Multiple TAF_{II}s Directing Synergistic Activation of Transcription

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Coordinate activation of transcription by multiple enhancer binding factors is essential for the regulation of pattern formation during development of *Drosophila melanogaster*. Cell-free transcription reactions are described that recapitulate transcriptional synergism directed by the *Drosophila* developmental regulators Bicoid (BCD) and Hunchback (HB). Within the basal transcription factor complex TFIID, two specific targets, $TAF_{II}110$ and $TAF_{II}60$, served as coactivators to mediate transcriptional activation by these two enhancer binding proteins. A quadruple complex containing TATA binding protein (TBP), $TAF_{II}250$, $TAF_{II}110$, and $TAF_{II}60$ mediated transcriptional synergism by BCD and HB, whereas triple TBP-TAF_{II} complexes lacking one or the other target coactivator failed to support synergistic activation. Deoxyribonuclease I footprint protection experiments revealed that an integral step leading to transcriptional synergism involves the recruitment of TBP-TAF_{II} complexes to the promoter by way of multivalent contacts between activators and selected TAF_{II}s. Thus, the concerted action of multiple regulators with different coactivators helps to establish the pattern and level of segmentation gene transcription during *Drosophila* development.

The segmented body pattern of *Drosophila melanogaster* is established by a hierarchic network of maternal and zygotic segmentation gene activities that progressively subdivide the embryo into its final metameric pattern. Genetic and molecular studies have revealed that the temporally and spatially restricted patterns of segmentation gene transcription are controlled by a cascade of transcription factors (*1*). However, the underlying molecular mechanisms that control the transcription of segmentation genes remain unclear. Although the gene regulatory factors are present in rather diffuse and overlapping concentration gradients along the axis of the embryo, spatially restricted patterns of transcription domains with sharp boundaries are generated (*2*). How can diffuse overlapping concentration gradients of transcription factors generate the complex yet precise mosaics of gene expression observed? One model postulates that the expression of segmentation genes is activated by transcription factors encoded by maternal genes and that the sharp borders of expression within segments become established as a result of repressors encoded by zygotic genes (*3*). However, the interplay of activators and repressors cannot fully explain how the levels and the initial expansion of segmentation gene transcription become established. Genetic and molecular analysis of *Drosophila* segmentation genes suggest instead that another key feature of mechanisms that dictate transcriptional levels and segmented patterns of expression involves synergistic activation of transcription by multiple transactivators (*4–6*). It is, therefore, of interest to determine how the coordinate interaction of multiple activators can specify and regulate transcription of segmentation genes.

When eukaryotic enhancer binding factors are tethered to the template DNA by their DNA binding domains, appropriately exposed activation domains contact one or more components of the basal transcriptional apparatus (*7*). Many sequence-specific activators interact directly with one or more subunits of the basal transcription factor IID (TFIID). TFIID is composed of the TATA binding protein (TBP) and eight or more TBP-associated factors (TAF_{II}s) (*8*). Different classes of activators (such as acidic, glutamine rich, isoleucine rich) contact distinct target coactivators within the TFIID complex to mediate transcriptional activation (*9–12*). It was, therefore, of interest to determine whether transcription factors responsible for *Drosophila* body pattern formation also require TAF_{II}s as coactivators. In particular, we hoped to discern the role (if any) of TAF_{II}s in the mechanisms of transcriptional amplification that allow multiple activators to switch gene expression from an off-state to a highly increased on-state.

To study the mechanisms of transcriptional synergy, we analyzed the relation between multiple enhancer binding proteins that regulate segmentation genes and putative coactivators required to mediate transcriptional activation by RNA polymerase II (Pol II). For these studies, we used the *Drosophila* gene *hunchback* (*hb*) (*13*). In the embryo, the transcription of zygotic *hb* is synergistically activated by the product of the maternal gene *bicoid*, a homeodomain protein (BCD), and of *hb* itself, a zinc finger protein (HB) (*14, 15*). This model system is ideal for in vitro studies because only two transactivators are necessary for the transcriptional synergism observed in vivo. All of the cis-regulatory sites required to program these two activators reside within a relatively short DNA sequence of 300 base pairs (bp) located proximal to the start site and TATA box of the *hb* core promoter (*15*).

Synergistic activation of the *hb* promoter by BCD and HB is TAF_{II}-dependent. For these in vitro transcription studies, we used a *Drosophila*-reconstituted transcription system composed of purified recombinant basal factors TFIIA, B, E, F, purified TFIIH, RNA Pol II, and either endogenous TFIID or recombinant TBP-TAF_{II} complexes (*16*). The template we used consisted of a plasmid containing the transcriptional control region (~300 bp) necessary and sufficient for proper *hb* expression in vivo. This control region contains one HB binding site intermingled with three BCD binding sites (Fig. 1A) (*15*). To assay activation, we used purified BCD and HB proteins that were produced in Sf9 cells infected with recombinant baculovirus expressing Flag epitope–tagged versions of these *Drosophila* activators (Fig. 1, B and C) (*17*). Primer extension analysis was used to measure in vitro transcription products in reconstituted reactions supplemented with either TBP or TFIID in the presence or absence of activators (Fig. 1, D and E) (*18*). Transcription reactions containing TBP failed to support activation by either BCD or HB proteins (Fig. 1D). By contrast, reactions containing endogenous TFIID supported a 5- to 10-fold activation by both BCD and HB activators individually (Fig. 1D). Control templates without HB and BCD binding sites failed to respond to either activator protein (*19*). Thus, HB and BCD, like most other tested eukaryotic activators, require one or more of the TAF_{II} subunits in TFIID to mediate transcriptional activation.

