

Intron of the mouse *Hoxa-7* gene contains conserved homeodomain binding sites that can function as an enhancer element in *Drosophila*

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ABSTRACT The 5' flanking sequences and the intron of the mouse *Hoxa-7* gene were searched for regulatory elements that can function in *Drosophila*. Only the intron is able to activate a *lacZ* fusion gene in various tissues of *Drosophila* embryos. This enhancer function requires a cluster of three homeodomain binding sites (HB1-element) that are also found in the introns of other *Hox* genes as well as in a putative autoregulatory element of *Ultrabithorax* (*Ubx*), the *Drosophila* homolog of *Hoxa-7*. If a single binding site in the autoregulatory element of *fushi tarazu* (*ftz*) is replaced by the HB1-element of *Hoxa-7*, the expression pattern is altered and newly controlled by the homeotic gene *caudal* (*cad*). These data suggest that HB1 is a potential target for different homeodomain proteins of both vertebrates and invertebrates.

Homeotic genes of vertebrates and invertebrates determine cell fates along the anterior-posterior body axis (1). The homeobox encodes a conserved DNA binding domain, the homeodomain (2–16), which is required for the function of the homeotic proteins as transcription factors *in vivo* (17–27). The high degree of evolutionary conservation of the homeodomain suggests that the DNA binding sites recognized by the homeodomains might also be conserved and that some of the regulatory circuits may be the same in both mammals and insects. It has been shown that the mammalian proteins can mimic the function of their *Drosophila* counterparts and produce the same phenotypes when expressed ectopically in flies (28–32). The mouse *Hoxb-6* gene, the homolog of *Antennapedia* (*Antp*), can induce antenna to leg transformations similar to *Antp* (28), whereas the mouse *Pax-6* gene, the homolog of the *eyeless* (*ey*) gene, is able to induce ectopic eye formation in the fly (32). In addition, *Hoxd-4* control regions were shown to exhibit enhancer activity in *Drosophila* similar to *Deformed* (*Dfd*), whereas a *Dfd* autoregulatory element was active in the expression domain of the paralog group 4 genes in the mouse, suggesting that this pathway may be conserved between vertebrates and invertebrates (33, 34). Since some of the homeobox genes are autoregulated (*Ubx*; ref. 35); *Dfd* (36); *ftz* (22); and crossregulated (*Antp* by *Ubx*; refs. 37 and 38), *Ubx* by *abd-A* (39, 40), the control regions of the mouse *Hox* genes were expected, by analogy to their *Drosophila* homologs, to contain functional homeodomain binding sites. Therefore, the genomic region of the mouse *Hoxa-7* gene (41, 42), the homolog of the *Drosophila* *Ubx* gene, was analyzed. It has been shown that 7 kb of genomic region are sufficient in transgenic mice for the early expression in the spinal cord and the somites (43, 44). The primary control elements for activation are located within a 469-bp fragment 1.6 kb upstream of the transcription start site, whereas the intron is needed for repression in sclerotomal cells in the somites (43–45). Thus, we tested whether the upstream and intronic regions exhibit

enhancer activity in *Drosophila*. A 680-bp and a larger 4-kb upstream fragment as well as an intron fragment were cloned in front of a *lacZ* reporter gene under the control of an hsp70 minimal promoter in the transformation vector Hz50pl (48), and *lacZ* expression was analyzed in transformed embryos.

MATERIALS AND METHODS

Fly Strains. The *rosy*⁵⁰⁶-strain (*ry*) was used as recipient for injections. *Antp*^{w10}, *Ubx*¹, *cad*¹, *cad*², and *cad*³ mutant alleles were used in this study. The following heat shock strains were used: HS-*Antp*^{H4} (17), HS-*Ubx*^{la22}/TM6B (18), HT*cad-1* (46), and HS-*ftz*^{T1} (U. Grossniklaus and K. Cadigan, personal communication).

