

Drosophila head segmentation factor Buttonhead interacts with the same TATA box-binding protein-associated factors and *in vivo* DNA targets as human Sp1 but executes a different biological program

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ABSTRACT The *Drosophila* gene *buttonhead* (*btd*) is required for the establishment of three embryonic head segments. It encodes a zinc-finger-type transcription factor expressed in the corresponding head segment anlagen in the blastoderm stage embryo. The DNA-binding properties of the *btd* protein (BTD) are indistinguishable from the human transcription factor Sp1. Furthermore, BTD and Sp1 are capable of activating transcription in transfected cultured cells through interaction with the same DNA target sites. Herein we show that BTD and Sp1 functionally interact with the same TATA box-binding protein-associated factors and support *in vitro* transcription activation through these contacts. Transgene expression of BTD results in the rescue of the head segments that fail to develop in *btd* mutant embryos, whereas Sp1 or Sp1 containing the zinc finger region of BTD rescues mandibular segment development. The results suggest that BTD contains functional domains other than an equivalent DNA-binding region and interaction sites of the TATA box-binding protein-associated factors, which are necessary to establish head segments that fail to develop in response to Sp1.

Head segments of the *Drosophila* embryo are generated in response to the overlapping activities of the segmentation genes *orthodenticle* (*otd*), *empty spiracles* (*ems*), and *buttonhead* (*btd*) (1). These genes are expressed in three overlapping head segment anlagen of the blastoderm embryo that fail to be established in the corresponding mutants (2–5). *btd* is required for antennal, intercalary, and mandibular segment formation (1). It encodes a transcription factor, BTD, with a modular design similar to the human transcription factor Sp1 (5). BTD is able to bind to the G + C-rich DNA target site of Sp1 and to activate transcription in an Sp1-like manner in *Drosophila* Schneider cells (5).

Transcriptional activation by human Sp1 involves the general RNA polymerase II transcription factor TFIID (6). TFIID is composed of the TATA box-binding protein (TBP) and a set of TBP-associated factors (TAFs) (for review, see refs. 7 and 8). TAFs provide interfaces for enhancer-bound transcription factors that contact the basal transcription apparatus and direct the activation of transcription. Sp1 selectively interacts with TAF_{II}110 (9), one of the eight TAFs of *Drosophila* that include TAF_{II}250, TAF_{II}150, TAF_{II}110, TAF_{II}80, TAF_{II}60, TAF_{II}40, TAF_{II}30 α , and TAF_{II}30 β (10). Partial TFIID complexes composed of TBP, TAF_{II}250, TAF_{II}150, and TAF_{II}110 are sufficient to support transcriptional activation *in vitro*, whereas partial complexes lacking TAF_{II}110 or mutant Sp1 lacking the interaction surface fail to activate transcription (11, 12). Furthermore, different transcription factors, such as