We tested whether this in vitro system could recapitulate the transcriptional synergism between BCD and HB that has been observed in vivo (*14*). Because we were interested in the contribution of activator-coactivator interactions to transcriptional synergism, the reactions were carried out with near-saturating levels of activators in order to minimize any potential cooperative DNA binding interactions between BCD and HB at the promoter. Under these conditions, either HB or BCD alone gave rise to a six- to sevenfold level of activation

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(Fig. 1E). However, in the presence of both activator proteins, we observed a large enhancement of transcription (greater than 65-fold) when TFIID was used in the reconstituted reactions (Fig. 1E). When TBP was used instead of TFIID, no activation or synergism was observed. Thus, both simple activation as well as synergistic activation by BCD and HB is TAF_{II}-dependent.

HB and BCD target different TAF_{II}s in the TFIID complex. Different classes of activators recognize and interact selectively with distinct TAF_{II}s to mediate transcriptional activation (*8–12*). We wanted to determine which TAF_{II}s were targets for BCD and HB. The activation domain of HB is not fully characterized, but on the basis of the primary sequence it may be located between the NH_2- and COOH-terminal zinc finger DNA binding domains of the protein (*13*). BCD most likely contains multiple activation domains; these include a glutamine-rich region that is necessary for transcriptional activity in the embryo and also a COOH-terminal acidic domain that is active in yeast but dispensable in *Drosophila* (*20*). BCD also contains a third putative activation domain that is alanine rich and is located between the glutamine-rich domain and the acidic region (*21*). For the remainder of the experiments, we used full-length HB protein in combination with a truncated version of BCD (BCD-Q) that contains only the glutamine-rich activation domain (*22*). Direct protein-protein binding assays were used to identify which TAF_{II}s in the TFIID complex bind selectively to the activation domains of HB and BCD-Q.

Epitope-tagged BCD-Q and HB were immunopurified and the resulting protein affinity resins were incubated with ^{35}S-methionine–labeled reticulocyte lysate–expressed proteins (*23*). Affinity beads saturated with purified BCD-Q selectively bound to $dTAF_{II}110$ but not to $TAF_{II}60$, $TAF_{II}80$, $TAF_{II}250$, or TBP (Fig. 2A). Affinity resin containing HB protein bound specifically to $dTAF_{II}60$ but not to $TAF_{II}110$, $TAF_{II}80$, $TAF_{II}250$, or TBP (Fig. 2B). Control affinity beads without either BCD or HB protein failed to retain any of the tested proteins (Fig. 2B). Several other TAF_{II}s were also tested but did not bind to either BCD-Q or HB (*19*). Thus, HB must contain one or more activation domains that can recognize and interact selectively with $TAF_{II}60$. Consistent with other glutamine-rich activators, the glutamine-rich domain of BCD interacts selectively with $dTAF_{II}110$ (*10*, *19*, *24*). Thus, BCD-Q and HB can target distinct components of the TFIID complex and therefore may contribute to multivalent interactions that function in synergistic activation.

A complex containing both $dTAF_{II}60$ and $dTAF_{II}110$ is required to mediate BCD-Q– and HB-dependent synergistic activation. We used the in vitro assembly of partial TFIID complexes (*11*) to test which specific activator-TAF_{II} interactions were required to mediate transcriptional synergism. Four distinct TBP-TAF_{II} complexes were assembled (Fig. 3A) (*25*) and tested for their ability to support transcriptional activation and synergism (*26*). Reconstituted transcription reactions supplemented with the TBP, $TAF_{II}250$, and $TAF_{II}60$ complex supported activation by HB but not BCD-Q (Fig. 3, B and C). The triple complex of