Plasmid Constructs. Further details of the cloning procedures are available on request (47). All constructs were cloned in the same orientation into Hz50pl (48) except HA1, which was cloned in RS27 (47). p40 contains a 4-kb *PstI* fragment, p7 a 680-bp *AluI*–*Sau3A* fragment, and PB6 a *PstI*–*BamHI* fragment from pm6Δ (43).

β-Galactosidase (β-gal) activity staining was performed as described by Freeman *et al.* (49).

Antibody stainings were performed as described by Frasch *et al.* (50) using the Vectastain kit (Vector Laboratories). A monoclonal serum against β-gal was used from Promega. The antiserum against the *engrailed* (*en*) gene product was a gift from N. Patel. The antiserum against CAD was described by Mlodzik *et al.* (51).

Heat Shocks. Virgins of the heat shock lines were crossed with males homozygous for the *lacZ* transgene. Two- to 6-h-old embryos were collected and heat-shocked for 20 min at 37°C, incubated at 18°C for 7 h, and fixed.

Mobility-Shift Assays. ANTP and FTZ shifts were described previously (2, 52). The samples were electrophoresed at 4°C on native, 6% polyacrylamide gels. ANTP and FTZ homeodomains were purified from bacterial extracts (2, 52). CAD protein was a gift of R. Rivera-Pomar and H. Jäckle (53). The sequence of the HB1 oligonucleotides were as follows: HB1-5, 5'-TTGCTCATAAACTTTTATGGCCCAATTAATGGTTC-3'; and SP3, 5'-GAACCCATTAATTGGGCCATAAAAAGTTTATGA-3'.

RESULTS

The *Hoxa-7* 5' Control Region Is Not Functional in *Drosophila*. We found that the fragments from the upstream region (p7 and p40, Fig. 1A) inserted into the Hz50pl vector only exhibited enhancer activity within epidermal cells in the maxillary segment at the time of head involution (Fig. 2A). This activity, although on average weaker, resembles that

Abbreviations: β-gal, β-galactosidase; HB1-element, homeodomain binding sites.

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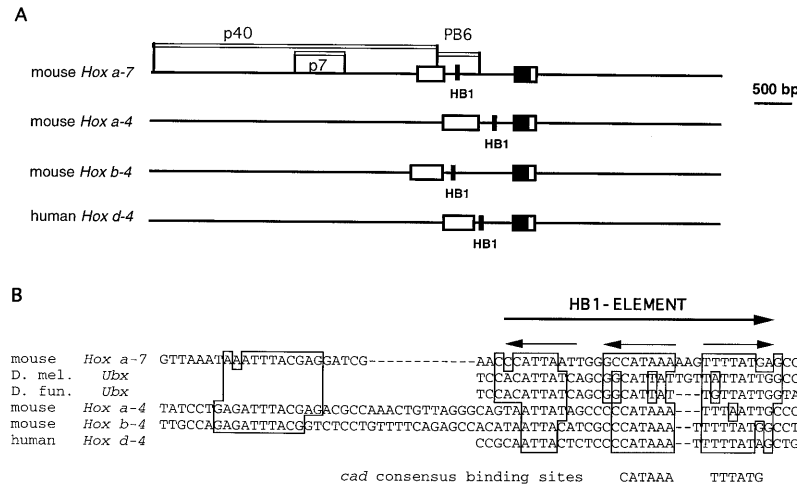


FIG. 1. A conserved cluster of homeodomain binding sites in vertebrate and invertebrate homeobox genes. (A) Location of the p40, p7, and PB6 fragments and the HB1 elements. The ORFs of the *Hox* genes are indicated by boxes, the homeoboxes are black. (B) Three conserved homeodomain binding sites (HB1-element) are present in the introns of the mouse *Hoxa-7*, *Hoxa-4*, *Hoxb-4*, and the human *Hoxd-4* genes as well as in the *Ubx* gene of two *Drosophila* species. The sequence of *Hoxa-7* is shown in the opposite orientation. An additional conserved element is similar to the *in vitro* consensus site for the *Pax-6* paired domain (61).

attributed to the *Hox d-4* upstream element that was previously studied (33). Analyzing transformants of the Hz50pl vector without any insert revealed that almost all lines (29/33) showed the same expression pattern (Fig. 2B). This result shows that this enhancer activity depends on vector sequences alone and suggests that the *Hoxa-7* upstream enhancers are not functional in *Drosophila*.