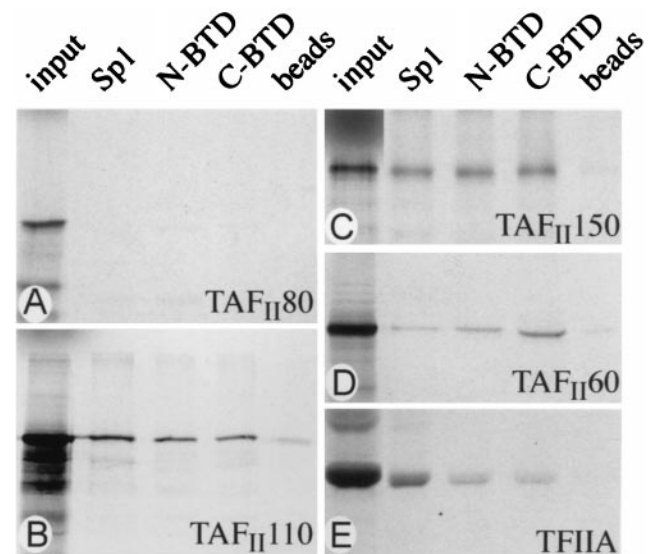


FIG. 1. Comparison of BTD and Sp1 *in vitro* interactions with TAFs. Flag-tagged BTD and Sp1 were expressed and purified from Sf9 cells and incubated with [³⁵S]methionine-labeled recombinant TFIIA or TAFs. An example of noninteracting TAFs, such as TAF_{II}80, is shown in *A*. C-terminal BTD (C-BTD), N-terminal BTD (N-BTD), and Sp1 interact strongly with TAF_{II}110 (*B*) and TAF_{II}150 (*C*) and weakly with TAF_{II}60 (*D*) and TFIIA (*E*). Input refers to 10% of the labeled TAFs that was used for the binding reaction. Beads refers to a control incubation of unreacted resin with labeled TAFs and TFIIA.

NTF-1, functionally interact with other TAFs (12). This suggested that the different subunits of TFIID, each with distinct structural characteristics, provide specific substrates for enhancer-bound transcriptional activators to contact the basal transcription apparatus and thereby direct the activation of gene expression.

Herein we show that BTD and Sp1 interact with the same TAF subunits and support *in vitro* transcription activation via these contacts. In addition, the DNA-binding regions of the two proteins are equivalent. However, transgene-dependent Sp1 expression in place of BTD rescues only some aspects of mandibular development in *btd* mutant embryos. The results suggest that the generation of the intercalary and the antennal segment requires functional interaction domains of BTD that are not present in Sp1.

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MATERIALS AND METHODS

Drosophila Strains and Mutant Embryos. The following fly strains were used in this work: Oregon R; *y w btd^{XG}/FM7c, fiz-lacZ; svb^{YP17b} btd^{XG}/FM7c* (5, 13, 14); *TAF_{II}110ΔC/TM6B* (15). Homozygous lines of the genomic *btd* (*P_{btd}*) and *Sp1* (*btd>Sp1*) rescue constructs (5) and the new constructs were also used. The *svb btd* double mutant allows the detection of *btd* mutant cuticles when crossing this strain to the transgene (13). The following strains were constructed: a fly strain homozygous for *btd>Sp1* on the second and third chromosome and *svb^{YP17b} btd^{XG}/FM7c; P_{btd}*. When crossing the latter flies to the *TAF* mutant, the *svb* locus allowed us to recognize *btd* mutant embryos that are rescued by one copy of *P_{btd}* and in 50% of the cases are also heterozygous for *TAF_{II}110ΔC*.

Generation and Analysis of the Rescue Construct. *Sp1^{BTDzf}* (*Sp1* with the *BTD* zinc finger region) consists of a 1.8-kb filled-in *Bam*HI fragment and a 670-bp filled-in *Bgl*II–*Hind*III fragment from pSp1–778c (16) that was ligated with a 300-bp filled-in *Ava*II–*Sfi*I fragment from pKSbtd (17). After addition of *Xba*I linkers, the fragment was cloned into pBluescript II KS+ (Stratagene). After checking the sequence, the construct was subcloned into the *P* element vector pCbtdRV-2ndBΔXba, which provides the 5.2-kb *btd* cis-acting element (17). To generate transgenic flies, constructs were injected into *w sn³* embryos by using pΔ2–3 (18) as helper (19). Transgenic progeny were balanced over CyO or TM3. At least three independent transgenic lines were analyzed.

