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Embryonic expression and characterization of a Ptx1 homolog in *Drosophila*

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Abstract

We describe the molecular characterization of the *paired*-type homeobox gene *D-Ptx1* of *Drosophila*, a close homolog of the mouse pituitary homeobox gene *Ptx1* and the *unc-30* gene of *C. elegans*, characterized by a lysine residue at position 9 of the third α-helix of the homeodomain. *D-Ptx1* is expressed at various restricted locations throughout embryogenesis. Initial expression of *D-Ptx1* in the posterior-most region of the blastoderm embryo is controlled by *fork head* activity in response to the activated Ras/Raf signaling pathway. During later stages of embryonic development, *D-Ptx1* transcripts and protein accumulate in the posterior portion of the midgut, in the developing Malpighian tubules, in a subset of ventral somatic muscles, and in neural cells. Phenotypic analysis of gain-of-function and lack-of-function mutant embryos show that the *D-Ptx1* gene is not involved in morphologically apparent differentiation processes. We conclude that *D-Ptx1* is more likely to control physiological cell functions than pattern formation during *Drosophila* embryogenesis. © 1997 Elsevier Science Ireland Ltd.

Keywords: *D-Ptx1*; Embryonic expression patterns; Paired-type homeodomain; *Ptx1* homolog

1. Introduction

Genetic and molecular analysis of pattern formation during early *Drosophila* embryogenesis identified a considerable number of transcription factors that control this process. Most of these transcription factors are integral components of the segmentation gene cascade in the pre-blastoderm and blastoderm embryo, and many of them appear to be redeployed at later stages in organogenesis and tissue development (Akam, 1987; Ingham and Gergen, 1988; St Johnston and Nüsslein-Volhard, 1992; Hoch and Jäckle, 1993; Pankratz and Jäckle, 1993). The first and most prominent transcription factors that emerged from these studies were those encoded by the homeotic gene clusters of the Antennapedia (ANT-C) and bithorax complex (BX-C), which shared the conserved 60-amino acid DNA binding homeodomain (reviewed by Gehring, 1987; Scott et al., 1989). In vertebrates, homologous genes are clustered at four genomic loci (McGinnis and Krumlauf, 1992), each containing 9–11 homeobox or Hox genes. Comparing the sequences and organization of the genes in each cluster revealed a high degree of conservation of these genetic systems in different animal species. Gain- and loss-of-function experiments in genetically characterized species showed that these genes are required for the proper development of specific body structures (Lewis, 1978; reviewed by Krumlauf, 1994). In addition to these conserved Hox gene clusters, a large number of Hox genes have been identified that appear to function as regulators of specific cell fate decisions (for reviews see Scott et al., 1989; Laughon, 1991; Gehring, 1993; Kappen and Ruddle, 1993; Schubert et al., 1993; Duboule, 1994). A recent count listed at least 170 known Hox genes in vertebrates alone (Stein et al., 1996).

Despite the high degree of overall conservation among homeodomains, they can be subdivided into distinct groups

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(reviewed by Bürglin, 1993). Within the large Paired group of homeodomains (Bopp et al., 1986), a small, Bicoid-related subgroup is characterized by a lysine in position 9 of the third α-helix (Frigerio et al., 1986). The presence of this lysine residue within the motif VWFKNRR is typical for homeodomain proteins that act in the anterior region of both invertebrate and vertebrate embryos. They include the *Drosophila* proteins Bicoid (Bcd) (Frigerio et al., 1986; Berleth et al., 1988), Orthodenticle (Otd) (Finkelstein et al., 1990) and D-Goosecoid (D-Gsc) (Goriely et al., 1996; Hahn and Jäckle, 1996) as well as their vertebrate homologs, such as Otx1, Otx2 (Simeone et al., 1993) and Goosecoid (Gsc) (Blumberg et al., 1991). In addition, the recently identified *unc-30* gene of *C. elegans* and the mouse pituitary gene *Ptx1* are part of the *bicoid*-related subgroup. Expression of *unc-30* is found in the GABAergic type D motor neurons and appears to be required for proper axonal growth and formation of synapses (Jin et al., 1994), while *Ptx1* is not expressed in neural tissues but acts as a Bicoid-related transcription factor in the transcriptional activation of the pro-opiomelanocortin (POMC) gene in corticotrophs, a subset of anterior pituitary cells (Lamonerie et al., 1996). Its early expression throughout Rathke's pouch, the pituitary anlage, long before POMC is expressed, suggests that *Ptx1* may play a different role before its expression is restricted to the corticotroph cell lineage.