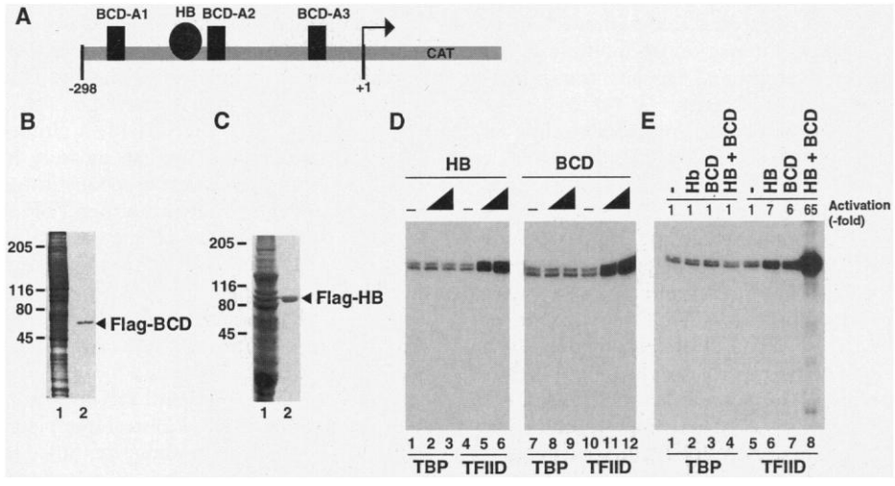

Fig. 1. TFIID is required for BCD- and HB-dependent synergistic activation. (**A**) Schematic representation of the *hb* enhancer-promoter reporter plasmid used in the in vitro transcription experiments. The gray rectangle represents the *hb* enhancer-promoter which is fused at position +115 to the chloramphenicol acetyltransferase (CAT) reporter gene (*15*). Black rectangles indicate the position of three high-affinity BCD binding sites, and the circle the position of the HB binding site. (**B** and **C**) Purification of BCD and HB. Recombinant Flag epitope–tagged BCD or HB was expressed in Sf9 cells, purified from cell extracts with Flag M2 antibody resin, and subsequently eluted from the beads with peptides mimicking the Flag epitope. Aliquots of the cell extracts before purification (lane 1) and of the eluted proteins (lane 2) were separated by SDS-PAGE, and proteins were detected by staining with Coomassie blue. The position and molecular sizes (in kilodaltons) of protein standards are indicated on the left. (**D**) BCD and HB require TFIID in order to activate transcription. Autoradiogram of in vitro transcription reactions with the reconstituted *Drosophila* transcription system containing either dTBP (lanes 1 to 3 and 7 to 9) or endogenous TFIID (lanes 4 to 6 and 10 to 12). Transcription from the *hb* enhancer-promoter reporter plasmid was assayed by primer extension in the absence (lanes 1 and 7) or presence of HB (lanes 2, 3, 5, and 6; lanes 2 and 5, 5 ng; lanes 3 and 6, 25 ng) or BCD (lanes 8, 9, 11, and 12; lane 8 and 11, 2.5 ng; lanes 9 and 12, 12.5 ng. (**E**) HB and BCD synergistically activate transcription. In vitro transcription experiments are shown with the same transcription system as in (D) containing either no activator (lanes 1 and 5), HB (lanes 2 and 6, 5 ng), BCD (lanes 3 and 7, 2.5 ng), or both transactivators (lanes 4 and 8, 5 ng HB + 2.5 ng BCD) in the presence of either TBP (lanes 1 to 4) or endogenous TFIID (lanes 5 to 8).

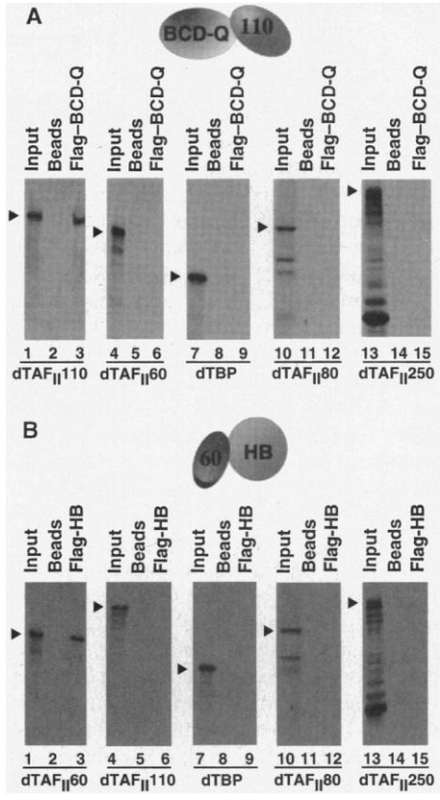

Fig. 2. BCD-Q interacts with $TAF_{II}110$, HB with $TAF_{II}60$. (**A** and **B**) Anti-Flag M2 antibody resin (A and B: lanes 2, 5, 8, 11, and 14) or beads loaded with either Flag–BCD-Q (A: lanes 3, 6, 9, 12, and 15) or Flag-HB (B: lanes 3, 6, 9, 12, and 15) were incubated with the ^{35}S-labeled reticulocyte lysate–expressed TAF_{II} indicated at the bottom of each panel. Protein complexes were washed, separated on SDS-PAGE, and bound TAF_{II}s were detected by autoradiography. (A and B) Lanes 1, 4, 7, 10, and 13 represent 25% of the starting material used in the binding reactions. The results of these experiments are illustrated above each panel.

TBP, $TAF_{II}250$, and $TAF_{II}110$ mediated transcription by BCD-Q but not HB. The quadruple complex of TBP, $TAF_{II}250$, $TAF_{II}110$, and $TAF_{II}60$ mediated activation by both BCD-Q and HB individually (Fig. 3, B and C), whereas a complex of TBP and $TAF_{II}250$ failed to support activation by either activator (*19*). These results confirm that transcriptional activation by HB requires the coactivator $TAF_{II}60$, whereas activation by BCD-Q requires the coactivator $dTAF_{II}110$.

Next, we tested whether any of the partial complexes could support synergistic activation by the combined action of BCD-Q and HB on the *hb* promoter template. We used two different concentrations of activators to assess the extent of synergistic activation in vitro (Fig. 3, B and C). Neither of the two trimer TBP-TAF_{II} complexes supported synergistic activation with either low or high concentrations of BCD-Q and HB protein (Fig. 3, B and C). In contrast, the complex containing TBP, $TAF_{II}250$, $TAF_{II}110$, and $TAF_{II}60$ supported a large enhancement of transcription in the presence of both activators (Fig. 3, B and C). Whereas a single activator gave rise to approximately 3- to 4-fold activation at the low concentration of activator protein, the presence of both activators at the same low concentrations resulted in a 53-fold activation above basal (Fig. 3B). Thus, the presence of BCD-Q and HB in a reconstituted reaction containing a complex of $TAF_{II}110$ and $TAF_{II}60$ gave rise to a level of transcription that was greater than the product of the transcription activity observed with each activator alone. With near-saturating levels of activator proteins, we observed an 11- to 12-fold activation with either activator alone, whereas the two proteins together gave approximately 113-fold enhancement of transcription (Fig. 3C). Thus, even with high concentrations of the individual activators, the combined action of these two proteins direct a level of transcriptional activation that is well beyond additive and very nearly multiplicative. Thus, the transcriptional synergism resulting from the coordinate action of HB and BCD-Q is dependent on the presence of multiple TAF_{II}s that can serve as targets within the TFIID complex. Truncated versions of BCD and HB, containing a DNA binding domain but lacking activation domains, were unable to support either simple activation or synergistic activation, confirming that the presence of activation domains in both BCD-Q and HB is required for transcriptional synergism (*19*). In transcription reactions with the two different triple complexes mixed, no transcriptional synergism was observed (*19*), suggesting that $TAF_{II}60$ and $TAF_{II}110$ must be present in the same TBP-TAF_{II} complex in order to mediate synergistic activation of transcription.

