Targeting gene expression to the head: the *Drosophila orthodenticle* gene is a direct target of the Bicoid morphogen

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SUMMARY

The Bicoid (Bcd) morphogen establishes the head and thorax of the *Drosophila* embryo. Bcd activates the transcription of identified target genes in the thoracic segments, but its mechanism of action in the head remains poorly understood. It has been proposed that Bcd directly activates the cephalic gap genes, which are the first zygotic genes to act in this region of the head (reviewed by Jurgens and Hartenstein, 1993). The molecular hierarchy that subdivides the trunk of the *Drosophila* embryo along its anteroposterior axis has been studied in great detail (reviewed by St. Johnston and Nusslein-Volhard, 1992; Pankratz and Jackle, 1993). In the first step of this cascade, gradients of maternal proteins activate the expression of the zygotic gap genes. The gap genes, which specify broad regions of the trunk, in turn activate the pair-rule genes, which establish the early parasegmental subdivisions. A similar molecular hierarchy subdivides the posterior (gnathal) region of the head (reviewed by Jurgens and Hartenstein, 1993).

Segmentation of the anterior (cephalic) region of the head is less well understood. The cephalic gap genes, which include orthodenticle (*otd*), empty spiracles (*ems*), buttonhead (*btd*), and sloppy paired (*slp*), are the first zygotic genes to act in this region. In the blastoderm embryo, these genes are expressed in broad stripes which include the primordia of multiple head segments (Dalton et al., 1989; Finkelstein and Perrimon, 1990; Wimmer et al., 1993; Grossniklaus et al., 1994). Since the pair-rule genes do not appear to function in cephalic segmentation, it has been proposed that anterior head segments are established directly by the cephalic gap genes (Cohen and Jurgens, 1990). Homologues of *otd* and *ems* have been identified in vertebrates (the *Otx* and *Emx* genes), where they are also required for anterior regionalization (reviewed by Finkelstein and Boncinelli, 1994).

In the *Drosophila* embryo, the maternal morphogen Bcd is required for both head and thorax development. *bcd* RNA is localized to the anterior pole of the oocyte, and Bcd protein diffuses to form an anteroposterior concentration gradient. In embryos lacking maternal *bcd* activity, the head and thorax are replaced by duplicated tail structures (Frohnhofer and Nusslein-Volhard, 1986). The *bcd* gene encodes a homeodomain protein (Frigerio et al., 1986; Berleth et al., 1988) that is capable of activating transcription in vitro (Struhl et al., 1989; Driever and Nusslein-Volhard, 1989).

According to an early model, the affinity of Bcd-binding sites in the promoters of target genes determines the posterior extent of their expression (Struhl et al., 1989; Driever et al., 1989). This hypothesis, referred to as the Gene X model, predicts that genes expressed specifically in the head primordium will contain low affinity Bcd sites, so that high levels of Bcd protein are required for their activation. Higher affinity Bcd sites would permit gene expression extending into the thoracic primordium. Other parameters, such as the spacing between Bcd sites (Hanes et al., 1994) and cooperative binding (Ma et al., 1996) have also been proposed to affect *bcd* target gene regulation. However, the importance of all these factors in the regulation of actual *bcd* target genes has not been determined.

INTRODUCTION

The molecular hierarchy that subdivides the trunk of the *Drosophila* embryo along its anteroposterior axis has been studied in great detail (reviewed by St. Johnston and Nusslein-Volhard, 1992; Pankratz and Jackle, 1993). In the first step of this cascade, gradients of maternal proteins activate the expression of the zygotic gap genes. The gap genes, which specify broad regions of the trunk, in turn activate the pair-rule genes, which establish the early parasegmental subdivisions. A similar molecular hierarchy subdivides the posterior (gnathal) region of the head (reviewed by Jurgens and Hartenstein, 1993).

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To understand how bcd generates head-specific expression, it is critical to determine which zygotic genes expressed in the head are directly regulated by bcd. The gap gene hunchback (hb), required for thoracic and gnathal development (Lehmann and Nusslein-Volhard, 1987), is a direct target of bcd (Struhl et al., 1989; Driever and Nusslein-Volhard, 1989). A second gap gene, tailless (tll), required for the formation of the unsegmented termini of the embryo (Strecker et al., 1988), also contains functional Bcd sites in its promoter region and is probably directly regulated by bcd (Liaw and Lengyel, 1992). Finally, the gap gene giant is a candidate bcd target gene (Kraut and Levine, 1991). The targets of bcd regulation in the cephalic segments, however, remain undetermined.