Protein Binding and *in Vitro* Transcription Assay. The baculovirus transfer vector pSLFlag (20) was used to generate flag-tag fusion constructs for overproduction of proteins. The following constructs were generated. For the full-length *BTD*, a 2.2-kb *Sma*I–*Sp1* fragment from pKSbtd (17) was cloned into pSLFlag digested with *Nde*I and *Sma*I and blunt-ended with mung bean nuclease. For a full-length *Sp1*, a filled-in 2.4-kb *Not*I–*Nde*I fragment from pSp1–778c (16) was cloned into pSLFlag digested with *Nde*I and *Sma*I and blunt-ended with mung bean nuclease. For N-terminal *BTD* without the zinc finger region, a 1,030-bp filled-in *Sma*I–partial *Bsp*EI fragment from pKSbtd was cloned into pSLFlag digested with *Nde*I and *Sma*I and blunt-ended with mung bean nuclease. For the C-terminal *BTD* without a zinc finger region, a 880-bp blunt-ended *Bgl*II–*Ssp*I fragment from pKSbtd was cloned into pSLFlag digested with *Nde*I and *Sma*I and filled in. All constructs were checked by sequencing. Generation of recombinant baculovirus with Baculogold viral DNA (PharMingen), expression of Flag-epitope-tagged proteins, and their purification from Sf9 cells was performed as described (21, 22). Interaction studies were performed with approximately 50 ng of Flag-epitope-tagged proteins immobilized on Flag-M2 antibody resin (Eastman Kodak) and [³⁵S]methionine-labeled proteins that were generated in a TNT-coupled *in vitro* tran-

scription system containing either TBP (lanes 1–4) or TFIIID (lanes 5–8), which were programmed with the reporter plasmid (GC)₃BCAT (12) and either *Sp1* (lanes 2 and 6) or *BTD* (lanes 4 and 8). TBP was unable to mediate *Sp1*- and *BTD*-dependent transcription activation (lanes 1–4), whereas TFIIID strongly stimulated transcription activation (lanes 6 and 8). (C) Silver-stained SDS/polyacrylamide gel of *in vitro*-assembled partial TBP–TAF complexes composed of TAF_{II}250, TAF_{II}150, TAF_{II}60, and TBP (lane 1); TAF_{II}250, TAF_{II}110, TAF_{II}60, and TBP (lane 2); or TAF_{II}250, TAF_{II}150, TAF_{II}110, and TBP (lane 3). Molecular weights (in kDa) are indicated to the right. (D) Autoradiographs of *in vitro* transcription reaction products, programmed with partial TBP–TAF complexes shown in C (indicated at the bottom) with or without 10 ng of purified *Sp1* (lanes 1–6) or 10 ng of *BTD* (lanes 7–12). Transcription products were assayed by primer extension; the position of the reverse transcript is indicated by an arrow. The combination of TBP, TAF_{II}250, TAF_{II}110, and TAF_{II}150 (lanes 6 and 12) causes strong and synergistic activation of transcription, whereas comparatively weak activation is observed with other partial complexes.