Thus far we have limited our analysis to a single endogenous template, the *hb* enhancer-promoter region. To assess the potential contribution of promoter architecture to transcriptional synergism, we also tested two additional templates containing enhancer elements bearing only one BCD and one HB binding site derived either from the *even skipped*–stripe 2 enhancer or from the *hb* enhancer (*27*). Like the *hb* enhancer these two minimal enhancers supported a high level of synergistic activation only in the presence of both BCD-Q and HB and the quadruple complex containing both $TAF_{II}110$ and $TAF_{II}60$, whereas simple activation was observed when either of the two triple TBP-TAF_{II} complexes was used (*19*). These findings confirm that BCD and HB can activate transcription synergistically by way of specific contact with $TAF_{II}110$ and $TAF_{II}60$, respectively, in the context of different enhancer arrangements.

TAF_{II}s direct activator-dependent recruitment of TFIID to the promoter. Although the in vitro transcription studies establish that multiple activator-TAF_{II} contacts function in mediating transcriptional activation and synergism, they cannot decipher the likely mechanism by which specific protein-protein interactions govern different steps during the transcription cycle. We hoped to address the role of activator-coactivator interactions in recruiting TFIID to the promoter. For these studies, we performed deoxyribonuclease I (DNase I) footprint protection experiments with a radiolabeled enhancer fragment derived from the *hb* control region (residues −231 to +115) together with purified HB and BCD-Q protein in the presence of various partial TBP-TAF_{II} complexes (*28*). Our DNA binding studies were carried out with saturating amounts of BCD-Q and HB such that each activator alone could achieve greater than 90% occupancy of their cognate binding sites (Fig. 4). Consistent with previous reports, HB protein recognized and bound to a single site (−175 to −200) within the *hb* enhancer region (Fig. 4, B and C), whereas BCD-Q bound to two independent sites, A3 (residues −70 to −60; Fig. 4, D and E) and A2 (−160 to −170; Fig. 4, D and E) (*15*). We also carried out DNase I footprint protection assays in the presence of both proteins to determine the extent, if any, of cooperative DNA binding between BCD and HB. Our results revealed no detectable cooperativity in the binding of these proteins to the multiple DNA binding sites within the *hb* promoter (*19*).

We tested increasing concentrations of various TBP-TAF_{II} complexes for binding to the TATA region. We expected to see a somewhat extended footprint encompassing the TATA box (Fig. 4A) because we used TBP-TAF_{II} complexes rather than TBP alone (*29*). For detection of a protected region overlapping the TATA box without an activator, a very high concentration (160 nM) of TBP-TAF_{II} complexes was required (Fig. 4A). However, in the presence of activators, binding of TBP-TAF_{II} complexes to the promoter DNA was ob-

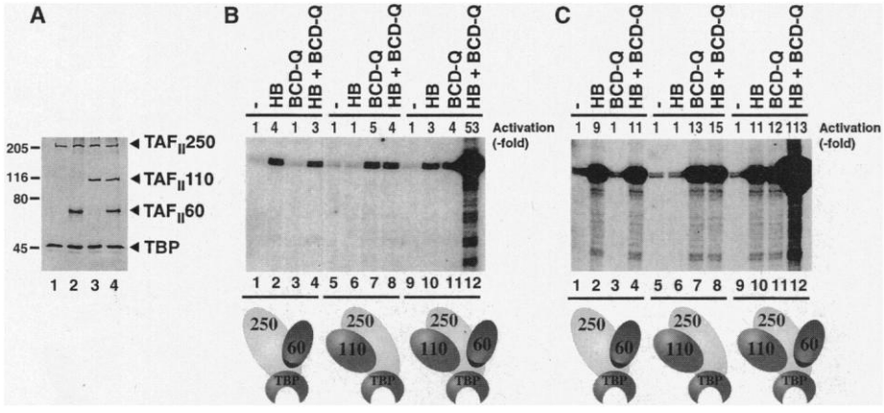

Fig. 3. Partial TFIID complexes containing dTBP, $TAF_{II}250$, $TAF_{II}60$, and $TAF_{II}110$ support BCD-Q– and HB-dependent synergistic activation. (**A**) Silver-stained gel of in vitro assembled partial TFIID complexes containing dTBP and $TAF_{II}250$ (lane 1); TBP, $TAF_{II}250$, and $TAF_{II}60$ (lane 2); TBP, $TAF_{II}250$, and $TAF_{II}110$ (lane 3); or TBP, $TAF_{II}250$, $TAF_{II}60$, and $TAF_{II}110$ (lane 4). Aliquots of the assembled complexes were separated by SDS-PAGE and visualized by silver staining. The position and molecular sizes (in kilodaltons) of marker proteins are indicated on the left. (**B**) HB and BCD-Q require $TAF_{II}60$ and $TAF_{II}110$ in order to activate transcription. Four nanomolar of the assembled partial TFIID complexes TBP-$TAF_{II}250$-$TAF_{II}60$ (lanes 1 to 4), TBP-$TAF_{II}250$-$TAF_{II}110$ (lanes 5 to 8), and the quadruple complex TBP-$TAF_{II}250$-$TAF_{II}60$-$TAF_{II}110$ (lanes 9 to 12) were tested in the reconstituted *Drosophila* transcription system lacking endogenous TFIID. Transcription was analyzed by primer extension in the absence of activators (lanes 1, 5, and 9) or in the presence of 2 ng of HB (lanes 2, 6, and 10) or 1 ng of BCD-Q (lanes 3, 7, and 11) or 2 ng of HB and BCD-Q (lanes 4, 8, and 12). (**C**) The same as in (B) except that 10 ng of HB (lanes 2, 4, 6, 8, 10, and 12) and 5 ng of BCD-Q (lanes 3, 4, 7, 8, 11, and 12) were used.