The anterior domains of expression of otd, ems, btd, and slp appear very early in embryogenesis, and are absolutely dependent on maternal bcd function (Dalton et al., 1989; Finkelstein and Perrimon, 1990; Wimmer et al., 1993; Grossniklaus et al., 1994). In addition, increases in bcd dosage cause a posterior expansion of the expression domain of each gene. Although bcd is the only gene product absolutely required for cephalic gap gene expression, at least two additional genetic inputs are necessary for the correct specification of the boundaries of expression. The first is the terminal maternal system, required for the formation of the anterior and posterior termini of the embryo (reviewed by Sprenger and Nusslein-Volhard, 1993). The terminal cascade prevents otd expression near the anterior pole of the embryo, and also contributes to the activation of specific cephalic gap genes within their normal expression domains (Finkelstein and Perrimon, 1990; Grossniklaus et al., 1994; Gao et al., 1996). It has been suggested that the terminal system affects Bcd target gene expression indirectly, by phosphorylating Bcd and decreasing or increasing its potency as a transcription factor (Ronchi et al., 1993; Grossniklaus et al., 1994). The second genetic requirement for correct cephalic gap gene activation is the gap gene hb. In embryos lacking maternal and zygotic hb activity, cephalic gap gene expression shifts anteriorly (Simpson-Brose et al., 1994; Gao et al., 1996).

Despite these genetic studies, the molecular mechanism of cephalic gap gene activation remains undetermined. To understand how gene expression is targeted to the anterior head, we are focusing on the regulation of the otd gene. Our first goal is to determine whether bcd activates otd directly, and to understand why this activation is limited to the head primordium. Our second goal is to understand the contribution of other genes in establishing head-specific expression. Since no bcd homologue exists in vertebrates, this second objective is particularly important.

In this study, we show that a 900 bp fragment upstream of the otd gene is sufficient to generate otd-like head expression. This fragment drives only early head expression, indicating that additional regulatory elements are required for later otd expression in the embryonic brain. Dissection of the 900 bp fragment shows that it contains two smaller elements, each sufficient to drive head expression. The sequence of the first element reveals three candidate Bcd-binding sites. We demonstrate that this element binds Bcd in vitro and that the three Bcd sites are essential for the function of this element in vivo. Consistent with the Gene X model, this regulatory element has a lower affinity for Bcd than an equivalent enhancer from the hb promoter. The second element does not bind Bcd. Instead it contains a sequence repeat resembling a motif required to target the expression of vertebrate Otx genes to cephalic mesenchyme (Kimura et al., 1997). We discuss the implications of our results for understanding head formation in Drosophila and higher animals.

**MATERIALS AND METHODS**

**Fly stocks and P-element-mediated germ line transformation**

Transgenes were introduced into yw homozygous flies. The dominant allele tor^{POD1} causes constitutive tor activation and expansion of the embryonic termini (Strecker et al., 1989). The multiple copy bcd stock used was BB bw/Cyo, in which the BB bw chromosome contains two copies of the bcd gene (Namba and Minden, 1997). For additional information regarding mutant alleles, see Lindley and Zimm (1992).

Embryos were injected as previously described (Spradling and Rubin, 1982). For each construct tested, at least three independent transformant strains were generated and tested.

**Plasmid construction, in situ hybridization, and X-gal staining**

Constructs including the otd transcription start site were made using the P element vector pCaSpeR-AUG-bgal (Thummel et al., 1988). Constructs lacking this region were generated using the vector pCaSpeR hs43 lacZ, which includes an hsp70 minimal promotor (Thummel and Pirrotta, 1992). Regulatorary fragments were inserted into the polylinker located 5’ of the lacZ gene in either vector. Larger deletions were generated using the restriction sites described in the figure legends. All other deletions were constructed by PCR using appropriate primers and verified by sequence analysis.

Whole-mount in situ hybridization was performed using digoxigenin-UTP-labeled lacZ or otd antisense RNA probes as previously described (Gao et al., 1996). X-gal staining was performed according to the method of Brand and Perrimon (1993).