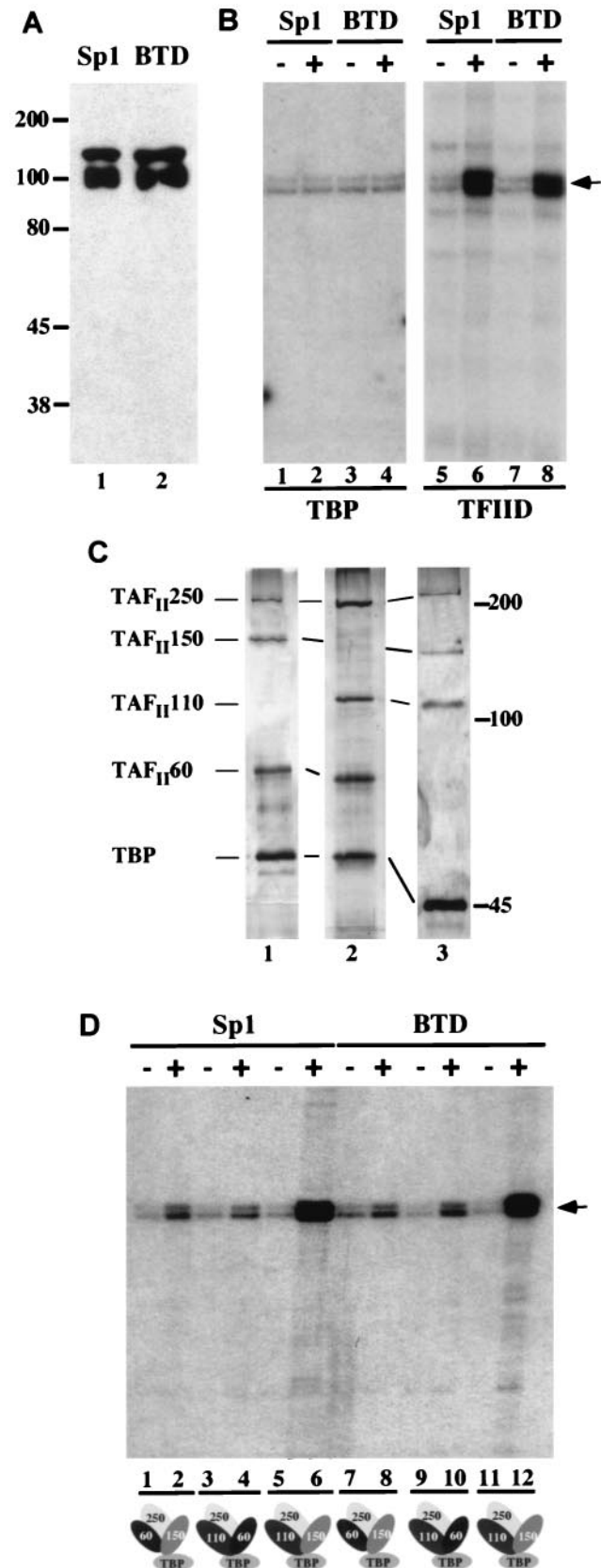


FIG. 2. Transcriptional activation by *BTD* and *Sp1* *in vitro*. (A) Western Blot showing purified *Sp1* (lane 1) and *BTD* (lane 2) produced in baculovirus-infected Sf9 cells. The purified proteins were detected by anti-Flag antibody; molecular weights (in kDa) are indicated. (B) *Sp1*- and *BTD*-dependent transcriptional activation *in vitro*. *In vitro* transcription reactions were composed of a reconstituted *Drosophila* transcription system containing either TBP (lanes 1–4) or