served at much lower concentrations. For example, in the presence of HB, an extended TATA box footprint was observed in the presence of the triple complex of TBP, $TAF_{II}250$, and $TAF_{II}60$ (at ~12 nM) (Fig. 4B), yet no protection over the TATA box was observed when the TBP, $TAF_{II}250$, and $TAF_{II}110$ complex was used (Fig. 4C). In the presence of BCD-Q, we observed a TATA region footprint with the triple complex TBP, $TAF_{II}250$, and $TAF_{II}110$ (Fig. 4D) but not with the complex of TBP, $TAF_{II}250$, and $TAF_{II}60$ (Fig. 4E). The concentration of TBP-TAF_{II} complexes necessary to detect TATA box binding in the presence of saturating levels of BCD-Q was somewhat lower than in the presence of HB, presumably because there are two BCD DNA binding sites but only one HB DNA site present on the template. These results are consistent with our in vitro transcription findings and confirm that recruitment of TFIID that leads to transcriptional activation is dependent on specific activator-coactivator interactions.

We tested the quadruple TBP-TAF_{II} complex containing both $TAF_{II}110$ and $TAF_{II}60$. Binding of this complex to the TATA box was enhanced substantially when either HB or BCD occupied their respective binding sites on the template (Fig. 5, A and B). However, with both activators, the concentration of the quadruple complex required to produce a distinct footprint was ~50-fold lower (80 pM) than the concentration needed (4 nM) to bind DNA in the presence of a single activator (Fig. 5C). Moreover, this enhanced ability of BCD-Q and HB to recruit TFIID to the promoter was not observed when either of the two triple TBP-TAF_{II} complexes was used (Fig. 5, D and E). Instead, we observed TATA region protection only at the highest concentration of TBP-TAF_{II} complexes (Fig. 5D) in a manner similar to reactions containing only one or the other activator alone (Fig. 5, A and B). Moreover, the recruitment of TBP-TAF complexes by HB and BCD-Q, like transcriptional activation, depends on the activation domains because truncated versions of these proteins containing only the DNA binding domain failed to recruit TBP-TAF complexes to the template DNA (*19*). These results suggest that the same activator-TAF_{II} interactions required for transcriptional synergism also mediate an enhanced recruitment of TBP-TAF_{II} complexes to the promoter DNA. Therefore, it seems likely that synergistic activation requires the targets of independent activators to be assembled into a complex containing multivalent contacts.

In vivo analysis of transcriptional events that control *Drosophila* development have underscored the importance of synergistic activation directed by the interplay of multiple enhancer binding proteins in the embryo (*4–6*, *14*). One of the clearest examples of coordinate activation by two distinct enhancer binding factors working in concert is the pattern of *hb* expression directed by the maternal factor, BCD, and the HB protein itself (*14*). One simple and widely accepted model postulates that the binding of BCD and HB to their cognate recognition sites within the *hb* enhancer may be cooperative. For example, the binding of BCD could facilitate the binding of HB to the template, or vice versa. Many examples of synergistic transcriptional activation revealed by in vivo studies with transgenic flies have been interpreted to result from cooperative DNA binding (*4–6*, *14*, *15*, *30*). However, we were unable to obtain any evidence that HB and BCD proteins interact with each other to direct cooperative binding at the promotor (*19*). Thus, it was important to determine whether other mechanisms accounted for the observed synergism of transcription by these two activators.

An alternative mechanism would be multiple enhancer factors that bound independently to their DNA recognition sites to interact simultaneously with multiple components of the basal transcription machinery and efficiently mediate cooperative binding by way of activator-coactivator interactions. In this model, multiple enhancer binding factors tethered to the DNA induce synergistic activation by enhancing the recruitment of essential basal factors to the template. To test such a protein-protein–driven mechanism, we have recapitulated and dissected the requirements for synergistic activation by BCD and HB in vitro using a purified transcription system. Our results establish that a primary mechanism for synergistic transcriptional activation of the wild-type *hb* promoter-enhancer template involves specific binding between activation domains of BCD and HB with select coactivator targets in the TFIID complex. In vitro transcription reactions in combination with protein binding assays