**Sequence analysis and mutagenesis**

Sequence analysis was performed using automated Taq DyeDeoxyTm Terminator cycle sequencing reagents and an ABI PRISM 377 DNA Sequencer. Candidate binding sites for specific regulatory proteins were determined using the TFSEARCH program (can be obtained from http://pdaapl.trc.rwcp.or.jp/research/db/TFSEARCH.html).

Inactivation of the three candidate Bcd binding sites in the 186 bp fragment was performed by PCR using appropriate oligonucleotides. Two base pairs in each site were changed as follows: B1 from CTTAATCT to CTTAATGC, B2 from TTATCGC to TTATAGCC, and B3 from GTTATATGC to GTTATGTC. The altered base pairs are required for Bcd binding in vitro (Hanes and Brent, 1991). The oligonucleotides used were 26mers each containing the two base changes. All the mutagenized constructs were verified by sequence analysis.

**Gel retardation assays**

Gel retardation assays (Figs 6A and 7A-C) were performed in 20 μl of 1x BB buffer (prepared according to Ma et al., 1996) containing 0.1 mg/ml dl-DC (Pharmacia) for 20 minutes at room temperature. Longer reaction times yielded similar results, suggesting that binding assays had reached equilibrium by 20 minutes (also see Ma et al., 1996). 0.1 mg/ml bovine serum albumin (BSA) was also added to the reactions described in Fig. 6A. This full length recombinant Bcd protein, generously provided by X. Ma, was produced in insect Sf-9 cells using the baculovirus expression vector pJW Nhel. To prevent nonspecific binding, 5 μg/lane of single-stranded DNA was added. The concentration of [32P]-labeled probes was 4.5x10^{-11} M. 3 μl of 20% Ficoll was added to each reaction before loading onto a native polyacylamide gel in 0.5x TBE buffer. Unlabeled Bcd site competitor DNA was generated by annealing two 23 mer...
oligonucleotides, Bcd1A (CTAGACGAGTCTAATCCCATGAT) and Bcd1B (CTAGATCATGGGATAGACTCGT) (gifts from D. Wilson and C. Desplan). These oligonucleotides include a 19 bp region of overlap containing the 9 bp Bcd consensus binding site (TCTAATCCCC).

Because of difficulties in obtaining sufficient quantities of full length Bcd, we used a truncated Bcd protein (amino acids 89-154, including the homeodomain) purified from bacteria for the experiments described in Fig. 7. Previous studies showed that this protein has similar binding properties to full length Bcd (Rivera-Pomar et al., 1995). We also performed smaller scale experiments with the full length protein that confirmed the results shown in the figure (data not shown). In the experiments shown, labeled and unlabeled fragments were added simultaneously and reactions performed for 20 minutes at room temperature.

RESULTS

Embryonic regulatory elements lie upstream of the otd gene

To localize the control elements required for embryonic head expression, we constructed a series of lacZ reporter fusions spanning the otd genomic region. Each of these constructs was introduced into flies by P-element-mediated transformation, and lacZ expression monitored during embryogenesis. We found that a 7.6 kb fragment extending upstream of the otd transcriptional start site was sufficient to recapitulate the endogenous pattern of otd head expression. We also tested additional regions upstream of the 7.6 kb fragment and downstream of the otd gene, and found that they did not drive head-specific expression (data not shown).

We then compared the pattern of endogenous otd expression to that driven by the 7.6 kb regulatory fragment (Fig. 1B-I). As described previously (Finkelstein and Perrimon, 1990; Gao et al., 1996), otd is expressed initially at relatively low levels in an anterior cap-like region of the syncytial blastoderm embryo (Fig. 1B). The posterior boundary of this early expression domain is not sharp, but is graded in intensity. Expression quickly fades from the anterior terminus, leaving a stripe extending from 75-92% egg length (EL) in the cellular blastoderm embryo (Fig. 1C). During this period, ventral expression also disappears (not shown; see Gao et al., 1996). By this stage, the anterior and posterior boundaries of otd expression are sharply defined. During germ band extension, otd expression becomes more complex, appearing at the ventral midline (Fig. 1D) and in other regions of the embryo. In the germ band-retracted embryo, expression can be seen in the anterior brain and in midline CNS cells (Fig. 1E). This expression persists through embryogenesis.