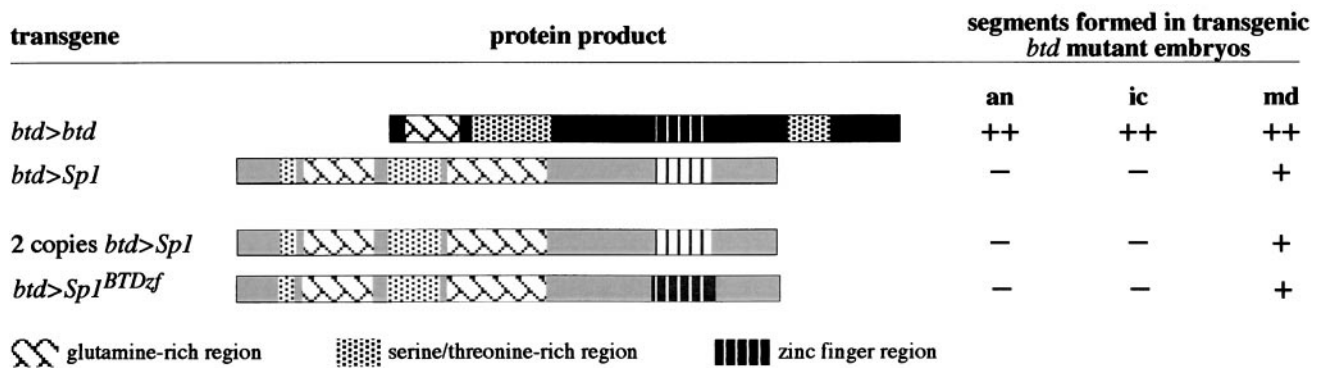


Fig. 3. Schematic representation of transgenes and their expression response in *btd* mutant embryos. Transgene constructs (Left) and rescue response of transgene-bearing *btd* mutant embryos are shown as assayed by Engrailed expression, the presence of sensory neurons in the head region, and cuticular head markers (ref. 28; for details, see Fig. 4). *btd>btd* and *btd>Sp1* rescue is shown for comparison (5, 17). ++, a full rescue of *btd* mutant embryos [i.e., the corresponding set of segment markers could be scored in transgene-bearing *btd* mutant embryos ($n \geq 50$ embryos analyzed)]; +, a partial rescue (i.e., only a subset of segment markers was found); -, no rescue. Representative examples of rescued embryos are shown in Fig. 4.

scription/translation system (Promega) (22). Protein complexes were analyzed by SDS/PAGE and visualized by autoradiography. *In vitro* transcription reactions (22, 23) were programmed with 10 ng of BTD or Sp1 and 50 ng of reporter gene plasmid (12). The reconstituted *Drosophila* transcription system is as described (23).

Histochemistry and Cuticle Preparations. Immunological staining of whole-mount embryos was performed as described (24) with the Vectastain ABC Elite Kit (Vector, Boehringer Ingelheim) and the primary antibodies anti- β -galactosidase (Cappel), anti-En (4D9) (25), and mAb22C10 (26). Homozygous *btd* mutant embryos were identified with blue balancers (27). Stained embryos and cuticle preparations (13) were photographed with a Zeiss Axiophot microscope.

RESULTS AND DISCUSSION

Sp1 and BTD Interact with the Same TAFs. Biochemical interaction assays and functional *in vitro* studies provided critical support that transcriptional activation by Sp1 is mediated by binding to TAF_{II}110 (9). Protein-protein interactions (i.e., Sp1 and TAF_{II}110) were sufficient to support transcriptional activation *in vitro*, whereas partial complexes lacking TAF_{II}110 failed to do so (12). Because the C₂H₂ zinc finger DNA-binding motif and glutamine- and serine/threonine-rich N-terminal domains are conserved between Sp1 and BTD (16), we tested whether BTD exerts Sp1-like biochemical properties. For this, we performed binding studies involving components of the transcriptional apparatus, including TFIIA and the eight *Drosophila* TAFs (see Introduction; Fig. 1). To allow for identical posttranslational modifications, we produced Flag-epitope-tagged BTD and Sp1 expressed in Sf9 cells that were infected with recombinant baculovirus (Fig. 2A). The epitope-tagged proteins were immunopurified by immobilization on Flag M2 antibody resins. The protein-coated resins were incubated with [³⁵S]methionine-labeled TFIIA or TAFs that were generated by using a coupled *in vitro* transcription/translation system.

Affinity beads containing either the purified N-terminal or C-terminal region of BTD, excluding the zinc finger domain, or full-length purified Sp1 were able to specifically retain TAF_{II}110 and TAF_{II}150 (Fig. 1B and C). In addition, weak binding was observed with TAF_{II}60 and TFIIA (Fig. 1D and E), whereas the other four TAFs were not retained on the BTD- or Sp1-coated affinity resins (for example, see Fig. 1A). This indicates that both BTD and Sp1 can target the same components of the TFIID complex *in vitro*. We next asked whether the conserved contacts of BTD also support the

activation of transcription in a cell-free reaction system in an Sp1-like fashion.

Interacting TAFs Support BTD-Dependent *In Vitro* Transcription. To compare the transcriptional properties of Sp1 and BTD in a cell-free transcription assay, we used a reconstituted *Drosophila* transcription system composed of the purified recombinant basal transcription factors TFIIA, -B, -E, and -F, purified endogenous TFIID, RNA polymerase II, and either purified recombinant TBP or *in vitro*-assembled TBP-TAF complexes (Fig. 2). We programmed the cell-free reaction system with recombinant epitope-tagged BTD or Sp1 that was expressed and purified as described above (Fig. 2A). As a reporter gene, we used a plasmid construct containing five Sp1 *in vitro* DNA-binding sites upstream of the E1B TATA box that governs the transcription of the reporter gene encoding chloramphenicol acetyltransferase. Reporter gene transcription was monitored by primer extension analysis.