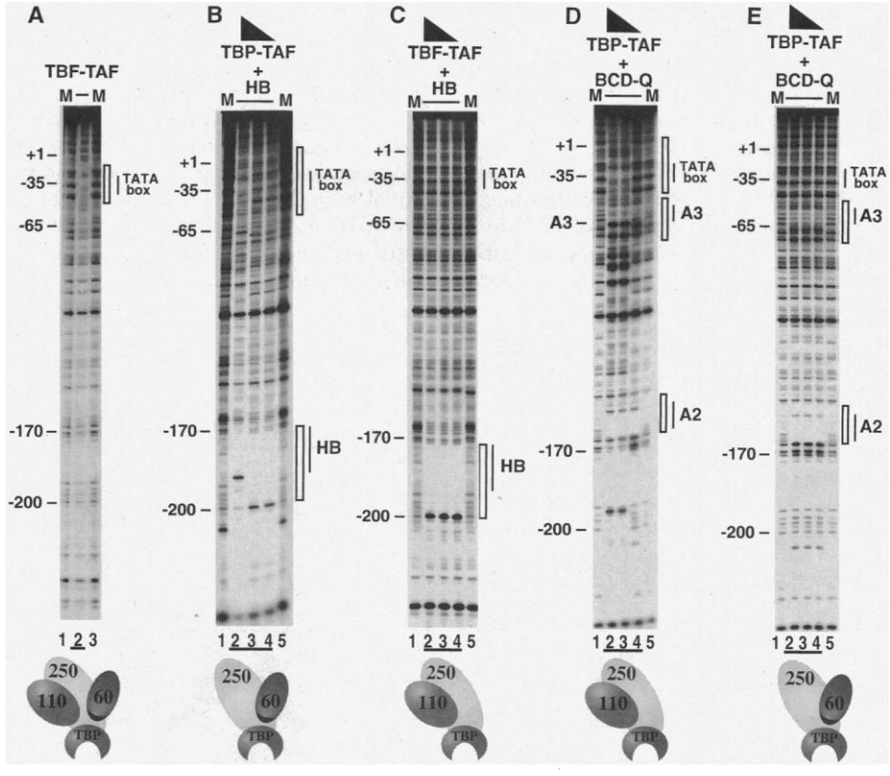

Fig. 4. Specific activator-coactivator interactions mediate recruitment of TBP-TAF_{II} complexes to the *hb*-promoter. (A to E) DNase I footprint experiments with 0.2 fmol of a template from the *hb*-control region (−231 to +115) radiolabeled on the transcribed strand, constant amounts of HB or BCD-Q, and variable amounts of the indicated assembled TBP-TAF_{II} complexes. (**A**) DNase I footprinting of 1.5 μg of the quadruple TBP-$TAF_{II}250$-$TAF_{II}60$-$TAF_{II}110$ complex in the absence of activators (lane 2). (**B**) DNase I digest with 5 ng of HB (lanes 2 to 4) in the presence of 14 nM (lane 2), 4 nM (lane 3), or 1.3 nM (lane 4) of the triple TBP-$TAF_{II}250$-$TAF_{II}60$ complex. (**C**) Same as (B) except that 14 nM (lane 2), 4 nM (lane 3), or 0.8 nM (lane 4) of the triple complex containing $TAF_{II}110$ were used. (**D**) Same as (C) except that 2.5 ng of BCD-Q (lanes 2 to 4) were used instead of HB. A2 and A3 indicate the position of BCD-Q binding sites. (**E**) Same as (B) except that 2.5 ng of BCD-Q (lanes 2 to 4) were used. M, DNase I digestions carried out in the absence of any protein (A, lanes 1 and 3; B to D, lanes 1 and 5). Rectangles, DNase I–protected regions; bars, consensus binding sites.

and DNase I footprint studies reveal that synergistic activation requires simultaneous interactions of BCD and HB with $TAF_{II}110$ and $TAF_{II}60$, respectively (Fig. 6). Transcription reactions with only one activator lead to simple activation but no synergism. Likewise, transcription reactions reconstituted with TBP-TAF_{II} complexes lacking either $TAF_{II}110$ or $TAF_{II}60$ result in simple activation but no synergism. However, when both target coactivators interact simultaneously with the two activators tethered to DNA, an enhanced synergistic level of transcription is induced. Thus, it appears that the multivalent nature of the TFIID complex containing numerous potential interfaces for contact by different activation domains represents an important feature with the potential to mediate synergistic activation of transcription.

A key aspect of the transcriptional synergy appears to be the active and efficient recruitment of TBP-TAF_{II} complexes to the promoter by virtue of multiple contacts between activators and TAF_{II}s. We expected that efficient recruitment of TFIID may be a regulated step during the transcription cycle. In the case of BCD and HB, DNase I footprint experiments establish that when both activators are present on the template, binding of TFIID to the TATA box occurs even at very low concentrations, whereas when only one activator is present much higher concentrations of TFIID are required to occupy the TATA box region. Thus, the transcriptional synergism observed during in vitro transcription reactions can be accounted for largely by the markedly enhanced ability of HB and BCD to act coordinately to recruit TFIID by way of multiple contacts with individual TAF_{II}s tightly associated with TFIID. Given the cooperative nature of the interactions between TAF_{II}s and activators observed in our experiments, we expect that the binding of TFIID to TATA box elements of the promoters could reciprocally influence the binding of activators to enhancer elements. Thus, the transcriptional synergism we observe is a more elaborate version of cooperative DNA binding between transcription factors each able to contact DNA as well as one another to form a stable initiation complex.