In the blastoderm embryo, lacZ expression driven by the 7.6 kb fragment is indistinguishable from endogenous otd expression (compare Fig. 1B and 1F; 1C and 1G). Later in embryogenesis, lacZ expression in the anterior head and in midline cells is similar, but not identical to otd expression at equivalent developmental stages (compare Fig. 1D and 1H; 1E and 1I). Expression of the transgene is less localized within the head primordium, and significantly weaker in midline cells. This suggests that additional regulatory elements are required for correct late expression.

Identification of the regulatory region that controls early otd head expression

The results described above indicate that the 7.6 kb fragment contains the regulatory elements that control otd expression in the blastoderm head primordium. To map these elements more precisely, we constructed a series of 5’ and 3’ deletions of this fragment (Fig. 2).

Fig. 1. otd embryonic regulatory elements are contained within a 7.6 kb upstream region. (A) Map of the otd upstream regulatory region. Shown is a restriction map of the 7.6 kb upstream regulatory fragment. This fragment includes 513 bp of the otd 5¢ untranslated region (UTR). The transcriptional initiation site (indicated by an arrow) was defined by primer extension experiments (V. Iyer, Q. Gao, and R. Finkelstein, unpublished results) and adds 35 bp to the 5¢ UTR compared to the previously published sequence (Finkelstein et al., 1990). Abbreviations: RI, EcoRI; K, KpnI; RV, EcoRV; Bg, BglII; A, Asel; B, BamHI. (B-I) The 7.6 kb-lacZ fusion gene mimics embryonic otd expression. B-E show the pattern of otd RNA expression during embryogenesis, as detected by in situ hybridization. F-I show the expression pattern of the 7.6 kb-lacZ fusion gene, detected by in situ hybridization (F-H) or X-gal staining (I). Both otd and the 7.6 kb-lacZ fusion gene are initially expressed in a cap covering the anterior end of the syncytial blastoderm embryo (B,F). At this stage, the posterior border of expression is graded. As cellularization proceeds, expression disappears from the anterior terminus and the posterior border sharpens (C,G). In the germ band-extended embryo, both otd and the 7.6 kb-lacZ fusion gene RNA are expressed in the procephalic region and in midline precursor cells (D,H). The patterns of otd and lacZ expression are no longer identical by this stage, with the fusion gene expressed in a larger region of the head, and at lower levels in midline cells. In germ band-retracted embryos, expression is seen in the brain and ventral nerve cord (E,I). Again the expression patterns are no longer identical, with the fusion gene expressed in a larger region of the brain, as well as in two spots in the clypeolabral region. In all panels, anterior is to the left and dorsal is up.
Deleting 2.1 kb from the 5’ end of the 7.6 kb fragment had no discernible effect on early lacZ expression (data not shown). However, further deletion of a 1.8 kb EcoRV-BglII fragment completely eliminated head expression, indicating that essential control elements reside in this region. When fused to a minimal heterologous promoter, the 1.8 kb fragment drove strong lacZ expression in the blastoderm head primordium (Fig. 2C). This expression was indistinguishable, in intensity and position, from that produced by the 7.6 kb fragment. Expression driven by this fragment disappears by approximately 5 hours after egg laying (data not shown), demonstrating that a different control region is necessary to initiate or maintain otd expression in the head and brain.

We then determined the effects of additional deletions within the 1.8 kb fragment (Fig. 2B). The sequence of this fragment revealed five candidate Bcd sites and two possible Hb sites (see below). 5’ deletions, which removed two Bcd and one Hb site, very slightly decreased the level of lacZ expression but had no effect on its position (data not shown). Additional 5’ and 3’ deletions revealed however that a 900 bp fragment is essential for strong, head-specific lacZ expression. Fused to a heterologous promoter, this fragment generated otd-like head expression approximately equivalent in intensity to that driven by the 1.8 kb fragment (Fig. 2B,C). Since the 900 bp fragment is the smallest contiguous regulatory region capable of driving strong head expression, we will refer to it as the Early Head Enhancer (EHE).