Here we present the identification and molecular analysis of the *Drosophila* homolog of *Ptx1*. In contrast to *Ptx1* expression in the mouse, *D-Ptx1* transcripts are not expressed in the anterior region of the embryo. Instead, we find that it is initially expressed in the most posterior region of the blastoderm and later in specific patterns that are restricted to a small portion of the embryo. *D-Ptx1*-deficient embryos fail to develop a morphologically distinct phenotype, which suggests that the *Ptx1* homolog may serve physiological functions different from those of pattern formation.

2. Results and discussion

2.1. Identification and localization of the D-Ptx1 gene

In a search for genes that execute region-specific functions at *Drosophila* blastoderm, we identified a cosmid that included a transcription unit expressed in the most posterior region at this stage (Fig. 1A). Based on the high degree of sequence similarity between the protein encoded by this transcription unit and the mouse Ptx1 protein (see below), we called this transcription unit *Drosophila Ptx1* (*D-Ptx1*). Cytogenetic examination by in situ hybridization showed that *D-Ptx1* is located close to the right telomere of the third chromosome, at position 100B1,2 (Fig. 1B). In addition, the *D-Ptx1* bearing cosmid was mapped about 90 kb distal from the *tailless* (*tll*) gene on a chromosomal walk (Fig. 1C; Justice et al., 1995). To further delimit the *D-Ptx1* transcription unit within this cosmid DNA, we performed in situ hybridization to whole mount embryos, using fragments derived from the 40 kb cosmid. A 7.8-kb *Cla*I fragment was identified and used to isolate clones from an embryonic cDNA library. One of the cDNAs was close to the full-length mRNA of 3.5 kb (see below).

The genomic location of *D-Ptx1* was further mapped by the use of deficiency chromosomes generated by X-ray induced deletions of the P-element insertion *P[lArB]A177* (Fig. 1C; Justice et al., 1995). As illustrated in Fig. 1C, the distal breakpoint of *Df(3R)A177der20* maps close to the distal end of the 12-kb *Eco*RI fragment flanking the 5′ end of the longest *D-Ptx1* cDNA, whereas the *D-Ptx1* gene is deleted by *Df(3R)A177der22* and by *Df(3R)A177der25* which extends about 15–20 kb beyond the 3′ end of the cDNA. In contrast to the other deficiencies, *Df(3R)A177-der22* does not uncover the *tll* gene (Fig. 1C). Neither *D-Ptx1* transcript nor protein were detectable in homozygous *Df(3R)A177der25* or *Df(3R)A177der22* embryos, while both were expressed in homozygous *Df(3R)A177der20* embryos. Thus, we were able to investigate the gene's function by combining *Df(3R)A177der22* with *Df(3R)A177-der25* in *D-Ptx1*-deficient embryos (see below).

2.2. The D-Hox100K gene encodes a homolog of the mouse Ptx1 protein

Mapping all cDNAs with respect to the genomic DNA by Southern blot analysis and sequencing showed that the longest *D-Ptx1* cDNA consists of 5 exons, spanning a genomic region of 20 kb. Transcription starts at 60 kb distal to *tll* in a telomeric direction (Fig. 1C). Sequence analysis of this cDNA revealed that it consists of 3432 bp and includes 947 bp of untranslated leader sequence, an open reading frame of 1539 bp and a 946 bp untranslated trailer with a single polyadenylation signal followed by a poly(A) tail. The putative translation start site is preceded by AGGC, which fits only moderately the consensus sequence $^{C}/_{A}AA^{A}/_{C}$ (Cavener, 1987), but no other suitable translation start site is found within the coding sequence. The open reading frame encodes a protein of 513 amino acids that includes a homeodomain (Fig. 2A).

The D-Ptx1 homeodomain is most closely related to the homeodomains encoded by the recently identified mouse *Ptx1* (Lamonerie et al., 1996) and the *C. elegans unc-30* gene (Jin et al., 1994). The conserved amino acids include the lysine at position 9 of the third α-helix of the homeodomain, which defines the class of Bicoid-related homeodomains (Fig. 2B). A significant degree of conservation is also found with the Bicoid-related homeodomains of mouse Otx1 and Otx2, *Drosophila* Gsc and Bcd and other proteins that belong to the large group of Paired-related homeoproteins (Bopp et al., 1986; Bürglin, 1993), such as the *Drosophila* proteins Paired (Prd), Gooseberry (Gsb), Aristaless (Al) and Orthopedia (Otp) (Fig. 2B). Paired-related homeodomains have either a serine (Pax subgroup), glutamine