In vitro reactions containing endogenous *Drosophila* TFIID supported BTD-dependent activation of transcription, whereas reactions containing only TBP failed to do so (Fig. 2B). This result suggests that BTD, like Sp1, requires TAF subunits of the TFIID complex to mediate activation of transcription *in vitro*. To assess which TAF subunits mediate BTD-dependent activation of transcription, we used *in vitro*-assembled TBP-TAF complexes (Fig. 2C) instead of the endogenous TFIID, previously shown to mediate Sp1-dependent transcriptional activation (12). Reactions containing *in vitro*-assembled TBP-TAF complexes composed of TBP, TAF_{II}250, and various combinations of TAF_{II}60, TAF_{II}150, and TAF_{II}110 were assayed (Fig. 2D). Strongest activation of transcription in response to Sp1 and BTD was mediated by partial complexes containing TAF_{II}150 and TAF_{II}110 (Fig. 2D). This observation is consistent with the earlier finding that TAF_{II}110 and TAF_{II}150 mediate Sp1-dependent activation in a synergistic manner (12). The results suggest further that the presence of TAF_{II}60 did not provide additional transcriptional activation when acting in a combination with TAF_{II}110 or TAF_{II}150, meaning that there is no synergistic interaction between TAF_{II}110 or TAF_{II}150 and TAF_{II}60 (Fig. 2D, compare lanes 8, 10, and 12). These findings demonstrate indistinguishable properties of Sp1 and BTD with respect to their abilities to activate transcription in a TAF-mediated manner.

Sp1 and BTD Contact the Same Functional Targets. *btd* mutant embryos lack three adjacent head segments: the mandibular, the intercalary, and the antennal segments (1, 5). The development of these segments can be identified by a distinct set of head sensory organs in the embryo or the larval cuticle (1, 28, 29) and by the expression domains of the segment