The Intron of *Hoxa-7* Directs Expression in *Drosophila* Embryos. The enhancer activity of a 600-bp intron fragment of *Hoxa-7*, containing clusters of putative homeodomain binding sites, was analyzed in *Drosophila* (PB6, Fig. 1A). Many of the PB6 transgenic lines (6/11) showed maternal expression; early expression in the nurse cells subsequently in the oocytes (Fig. 2C) and in early embryos (Fig. 2D). All the lines showed zygotic expression starting shortly after germ band retraction. Strong staining appeared in lateral and dorsolateral cells in the epidermis and in the peripheral nervous system with enhanced expression in the three thoracic segments (Fig. 2E and G). Strong staining was also observed in the posterior abdominal segments in epidermal cells and in mesodermal cells around the invaginating hindgut (Fig. 2H).

To identify the sequences that can function as enhancer elements, a deletion analysis of the PB6 fragment was performed (Fig. 3). The enhancer activity of the maternal and the zygotic expression was found to be located in different regions. None of the 3' deletion constructs PS4, XS3, and FS2 showed maternal enhancer activity, whereas the zygotic activity was identical to PB6. However, XO3, a 3' deletion of 40 bp from XS3 including a cluster of three putative homeodomain binding sites, the HB1-element, resulted in the loss of the strong zygotic activity (compare Fig. 2G and H with J and K). XO3 showed activity only in the ventral nervous system and in some cells of the lateral ectoderm (Fig. 2J).

On the other hand, several (4/10) of the ON3 lines that contained a 5' deletion construct showed expression in the nurse cells, whereas only very weak zygotic expression was observed (Fig. 2M and N). S3, a 5' deletion lacking the HB1-element, showed neither maternal nor zygotic activity (data not shown). These results suggested that the HB1-element is required for both maternal and zygotic expression. But the HB1-element by itself was not sufficient to activate *lacZ* expression in any *Drosophila* tissue (Fig. 2I).

Finally, it was noted that both XO3 and ON3 showed enhancer activity in the ventral nervous system. The common sequence of ON3 and XO3, P1, was found to be sufficient for this activity (Fig.

2L). However, the construct HA1 in which the P1 sequence was cloned in front of an *hsp27* promoter, did not show this expression pattern (Fig. 2O). Therefore, we conclude that P1 is functional in the context of the *hsp70* promoter and inactive in the combination with the *hsp27* promoter.

Mobility-shift assays showed that three molecules of purified ANTP homeodomain are able to bind to the HB1-element *in vitro* (Fig. 4A). Therefore, we analyzed the zygotic expression of PS4 lines in the mutant background of homeotic selector genes. We found that the early expression in the lateral epidermis was missing in all three thoracic segments in *Antp*^{w10} mutant embryos (compare Fig. 2E with F). However, staining in this region was observed at later stages. In *Ubx*¹ mutant background, expression in the lateral epidermis was only slightly decreased, suggesting that some other activators are present within the *Ubx* expression domain (data not shown). The staining in the posterior segments that resembles the expression of the homeobox gene *caudal* (*cad*, 46, 51) was, however, not altered in *cad* mutant background. Overexpression of *Antp*, *Ubx*, and *cad* by heat shock on the other hand did not show any significant change in the expression pattern.