**The Early Head Enhancer responds to bcd and tor regulation**

We tested next whether expression driven by the EHE responds to maternal cues in a similar fashion to otd expression. As described above, early otd head expression depends on bcd and, to a lesser extent, tor activity. Decreasing or increasing maternal bcd dosage causes endogenous otd expression to shift anteriorly or posteriorly (Finkelstein and Perrimon, 1990; Fig. 3A,B). Constitutive tor activation results in a posterior expansion of the otd domain (Gao et al., 1996; Fig. 3C).

We introduced the 900 bp-lacZ reporter construct into genetic backgrounds in which maternal bcd or tor activity is altered. The boundaries of lacZ expression shifted in a similar manner to those of the endogenous otd domain (Fig. 3D-F). This indicates that the EHE contains regulatory elements that control the response of otd to these two maternal patterning systems.

**The Early Head Enhancer contains two independent control elements**

To understand how the EHE functions, we mapped it at higher resolution (Fig. 4A). Progressive 5’ deletions showed that a 186 bp element at its 5’ end is critical for maintaining the intensity of early head expression. This region contains the three putative Bcd sites in the EHE. Deletion of this element significantly decreased the intensity of lacZ expression, without significantly affecting its spatial extent (compare Fig. 4B and 4C). Further 5’ deletions, which removed a putative Hb site, had no obvious effect on the level or position of lacZ expression.

3’ deletions revealed a second important control element at the opposite end of the EHE. Removal of 173 bp from the 3’ end of the 900 bp fragment also reduced the intensity of lacZ expression (Fig. 4D). Again, the spatial extent of early head expression was not significantly altered.

Deletion of the region between the 186 bp and 173 bp
fragments had no obvious effect on lacZ expression in the head primordium. When these two fragments were fused and juxtaposed to a minimal promoter, they produced head expression similar in intensity and extent to that driven by the intact EHE (Fig. 4E). This construct also mimicked the response of the EHE to alterations in maternal bcd and tor expression (data not shown). These experiments revealed that the activity of the EHE resides primarily within two small regulatory elements, each sufficient to drive otd-like expression in the head primordium. As mentioned above, the 186 bp element contains three candidate Bcd binding sites. Each of these sites contains 6 of the 9 nucleotides defined as a high affinity Bcd site in the hb promoter (Fig. 5A; Dräger and Nusslein-Volhard, 1989a). In particular, each site contains the TAATC core critical for the recognition of purified Bcd protein in vitro (Hanes and Brent, 1991; D. Wilson and C. Desplan, personal communication). The presence of these sequences suggested that Bcd binds directly to the 186 bp fragment.

The 173 bp element does not contain candidate Bcd sites or consensus recognition sites for any of the proteins involved in early head development. However, it does contain a 6 bp motif (G G/C ATCT) tandemly repeated six times (Fig. 5B). A deletion which specifically removes all six copies of this repeat eliminated head-specific expression (data not shown). As described, the removal of the single putative Hb site did not obviously affect the function of the EHE. This is consistent with our previous observation that hb plays a relatively minor role in otd activation (Gao et al., 1996). In contrast, the loss of a putative dl site that lies between the 186 bp and 173 bp fragments prevented the ventral retraction of lacZ expression (Fig. 4E and Q. Gao, unpublished results). This is consistent with our previous finding that dl is required for this retraction (Gao et al., 1996). Finally, the EHE also contains possible binding sites for the product of the terminal gap gene huckebein (data not shown), which is involved in repressing otd expression at the anterior terminus of the embryo (Gao et al.,

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**Fig. 3.** The 900 bp fragment contains bcd and tor response elements. (A-C) otd expression and (D-F) lacZ expression, each detected by in situ hybridization. The embryos in A,D and B,E are from females carrying one copy or four copies of a wild-type bcd gene respectively. The embryos in C,F are from mothers carrying the gain-of-function allele tor^4021. (A,D) Decreasing maternal bcd dosage causes the otd blastoderm stripe to shift anteriorly (compare to Fig. 1C). A similar shift is seen in lacZ expression driven by the 900 bp fragment. (B,E) Increasing bcd dosage causes a posterior shift in otd and lacZ expression. (C,F) Constitutive tor activity causes otd expression to expand posteriorly. lacZ expression also shifts posteriorly, although to a lesser extent.