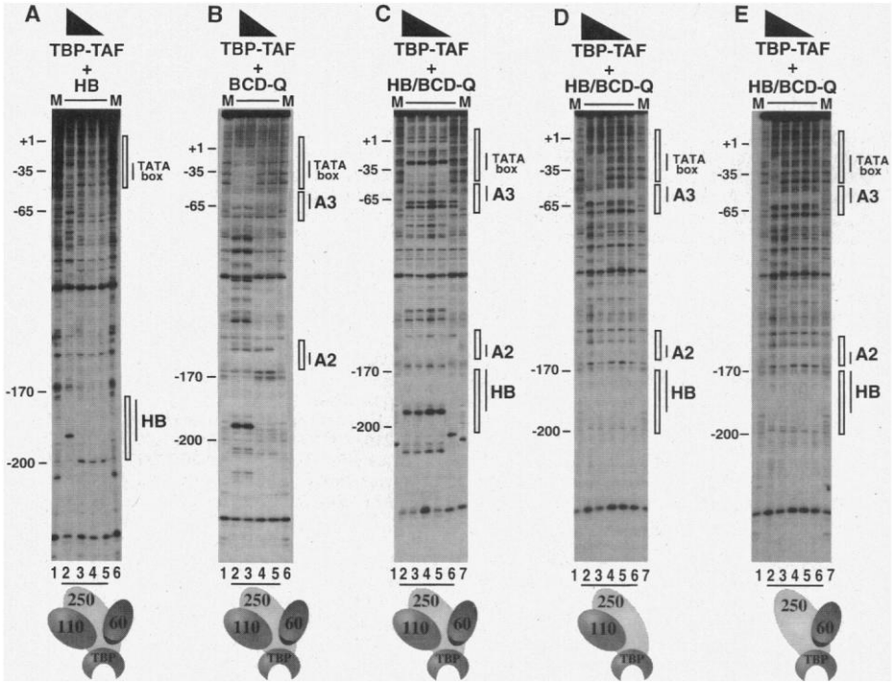

Fig. 5. Enhanced binding of a quadruple TBP-TAF_{II} complex containing $TAF_{II}60$ and $TAF_{II}110$ to the *hb*-promotor in the presence of both HB and BCD-Q. (A to C) DNase I footprint experiments with 0.2 fmol of the *hb*-control region (Fig. 4) and 14 nM (lane 2), 4 nM (lane 3), 0.8 nM (lane 4), 0.08 nM (lane 5), or 0.008 nM (C, lane 6) of the quadruple TBP-$TAF_{II}250$-$TAF_{II}60$-$TAF_{II}110$ complex. DNase I digestion in the presence of (**A**) 1 ng of HB (lanes 2 to 5), (**B**) 2 ng of BCD-Q (lanes 2 to 5), and (**C**) 1 ng of BCD-Q and 2 ng of HB (lanes 2 to 6). (**D**) DNase I footprint with 14 nM (lane 2), 4 nM (lane 3), 0.8 nM (lane 4), 0.08 nM (lane 5), or 0.008 nM (lane 6) of the triple TBP-$TAF_{II}250$-$TAF_{II}110$ complex in the presence of 1 ng of BCD-Q and 2 ng of HB (lanes 2 to 6). (**E**) Same as in (D) except that equal amounts of the triple TBP-$TAF_{II}250$-$TAF_{II}60$ complex were used. M, lanes representing DNase I digestions in the absence of protein. Rectangles and bars, DNase I–protected regions and consensus binding sites, respectively.

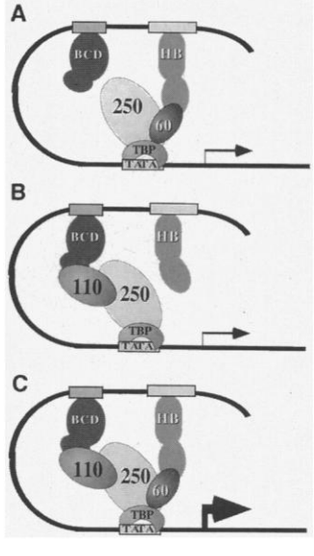

Fig. 6. Model for synergistic activation of transcription mediated by HB and BCD. (**A**) HB bound to its DNA binding site interacts with $TAF_{II}60$ in the triple complex containing TBP, $TAF_{II}250$, and $TAF_{II}60$ and supports transcriptional activation. The simultaneous presence of BCD on the DNA does not enhance the level of transcription (indicated by the arrow) because the TBP-TAF_{II} complex lacks the coactivator for BCD. (**B**) A triple complex containing TBP, $TAF_{II}250$, and $TAF_{II}110$ supports activation by BCD, but is unable to communicate with HB. (**C**) The simultaneous interaction of BCD and HB with $TAF_{II}60$ and $TAF_{II}110$ in the quadruple TBP-TAF_{II} complex supports a high level of synergistic activation of transcription.

