately responsive for TATTTAA and TATTA ATT; highly responsive for TATTTAAA, TATTTATAA, and TATTTAATTA). The yeast GCN4 and GAL4 proteins (both acidic transactivators) also differ from IE62 in their ability to interact functionally with certain TATA motifs. In fact, this finding was originally used to invoke models for either two different TATA promoters or two types of acidic activator domains with different selectivities (55). All evidence at present however, points to there being only one universal TBP species.

X-ray crystallographic and biochemical analyses indicate that TBP binds directionally to DNA as a monomer, but it exists in unbound form in solution as a stable homodimer (56–58). Unlike most DNA-binding transcription factors, TBP binds DNA in the minor groove in a two-step process (60) with relatively low discrimination of specific base pairs. In so doing TBP bends and conformationally distorts the helix, while undergoing conformational changes itself (56, 60, 61). Dissociation of unbound dimers and of DNA-bound monomers of TBP is relatively slow processes compared with the rate of association of monomeric TBP with DNA (62). Stabilization of TBP binding to either TATA elements by TFIIA (and TFIIIB) (48, 63, 64), or to nonspecific DNA sequences by SP-1 and other upstream or Inr protein interactions in the case of TATA less
promoters, are thought to be key rate-limiting steps in the formation and processing of initiation complexes. Indeed, binding between TBP and TFIIA has been found recently to be an essential step in transactivation by yeast acidic activator protein domains (9). TFIIA binding also appears to alter the conformation of bound TBP.

Our EMSA results (Fig. 9) indicate that all of the TATA-like motifs studied here (both consensus and non-consensus alike) exhibit similar affinity for TBP. These results closely resemble those obtained by Hahn et al. (41) for purified yeast holo-TFIID on larger DNA fragments by DNase I footprinting of a number of consensus and non-consensus TATA motifs. It seems, therefore, unlikely that either TBP or TBP-TAF complexes directly discriminate between different types of TATA motifs examined in this study. The highly uniform levels of transactivation of both our GAL4-responsive and VP16-responsive promoter panels containing different TATA motifs also suggest that neither basal levels (as supported by CAT activity and RNase protection data) nor upstream-element-mediated activation are influenced by particular TATA motifs in the panel tested. However, it could also be argued that the TATA motifs themselves become irrelevant when multiple upstream response elements are present so as to offset TATA box requirement for transcription by compensatory effects of other transcription factors binding to these upstream elements.

Considering the inability of the TBP protein to discriminate significantly among the different TATA motif oligonucleotides used in our in vitro EMSA binding studies, it appears unlikely that TBP interactions with the TATA motif alone can directly account for the specificity of IE62 transactivation. Similarly, neither the GAL4 fusion proteins containing the IE62 N-terminal acidic activation domain, nor Oct-1/TATAGARAT-targeted intact VP16, could discriminate functionally between the different TATA motifs. It also appears unlikely that TATA specificity is attained directly through the properties of any particular subclass of general factors or acidic activator domains.

Instead, these properties reside within the core non-activator domain segment of IE62 itself as indicated by our domain-swap experiment (Fig. 6). Although IE62 is also a sequence-specific DNA-binding protein, it preferentially recognizes consensus motifs containing ATCGTC elements, and it neither bound to the TATATAA oligonucleotide probes by EMSA in our work nor protected TATA motif regions in previous footprinting analyses (65). The homologous HSV ICP4 protein has also been shown previously by EMSA and DNA footprinting assays to form a cooperative tripartite DNA-bound complex with TFIIB and TBP (or holo-TFIID), but only on a target DNA probe containing both a TATA motif and an ICP4 binding site (49). Therefore, we are left to conclude that a direct interaction between IE62 with TBP, most likely as a complex with TFIIA or TFIIB or with a specific TAF or co-activator (66), can recognize subtle conformational or stability differences (at either the DNA or protein level) that are imparted by particular base pair arrangements within the TATA motifs, and that these altered interactions manifest themselves as different levels of transactivation.

Emerging evidence from in vivo studies in yeast with yTAF145 implicate distinctions among different core promoters (67). Our work reinforces this notion that there is a level of discrimination between different types of TATA motifs (particularly for transactivation) that is not currently understood. The VZV IE62 protein is particularly intriguing in this regard, because the effect occurs in the absence of upstream targeting motifs, and certain non-consensus TATA motifs, particularly TATAAAA, are sufficient to mediate activation. In addition, the ability of VZV IE62 to bind to TBP in vitro through an additional internal non-activator domain suggests that it combines the properties expected of both a transactivator and an adapter or co-activating factor.
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