**Fig. 4.** The 900 bp fragment contains two independent regulatory elements. (A) The top line is a schematic representation of the 900 bp fragment showing putative binding sites for Bcd, Hb, and DI. Below are a series of deletion constructs (1-12), all of which give otd-like head expression. These include three 5' deletions (1-3), six 3' deletions (4-9), and an internal deletion (10). Pluses indicate the approximate level of lacZ expression. (1-3) A 186 bp 5' deletion, which removes all three putative Bcd sites (construct 1), significantly reduces the intensity of lacZ expression but does not affect its spatial position (compare B and C below) Further deletion of putative Hb and DI sites (constructs 2 and 3) has no further effect on expression levels (data not shown). (4-9) Deletion of 173 bp at the 3' end of the 900 bp fragment (construct 4) also significantly reduces lacZ expression without altering its position (D). Larger 3' deletions (constructs 5-9) have no further effect on expression. (10) Fusion of the 186 bp and 173 bp fragments regenerates expression levels equivalent to those produced by the 900 bp fragment (E), (B-E) lacZ expression driven by the 900 bp fragment (B) and deletions there of (C-E). (B) The 900 bp fragment drives strong lacZ expression in the blastoderm head primordium. (C) The 186 bp 5' deletion (construct 1), which removes three putative Bcd sites, significantly reduces the intensity of lacZ expression, but does not alter its position. (D) A 173 bp 3' deletion (construct 4) affects lacZ expression approximately as in C. (E) An internal deletion that fuses the 186 bp and 173 bp fragments (construct 10) generates lacZ expression equivalent to that driven by the 900 bp fragment. This deletion, which removes a putative DI-binding site, reduces the ventral retraction of lacZ expression.
We did not analyze the function of these putative sites further. However, lacZ expression driven by the EcoRV-AvrII fragment, which lacks these sites, retracts only partially from the anterior pole (Fig. 7C).

The Early Head Enhancer binds Bcd with lower affinity than a hb enhancer

One of the goals of this study was to determine whether Bcd directly activates otd in the head primordium. We therefore tested whether purified Bcd protein binds to either of the two control elements within the EHE. Consistent with the sequence of the 186 bp element, gel retardation assays showed that it binds Bcd in vitro (Fig. 6A). No binding to the 173 bp element could be detected.

We also tested whether the three Bcd consensus sites in the 186 bp fragment are required for its activity in vivo. Using site-directed mutagenesis, we inactivated all three sites and verified that they no longer bind Bcd (Fig. 6A, lane 13). The mutated 186 bp fragment was reintroduced into flies, and lacZ expression monitored in the resulting embryos. Removal of the three Bcd consensus sites completely abolished head expression (compare Fig. 6B and 6C). This indicates that these three sites are essential for the head-specific regulatory activity of this fragment.

In its original form, the Gene X model predicted that a gene expressed specifically in the head primordium would have lower affinity Bcd sites than genes expressed more posteriorly. As mentioned earlier, in addition to the affinity of isolated Bcd sites, subsequent studies showed that intersite spacing, the number of sites, and cooperative binding effects all contribute to the affinity of regulatory regions for Bcd. We therefore compared the overall affinity of the 186 bp element for Bcd to that of a 250 bp enhancer from the hb promoter. The hb enhancer drives lacZ expression across both the head and thoracic primordia and binds Bcd with high affinity (Driever and Nusslein-Volhard, 1989). We found that significantly higher Bcd levels were required in gel retardation assays to shift the labeled 186 bp fragment than the labeled hb regulatory element (Fig. 7A, B). In a second assay, we compared the abilities of the two fragments to compete away Bcd protein from binding to Bcd sites. Consistent with the previous result, higher levels of the 186 bp fragment were necessary to inhibit Bcd binding (Fig. 7C). These experiments demonstrate that, consistent with the Gene X model, the overall affinity of the otd regulatory element for Bcd is lower than that of the hb enhancer.

Correct otd regulation requires the repression of posterior expression

The 1.8 kb regulatory fragment contains two candidate Bcd and...
orthodenticle is a direct target of Bicoid

one Hb site that lie upstream of the EHE. As mentioned earlier, deletion of the region containing these sites caused a slight decrease in the intensity of expression. To determine whether this region is sufficient to drive early head expression, we generated more fusion constructs and tested their functions in vivo.