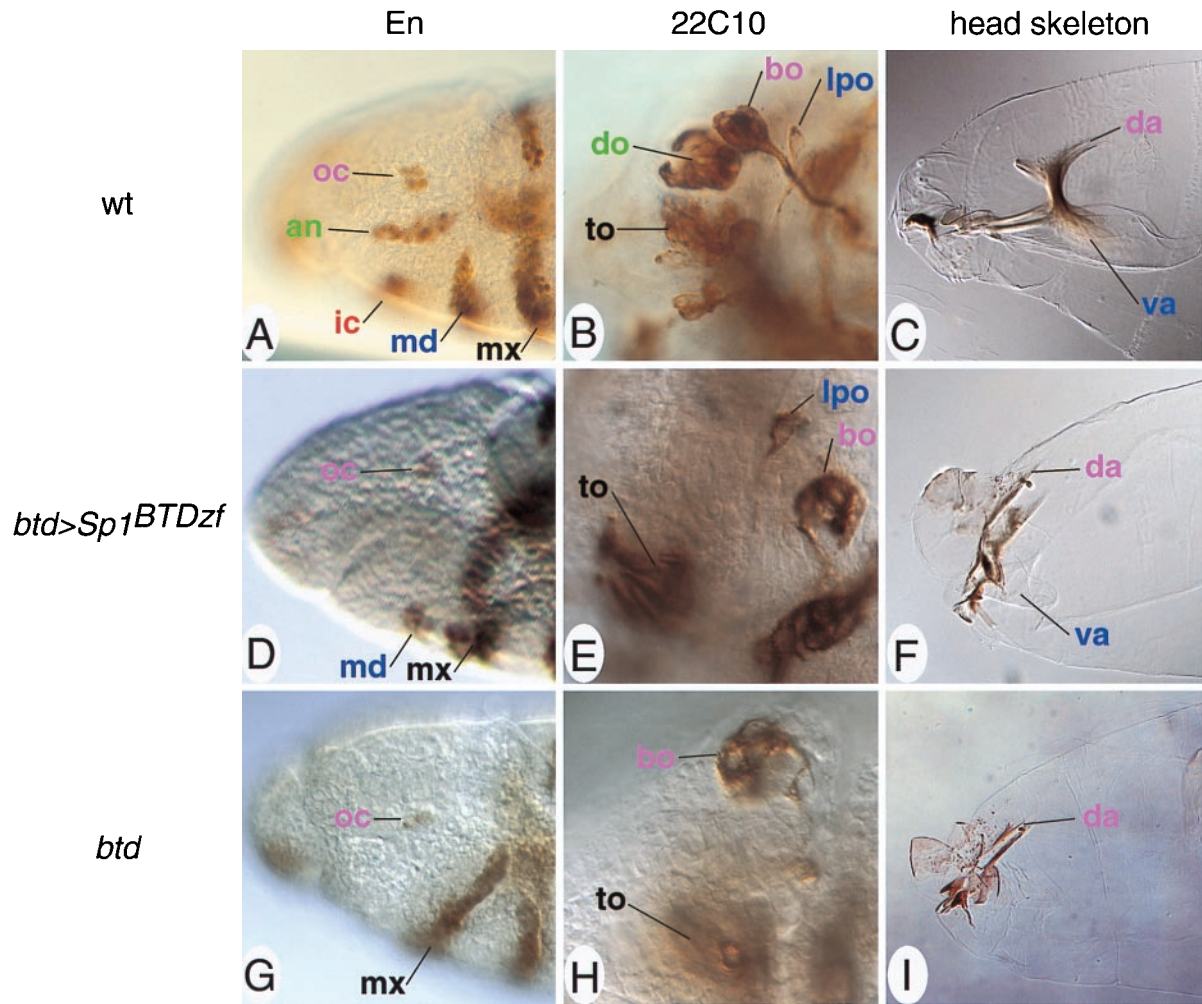


FIG. 4. Head rescue pattern of *btd>Sp1^{BTDz}*-bearing *btd* mutant embryos. (A–C) Head structures of wild-type embryos. (D–F) Head structures of *btd>Sp1^{BTDz}*-bearing *btd* mutant embryos. (G–I) Head structures of *btd* mutant embryos. (A, D, and G) Anti-En antibody labeling of stage 10 embryos. (Magnification, $\times 400$.) Only the mandibular segment is partially rescued (D); antennal and intercalary segments are missing (compare with A and G). (B, E, and H) mAb22C10 staining of stage 14/15 embryos showing head sensory organs at a representative focal plane. (Magnification, $\times 1,000$.) The lateropharyngeal organ of mandibular origin is rescued whereas the dorsal organ (an antennal organ) is not (E, compare with B and H). (C, F, and I) Head skeletons of stage 17 embryos show that intercalary and antennal cuticle structures are not rescued, whereas the ventral arm of mandibular origin is partially present in *Sp1^{BTDz}*-expressing embryos (F). (Magnification, $\times 400$.) Embryos are dorsal up and anterior to the left and staged according to ref. 32. Abbreviations and color code: ocular (oc) segmental structures (bo, Bolwig organ; da, dorsal arms) are labeled in violet; antennal (an) structures (do, dorsal organ) are green; intercalary (ic) structures are orange red; mandibular (md) structures (lpo, lateropharyngeal organ; va, ventral arms) are blue; maxillary (mx) structures (to, terminal organ) are black (for a detailed description of the segmental identity of organs, Engrailed stripes, and cuticle structures, see ref. 28).

polarity gene engrailed (*en*) in the germ-band-extended embryo (30). *btd* transgene expression under the control of the *btd* 5.2-kb *cis*-acting element (*btd>btd*, Fig. 3) provides the proper spatial and temporal expression in the three *btd*-dependent head segment anlagen and rescues the *btd* mutant head phenotype (17). In contrast, a transgene containing the *Sp1* cDNA in place of the *btd* cDNA (*btd>Sp1*, Fig. 3) provides a partial mandibular segment only (5), even when multiple copies of *btd>Sp1* were supplied (Fig. 3 and data not shown). Thus, Sp1 can activate BTD target genes required for mandibular development but is unable to support intercalary and antennal development (31).