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17. Expression plasmids encoding Flag epitope–tagged BCD or HB were generated by fusing a 1.8-kb Nde I–Bam HI complementary DNA (cDNA) fragment encoding full-length BCD or a 2.9-kb Nde I–Eco RI cDNA fragment encoding full-length HB derived from pARBCD or pARHB [T. Hoey and M. Levine, *Nature* **332**, 858 (1988); J. Treisman and C. Desplan, *ibid.* **341**, 335 (1989)] into the corresponding restriction sites of the baculovirus expression vector pSLFlag [S. Lichtsteiner and R. Tjian, *EMBO J.* **14**, 3937 (1995)], thereby fusing the cDNAs to the COOH-terminal end of the Flag epitope coding region. For generating recombinant baculovirus the resulting baculovirus expression plasmids pSLFlag-BCD or pSLFlag-HB were cotransfected with BaculoGold viral DNA (Pharmingen) into Sf9 cells. The resulting recombinant virus were plaque purified, amplified, and used for protein expression in Sf9 cells. Forty-eight hours after infection cell extracts were prepared by sonicating the cells in 0.4 M HEMG-NADM [0.4 M NaCl, 25 mM Hepes (pH 7.6), 0.1 mM EDTA, 6.25 mM $MgCl_2$, 15% glycerol, 1% NP-40, 1 mM dithiothreitol (DTT), 0.2 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride, and 5 mM sodium metabisulfide]. Extracts were centrifuged at 100,000*g*, incubated with Flag M2 antibody resin (Eastman Kodak, New Haven, CT) at 4°C for at least 4 hours. The protein resin was washed extensively with 1 M HEMG-NADM + 1% CHAPS. Proteins were released from the Flag M2 antibody resin by incubating the protein resin with peptides containing the Flag epitope (2 mg/ml in 0.4 M HEMG-NADM) for 30 min at 30°C. The eluted proteins were dialyzed against HEMG-DA containing 0.1 M KCl.
18. For in vitro transcription reactions we used a reconstituted *Drosophila* transcription system (*11*) composed of 0.5 ng of dTFIIA, 2.5 ng of dTFIIB, 0.2 ng of hTFIIE34, 0.75 ng of hTFIIE56, 10 ng of dTFIIF of recombinant basal factors, and ~10 ng of purified dTFIIH and 10 ng of purified dRNA Pol II were used in combination with either 1 ng of recombinant dTBP or ~25 ng of endogenous TFIID (S-300 fraction). Transcription was assayed by primer extension [J. W. Lillie and M. R. Green, *Nature* **338**, 39 (1989)] from reactions containing 25 ng of the reporter plasmid phbCAT-298 (*15*). Transcription reactions were assembled at 4°C in the following order: Template and activator were preincubated for 10 min and then basal factors were added. These mixtures were preincubated at 20°C for 15 min and transcription was initiated by adding nucleoside triphosphates to a final concentration of 2 mM. In vitro transcription reactions proceeded for 25 min at 20°C and reaction products (191 nucleotides) were detected by primer extension, visualized by autoradiography, and quantified by scintillation fluorography.
19. F. Sauer and R. Tjian, unpublished results.
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22. A BCD-Q baculovirus expression plasmid was generated by inserting a 0.91-kb Hin PI cDNA fragment encoding amino acids 2 to 305 into the baculovirus expression plasmid pSLFlag (*12*). BCD-Q was expressed in Sf9 cells and purified as described (*12*).
23. Protein-protein interaction assays were done as described (*12*). Briefly, 50 ng of Flag–BCD-Q or Flag-HB were immobilized on 2 μl of Flag M2 antibody resin (Eastman Kodak) and incubated in 0.1 HEMG-NADM with ^{35}S-methionine–labeled proteins produced with the TNT-coupled in vitro transcription-translation system (Promega) for 3 hours at 20°C. The resin was washed extensively with 0.4 M HEMG-NADM, resuspended in SDS sample buffer, and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE).
24. G. Cutler and R. Tjian, unpublished results.
25. Assembly of partial TBP-TAF_{II} complexes was done essentially as described (*11*) with the following modifications. Briefly, Sf9 cell extracts containing hemaglutinin (HA)–tagged $dTAF_{II}250$ were prepared in 0.4 M HEMG-NADM and loaded onto HA-antibody resin. Protein-resin was washed extensively with 1 M HEMG-NADM + 1% CHAPS until $dTAF_{II}250$ represented greater than 90% of the bound proteins judged by silver staining. This protein resin served as the basis for the complex assembly. The other components were added in the order of recombinant dTBP, $TAF_{II}110$, and $TAF_{II}60$ with extensive washes with 0.25 M HEMG-NADM + 1% CHAPS between each step. $dTAF_{II}60$ was expressed as His-tagged version in *Escherichia coli* and purified by Ni–ion affinity chromatography.
26. In vitro transcriptions with recombinant partial TBP-TAF_{II} complexes were done as described (*18*) except that the partial TBP-TAF_{II} complexes were preincubated with the activator and template at 4°C for 5 min before the remaining basal factors were added.
27. The reporter plasmids pBlue*eve*-HBCAT and pBlue*hb*-HBCAT were generated by cloning synthetic oligonucleotides derived from the *eve* stripe 2 enhancer element (−1110 to −1075) (*6*) or from the *hb* enhancer (−205 to −155) (*10*) flanked by a Hind III (5′ end) and a Bal I (3′ end) restriction site into the corresponding restriction sites of pBlue-HBCAT (*19*). The number of cloned inserts was verified by conventional dideoxy sequencing.
28. DNase I footprinting was done as described [T. Hoey, B. D. Dynlacht, M. G. Peterson, B. F. Pugh, R. Tjian, *Cell* **61**, 1179 (1990)]. For generating the template, a 269-bp Hin PI–Bam HI *hb* enhancer-promoter fragment (−154 to +115) derived from the plasmid phbCAT-231 (*15*) was fused to a synthetic oligonucleotide encompassing the *hb* enhancer from position (−231 to −154) in which two low-affinity BCD DNA binding sites were eliminated by base pair exchanges at position −220 and −210. The generated enhancer (−231 to +115) was inserted into the restriction sites Hind III and Bam HI of pBluescript KS+ (Stratagene) to create pBlueHB. pBlueHB was linearized by Hind III digestion, dephosphorylated, and 4 μg of the linear plasmid were end-labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP, labeling the *hb* enhancer-promoter fragment on the coding transcribed strand. A 50-μl footprinting reaction generally was composed of 0.2 fmol of labeled fragment, 25 mM Hepes (pH 7.6), 100 NaCl, 1 mM $MgCl_2$, 15% glycerol, 100 μM $ZnSO_4$, 1 mM DTT, various amounts of activators, and partial TBP-TAF_{II} complexes. Template and activators were preincubated for 10 min at 20°C, then complexes were added and the mixture was incubated for an additional 10 min at 20°C. Digestion was initiated by adding 50 μl of 10 mM $MgCl_2$-$CaCl_2$ solution containing DNase I (5 μg/ml; Worthington) and proceeded for 20 s.
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