Unexpectedly, we found that a 526 bp EcoRV-HincII fragment containing these sites drove both anterior and posterior lacZ expression (Fig. 8D,E). This expression resembled that of the terminal gap gene tll (Pignoni et al., 1990, 1992), suggesting that this fragment contains terminal system.

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**Fig. 7.** The 186 bp element binds Bcd with lower affinity than a regulatory element from the hb promoter. (A,B) Gel retardation assays performed with a truncated Bcd protein purified from bacteria (see Materials and Methods). Similar results were obtained with full length Bcd (data not shown). The 32P-labeled fragments are the 186 bp element (A) and a 250 bp enhancer from the hb gene, which contains at least 6 Bcd sites (B). Arrows indicate position of free probes. The concentrations of Bcd protein in the reactions shown in lanes 1-14 were 0 nM, 39 nM, 78 nM, 156 nM, 312 nM, 468 nM, 624 nM, 1.248 mM, 1.872 mM, 2.496 mM, 3.744 mM, 4.992 mM, 9.984 mM, and 19.968 mM respectively. (A) As Bcd concentration increases, progressive shifting of the hb regulatory fragment occurs. Multiple bands are generated by Bcd binding to each of the sites in the fragment, and possibly by the formation of super complexes of Bcd (Ma et al., 1996). Note that a Bcd concentration of 1.872 mM almost completely eliminates free probe. (B) Shifting of the 186 bp element by increasing concentrations of Bcd. A Bcd concentration of 3.744 mM, twice that required for the hb enhancer, is necessary to eliminate most of the free probe. (C) Competition experiment. 32P-labeled 186 bp fragment, in the presence of 5.0 mM truncated Bcd protein (approximately equivalent to the concentration used in the reactions in lane 12 of panels A and B), was incubated with increasing amounts of unlabeled competitor fragments. These competitor fragments, the 186 bp otd fragment or the hb enhancer fragment, were added at the same time as the labeled fragment. It can be seen that the hb fragment competes away Bcd protein more effectively than the 186 bp fragment. Equivalent results were obtained using the 32P-labeled hb probe with the same two cold competitors (data not shown).

**Fig. 8.** Correct otd expression requires posterior repression. In A, the top line shows the 1.8 kb regulatory fragment, which controls early otd expression in the head primordium (abbreviations as in Fig. 3A). Below are shown two 3’ deletion constructs. lacZ expression is schematized on the right. B-G show the results of in situ hybridization of various transgenic embryos with a digoxigenin-labeled lacZ probe. (A) As described earlier, the EcoRV-BglII 1.8 kb fragment generates otd-like anterior expression. A smaller EcoRV-HincII subfragment generates not only anterior, but also ectopic posterior expression. Restoration of the region between the HincII and AverII sites eliminates posterior expression, suggesting the presence of a negative regulatory region in that fragment. (B,C) The anterior, otd-like expression driven by the EcoRV-AverII subfragment generates only anterior, but also ectopic posterior expression. Restoration of the region between the HincII and AverII sites eliminates posterior expression, suggesting the presence of a negative regulatory region in that fragment. (B,C) The anterior, otd-like expression driven by the EcoRV-AverII subfragment in syncytial (B) and cellular (C) blastoderm embryos. Note that expression levels are significantly reduced compared to those driven by the entire 1.8 kb fragment (see Fig. 3B). (D,E) The ectopic posterior expression conferred by the EcoRV-HincII subfragment in syncytial (D) and cellular (E) blastoderm embryos. The posterior domain in D becomes replaced by discrete stripes of expression in E. (F,G) The EcoRV-HincII subfragment responds to terminal system regulation. Anterior and posterior expression both expand in syncytial (F) and cellular (G) blastoderm embryos derived from tor4021 females.
response elements. Consistent with this idea, the anterior and posterior expression domains specified by this fragment both expanded in embryos derived from torD females (Fig. 8F,G).

Since otd is not expressed at the posterior pole, we hypothesized that additional regulatory elements exist that prevent posterior expression. To test this idea, we examined expression driven by a larger regulatory fragment extending to the 5′ end of the EHE. This fragment drove expression only in the head primordium, indicating that it contains a negative regulatory element that represses posterior expression (Fig. 8B,C).