One possibility to account for the different *in vivo* properties of BTD and Sp1 is that they exhibit different DNA-binding characteristics that allow them to distinguish different sets of target sites. To assess this possibility directly, we generated a Sp1-derived protein that contains the BTD zinc finger DNA-binding domain (*Sp1^{BTDz}*). This hybrid gene was placed under the control of the *btd* enhancer sequence and expressed in transgenic *btd* mutant embryos. Expression of the

btd>Sp1^{BTDz} (Figs. 3 and 4 D–F, compare with wild type in Fig. 4 A–C and the *btd* mutant in Fig. 4 G–I) transgene resulted in an Sp1-like rescue of the mandibular segment. Thus, differences in target site specificity and/or binding affinity of the Sp1 and BTD DNA-binding domains are not critical with respect to the biological responses caused by the two proteins. Furthermore, taking into account their identical TAF requirement *in vitro*, the relevant differences between Sp1 and BTD should also not be dependent on their ability to stimulate transcriptional activation of the biologically relevant target genes.

Activation of *in Vivo* BTD Target Genes Is Not TAF-Specific.

We tested this proposal by a genetic interaction assay, involving a dominant negatively acting *Drosophila* mutant of TAF₁₁₁₀ (15) and a homozygous *btd* mutant that was rescued by a single copy of the *btd*-expressing transgene. The rationale behind this mutant combination was that the *btd* activity in such embryos was limiting and that an interference with the TAF₁₁₁₀–BTD interaction should further decrease *btd* action and thus impair *btd*-dependent head development. However,

trans-heterozygous *TAF_{II}110/btd* mutant embryos develop a normal head segmentation pattern (data not shown). BTD must therefore be able to properly activate its target genes by means other than synergistic interactions involving TAF_{II}110 and TAF_{II}150 exclusively. However, lowering the level of BTD or TAF_{II}110 further or reducing the TAF_{II}150 level in addition to BTD and TAF_{II}110 might result in head defects and thereby reveal the necessity of interactions between the two factors.

BTD Must Contain Information to Mediate Intercalary and Antennal Segment Development. Our data provide evidence that BTD and human Sp1 interact *in vitro* with the same subset of TAFs and use the same TAFs for transcriptional activation in a reconstituted cell-free assay. In addition, Sp1 containing the BTD DNA-binding domain supports partial mandibular development but fails to provide intercalary and antennal structures. The same is true for a BTD miniprotein composed of the BTD DNA-binding domain fused to the strong transcriptional VP16 activation domain (F. Schöck, unpublished results). Because Sp1 and the VP16 activation domain contact different TAFs [i.e., TAF_{II}40 and the basal factor TFIIB in the case of VP16 (33)], binding of the transcription factor and its contacts with basal transcription factors appear to be sufficient to establish mandibular pattern elements. This minimal requirement explains why BTD-dependent mandibular development can be achieved in response to a biologically unrelated transcription factor such as human Sp1. However, the transgene expression study also establishes that this property of BTD is not sufficient to establish segments that require *ems* (intercalary segment) or *ems* and *otd* (antennal segment) in addition to BTD. This suggests that BTD contains properties to interact with EMS and OTD or with their downstream factors. Furthermore, because Sp1 is not sufficient to establish all aspects of mandibular development, it also appears likely that the proper formation of this segment requires an additional BTD-specific component. We currently examine whether the unique BTD properties are due to specific interaction domains and whether such domains are required for interactions with, for example, EMS and OTD.

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