Elements Similar to HB1 Are Present in the Introns of other *Hox* Genes as well as in a Putative *Ubx* Autoregulatory Element. Sequences related to HB1 were found in the introns of *Hoxa-4*, *b-4*, and *d-4*. (Fig. 1B; refs. 54–56). They are located in the intron approximately 200 bp downstream of the 5' splice site (Fig. 6B), but the HB1-element of *Hoxa-7* is in the opposite orientation. An HB1 related sequence was also found in the control region of *Ubx*, the *Drosophila* homolog of *Hoxa-7*. This sequence is located downstream of the transcription start site and is conserved between *Drosophila melanogaster* and *D. funebris* (57). This sequence was shown to be required for the expression of *Ubx* in parasegment 7 in the visceral mesoderm (35). The strong sequence conservation of HB1 suggests an important functional role of this element.

Functional Analysis of the HB1-Element in the Context of the *ftz* Autoregulatory Element. To find out whether the HB1-element is a direct target for homeodomain proteins, it was analyzed in the context of the *ftz* autoregulatory element. The *ftz* gene is expressed in seven stripes in alternate parasegments. In a second-site suppression experiment, direct interaction between the FTZ protein and the *ftz* autoregulatory element has been demonstrated (22). This *ftz* enhancer contains redundant FTZ binding sites located in four regions (58). We defined a minimal element, AE20A (Fig. 5B), which