DISCUSSION

Identification of a regulatory region that specifically drives early head expression

The cephalic gap genes are initially expressed in broad stripes within the blastoderm head primordium. Later, each gene is transcribed in a more restricted region of the anterior head and brain. An important issue in anterior patterning is whether the early and late phases of cephalic gap gene expression are independently controlled.

Here, we showed that a 7.6 kb otd regulatory fragment accurately recapitulates early expression in the head primordium. This fragment continues to drive expression in a smaller region of the anterior head and brain later in embryonic development. Dissection of the 7.6 kb fragment led to the identification of a 900 bp region (the EHE) that contains the major control elements responsible for early head expression. Shortly after the cellular blastoderm stage, lacZ expression produced by the EHE disappears entirely. This demonstrates that there are indeed distinct control elements required for the re-initiation or maintenance of otd expression later in embryogenesis.

bcd and the mechanism of otd activation

One of the goals of this study was to determine if bcd directly regulates early otd expression. Dissection of the EHE showed that it contains a 186 bp fragment sufficient to generate otd-like expression in the head primordium. This fragment contains Bcd consensus sites that are required for its function in vivo and bind Bcd in vitro. This demonstrates that Bcd, or a protein with similar binding specificity, is required to activate otd expression in the head primordium. Since no such protein has been identified that is expressed early enough to function in otd activation, this result strongly suggests that Bcd participates directly in the regulation of a cephalic gap gene.

We have also shown that, consistent with the Gene X model, the 186 bp fragment binds Bcd with lower affinity than an analogous regulatory element from the hb gene. In future studies, it will be important to analyze the binding properties of the 186 bp fragment in more detail. It will be interesting to evaluate the contributions of spacing between Bcd sites, cooperative binding effects, and other parameters in determining the affinity of this regulatory element for Bcd. In addition, our results do not rule out the possibility that setting the posterior boundary of otd expression also requires repressors that prevent expression in the thoracic primordium. In this regard, it will be important to determine whether the 186 bp element can suppress the more posterior expression driven by the hb enhancer.

The terminal system and otd regulation

We also demonstrated that the terminal maternal system participates directly in otd activation. A 526 bp fragment upstream of the EHE drives expression at both the anterior and posterior poles of the embryo. This fragment also responds to alterations in maternal tor activity. It is therefore likely that a transcription factor in the terminal system cascade is directly involved in otd activation. Since otd is not normally expressed at the posterior pole, control elements must exist that prevent posterior expression. We show that at least some of these elements lie immediately downstream of the 526 bp fragment.

Why should a cephalic gap gene be regulated by the maternal terminal system? otd-related genes have been identified in every invertebrate yet analyzed, but bcd homologues have only been found in dipterans (Schröder and Sander, 1993). This implies that different maternal gene products must have contributed to otd activation in other invertebrates. It has been proposed that hb was the primary activator of the cephalic gap genes in primitive invertebrates (Simpson-Brose et al., 1994). However, we have shown that the contribution of hb to otd expression in the fruitfly embryo is relatively minor. A second possibility is that prior to the appearance of bcd, the terminal system played a more important role in otd activation than it does in Drosophila. Analysis of otd in other invertebrates will be required to test this hypothesis.

An evolutionary conserved head-specific regulatory element?

Perhaps the most surprising result of our study is the existence of the 173 bp regulatory element. This element is sufficient to generate early otd-like expression, but does not contain consensus Bcd sites or bind Bcd in vitro. This indicates that the 173 bp fragment must contain binding sites for a different activator of early head expression. However, since lacZ expression driven by the 173 bp fragment is eliminated in embryos lacking bcd (data not shown), this activator must, at least in Drosophila, be bcd-dependent. The only clue regarding the functional specificity of this activator is the reiterated sequence motif required for the activity of this regulatory element.

The mechanism of Otx gene activation in vertebrate embryos is poorly understood. In a recent study, the cis-acting elements necessary for Otx2 expression in the pufferfish and mouse were compared (Kimura et al., 1997). Two short sequence motifs, shared between mouse and fish, were shown to be required for expression in mesencephalic neural crest. One of these motifs (TAAAATCTG) shows similarity to the repeat unit we identified in the 173 bp fragment. It will be important to determine whether this repeat unit is sufficient to drive early head expression in fish. It will also be interesting to determine whether the vertebrate control element can direct head-specific expression in the fruitfly embryo.

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