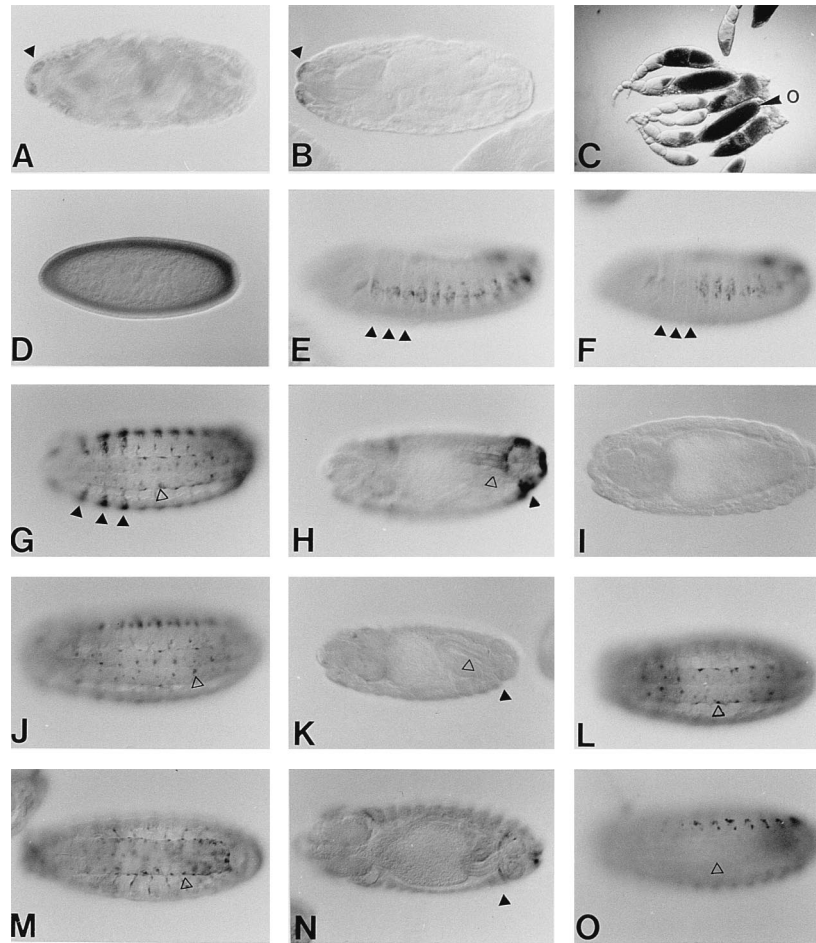


FIG. 2. Enhancer activity of the *Hoxa-7* intron in *Drosophila*. Stainings of embryos with antiserum against β -gal, if not indicated differently. (A) Stage 17 embryo of p40 showing epidermal expression in the maxillary segment (arrowhead). (B) Embryo of a control Hz50pl line without insert shows the same expression as A. (C) β -Gal activity staining of PB6 ovaries. Strong staining is obtained in the nurse cells from at stage 9 on and later in the oocyte (o). (D) Stage 5 PB6 embryo showing maternal expression. (E-N) Stage 13-15 embryos (E) PS4 embryo showing expression in the lateral epidermis. The thoracic segments are indicated by arrowheads. (F) PS4 embryo in a presumptive *Antp*^{w10} mutant background. Expression in the lateral epidermis in the three thoracic segments is missing (arrowheads). (G and H) XS3 embryos showing ventrolateral expression (G) and expression in the posterior segments (H). The zygotic staining in PS4, XS3, and FS2 lines is identical to PB6. (I) HB1 embryos show no expression. (J and K) XO3 embryos showing staining in cells of the ventral and peripheral nervous system and in some lateral epidermal cells (arrowhead) (J), but not in the cells around the posterior midgut (open arrowhead) and in the posterior segments (solid arrowhead) (K). (L) P1 embryo showing staining in the ventral nervous system. (M and N) ON3 embryo showing the same staining as P1 (M) as well as weak staining in the posterior segments (N). (O) HA1 embryo showing similar (nuclear) staining as HS1, but the P1 pattern (L) in the ventral nervous system is absent (arrowhead).

exhibits enhancer activity in alternate segments in the mesoderm (Fig. 6 A and B). This 154 bp long fragment contains only one FTZ binding site as previously shown by footprint analysis (59).

Mobility-shift assays indicate that three molecules of purified FTZ-homeodomain can bind to the HB1-element *in vitro* (Fig. 4B). Thus, the single FTZ binding site and a putative

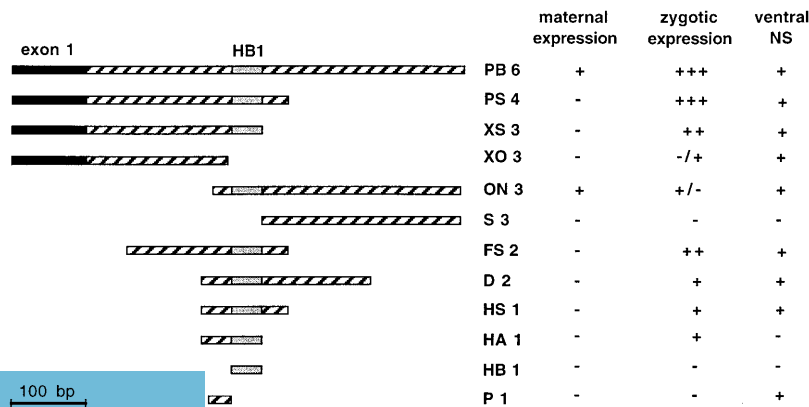


FIG. 3. Enhancer activity of deletion constructs. ■, Exon 1 part; ▨, intron; □, HB1-element. PS4, FS2, and HS1 have the same 3' ends, whereas D2, HS1, and HA1 have the same 5' ends.

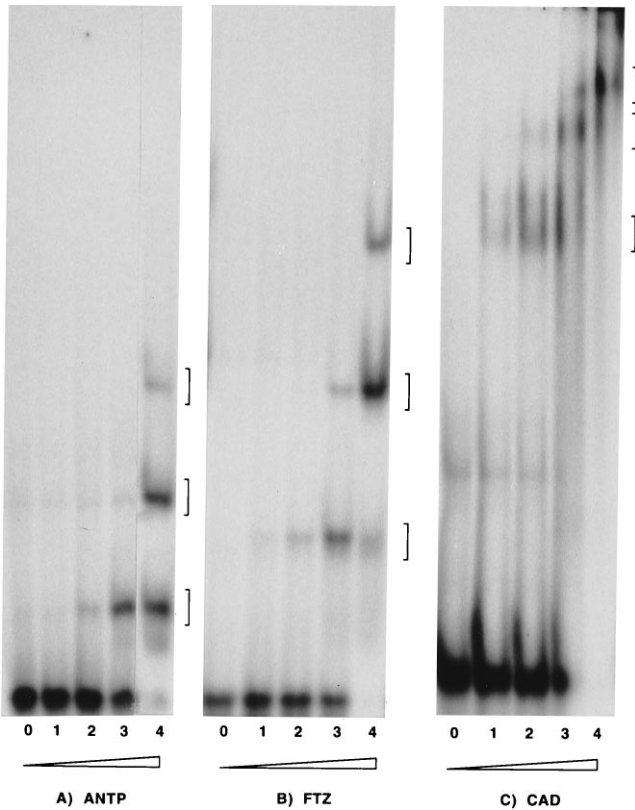


FIG. 4. Mobility-shift assays of homeodomain-DNA binding. (A) Binding of a 68-amino acid long (8 kDa) ANTP homeodomain to the HB1 sequence. (B) Binding of a 70 amino acid long FTZ homeodomain to HB1. (C) Binding of a 46 kDa CAD protein to HB1. Lanes 0 contain no protein. Concentration of the ANTP and FTZ homeodomains in lanes 1 is 0.8 nM, that of CAD is 100 nM. The concentration of ANTP and FTZ homeodomain increase with each lane by a factor of 4, that of CAD by a factor of 3. Three complexes are observed with all three proteins.

binding site for FTZ-F1 gene product (22, 58, 60) were replaced in AE20A by the three *ftz* binding sites of HB1 (4A7ftz, Fig. 5 A and B). If the HB1 binding sites were recognized by FTZ *in vivo*, expression in alternate segments as in AE20A would have been expected.

However, the 4A7ftz lines showed no *ftz*-like expression. Instead, at the stage of complete germ band extension, staining was seen in the last abdominal segment (Fig. 6 C and D). This pattern resembles the expression of the homeobox gene *cad* (Fig. 6 E and F) but the expression is restricted to the mesoderm. In 3A7ftz lines (Fig. 5A) the same expression pattern was superimposed on a *ftz*-like expression pattern (Fig. 6G). This enhancer contains additional FTZ binding sites and was therefore expected to activate *lacZ* in seven stripes. If HB1 is located 5' of AE21 or AE20A, replacing the 5' FTZ binding site of AE20 (Fig. 5A; A7ftz and 2A7ftz), no *cad*-like expression is observed (data not shown), suggesting that the position of the HB1 element is important.

A mobility-shift assay showed that three molecules of CAD protein are able to bind to the HB1-element *in vitro* (Fig. 4C). To demonstrate interaction *in vivo*, 3A7ftz and 4A7ftz lines were analyzed in *cad* zygotic mutant embryos (*cad*¹, *cad*², and *cad*³). In this background, the expression in 3A7ftz and 4A7ftz embryos is almost absent in the *cad* expression domain (Fig. 6 H and I). In contrast, ubiquitous expression of *cad* by heat shock (pHT*cad*-1), induced ectopic expression in lateral mesodermal cells in 4A7ftz (Fig. 6 J and K) but not in AE20A embryos (data not shown). This ectopic activation was not observed in control experiments using *Antp*^{H4} or *Ubx*^{la22} heat shock lines (data not shown).

The expression of the 4A7ftz embryos suggested that FTZ was not able to activate the transgene in these lines. When FTZ protein was overexpressed by heat shock induction in 4A7ftz lines (HS-*ftz*^{T1}), *ftz*-like expression was observed in addition to the *cad*-dependent expression (Fig. 6L). Therefore, we conclude that FTZ is still able to activate the transgene in 4A7ftz lines, but activation requires high levels of FTZ protein. Thus, the HB1-element contains potential direct target sites for both CAD and FTZ proteins.

DISCUSSION

The HB1-Element Is a Potential Target Motif for CAD and FTZ. The HB1-element is an evolutionarily conserved DNA sequence in the intron of *Hoxa-7*, in the leader of its *Drosophila* homolog *Ubx*, and in the introns of the paralog group 4 *Hox* genes. The analysis of the HB1-element in the *Ubx* gene revealed that the first two binding sites of HB1 are required for *Ubx* expression in the visceral mesoderm and it was suggested that they may function as an autoregulatory element (35). We

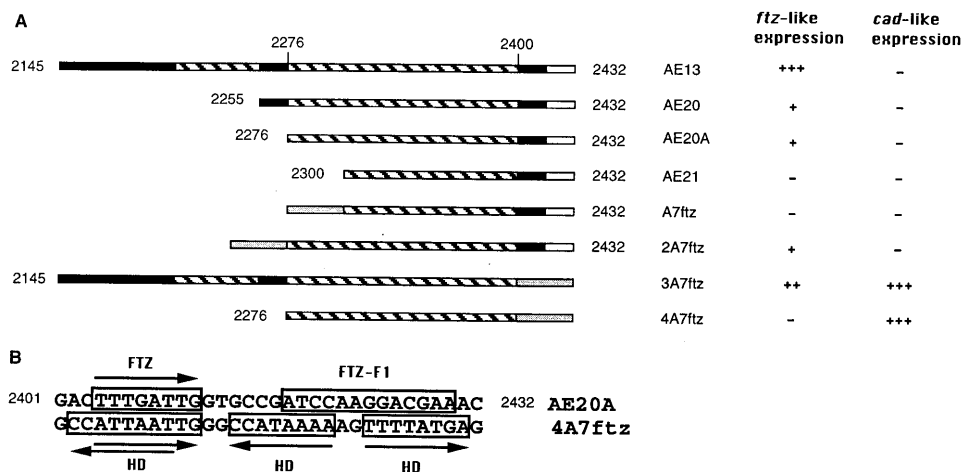


FIG. 5. Analysis of the HB1-element in the context of the *ftz* enhancer. (A) Deletion constructs of the *ftz* autoregulatory element and fusion constructs to HB1. ■, FTZ binding sites; □, *Hoxa-7* HB1-elements. AE13 contains several FTZ binding sites, AE20A a single binding site. Both exhibit enhancer activity in alternate segments in the mesoderm. AE21 is not functional. In 4A7ftz, the FTZ binding site and a binding site for FTZ-F1 (□) has been replaced by the HB1-element. (B) Sequence comparison of the *ftz* sequence in AE20A and the homeodomain binding sites (HD) are indicated.

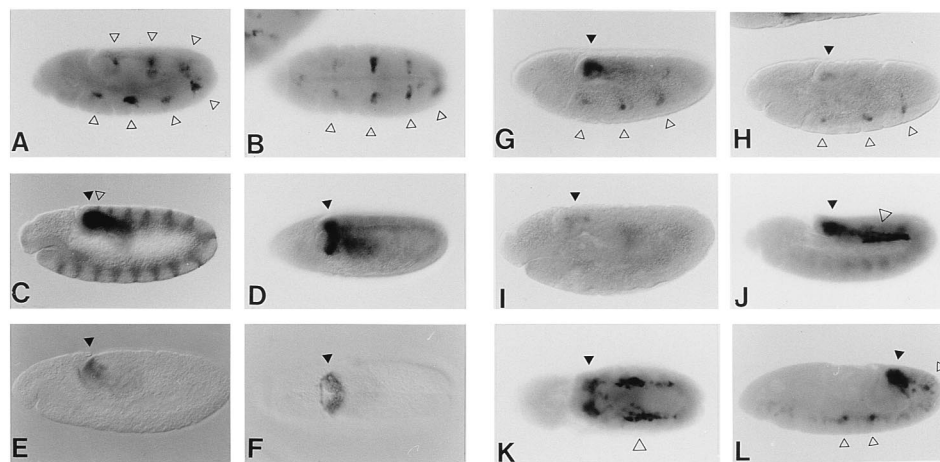


FIG. 6. Expression patterns of AE20A, 4A7ftz and 3A7ftz lines in *Drosophila* embryos. β -Gal activity stainings (A–D and G–L) and stainings with an antiserum against EN (C) and CAD (E and F). Lateral view (A) and ventral view (B) of a stage 11 AE20A embryo showing staining in alternate segments (arrowheads). Lateral view (C) and dorsal view (D) of an early stage 11 4A7ftz embryo. (C) The β -gal staining (solid arrowhead) appears more posteriorly of the last *en*-stripe (open arrowhead). (E and F) Stage 10 (E) and stage 11 (F) embryo stained with antiserum against the *cad* gene product (arrowheads). (G) Early stage 11 3A7ftz embryo showing *ftz*-like (open arrowheads) and *cad*-like (solid arrowhead) expression. (H) Early stage 11 3A7ftz embryo in a presumptive *cad*¹ mutant background. The *cad*-like expression (solid arrowhead) is almost absent, whereas the *ftz*-like expression (open arrowheads) is not affected. (I) 4A7ftz embryo in a presumptive *cad*³ mutant background showing reduced staining (solid arrowhead). (J) Lateral view and (K) dorsal view. Staining of a late stage 11 4A7ftz embryo after ubiquitous expression of *cad*. Ectopic expression appears in lateral mesodermal cells (open arrowhead). (L) Early stage 12 4A7ftz embryo after ubiquitous expression of *ftz* showing *ftz*-like expression (open arrowheads).

have shown that three molecules of ANTP, FTZ, or CAD homeodomains can bind to the HB1-element *in vitro* (Fig. 4) and that *ftz* regulates HB1 in the context of the 120-bp *ftz* autoregulatory sequence (Fig. 6L). It has been previously shown that the *ftz* autoregulatory enhancer is directly controlled by FTZ (16). Direct FTZ interaction with the binding site in AE20A is very likely, although it has not been demonstrated *in vivo*. Since there are no other binding sites present in 4A7ftz as previously shown by footprint analysis (59), we can assume that FTZ binds directly to the HB1-element. However, we found that high levels of FTZ are required to obtain *ftz*-like expression. Since a FTZ-F1 binding site was replaced in 4A7ftz by two homeodomain binding sites of HB1 (Fig. 5B), the FTZ-F1 binding site seems to be required for activation at physiological FTZ concentrations, suggesting that FTZ-F1 might serve as cofactor of FTZ to select binding sites *in vivo*.

We have shown that the 4A7ftz enhancer is regulated by *cad*. In previous studies analyzing the *ftz* zebra element, an expression pattern similar to the one of 4A7ftz was found and it was demonstrated that this activity requires two *in vitro* binding sites (TTTATG) for CAD (6). Two of these CAD binding motifs are also present in the HB1-element (Fig. 1B). Therefore, it is likely that CAD also binds directly to the HB1-element.

The Function of the HB1-Element in the Intron of *Hoxa-7* in *Drosophila* and the Mouse. The upstream region of *Hoxa-7* (p40 and p7), which contains functional elements regulating expression in the mouse embryo, was found not to confer any specific enhancer activity in *Drosophila*. The only activity of these constructs, similar to that of the conserved human *Hoxd-4* upstream enhancer (33), is also observed in the control experiment using the Hz50pl vector without insert. The intron of *Hoxa-7* on the other hand can function as enhancer in *Drosophila*. The observed expression pattern in *Drosophila* was responding to *Antp*, but only weakly to *Ubx* and not to *cad* as shown in mutants of these genes. In the mouse however, *Cdx-1*, the homolog of *cad*, is known to be a regulator of *Hoxa-7* (ref. 62; P. Gruss, personal communication), and our results with the 4A7ftz construct suggest that the HB1-element may be a potential target site for *Cdx-1*. Therefore, the HB1-element may be a conserved target for various homeodomain proteins of both vertebrates and invertebrates.

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