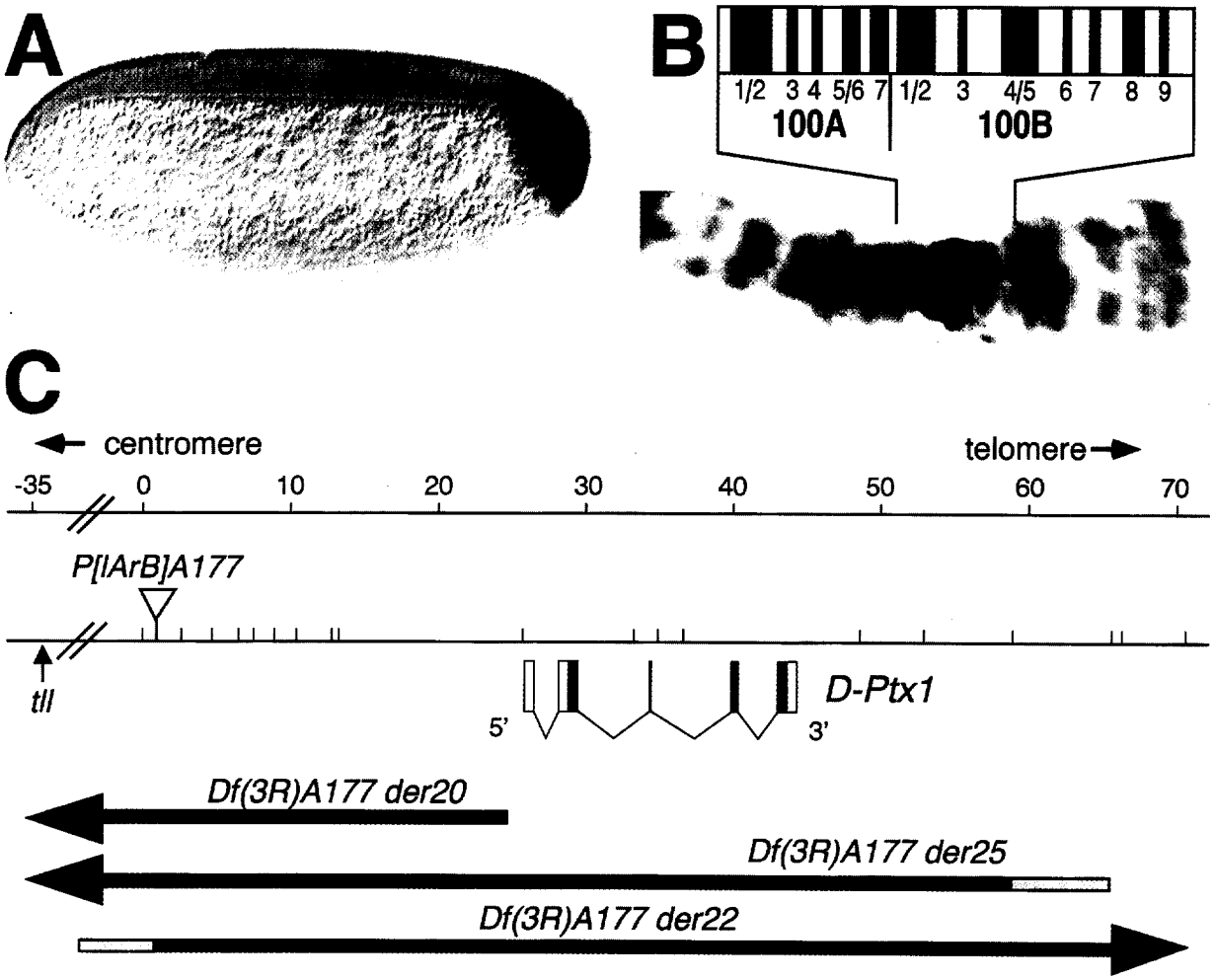

Fig. 1. Molecular characterization of the *D-Ptx1* locus. (A) In situ hybridization and expression of the DIG-labeled cosmid JHD5:32D1, which includes part of the *D-Ptx1* gene, at the onset of gastrulation. (B) In situ hybridization of *D-Ptx1* cDNA to salivary gland chromosomes localizes it to band 100B1,2 close to the right telomere of the third chromosome. (C) Physical map of the 100B1,2 region. The exons of the near full-length *D-Ptx1* cDNA (coding region in red, non-translated leader and trailer in yellow) are shown with respect to an *Eco*RI restriction map of a cloned genomic region above (scale in kb) and the deficiencies *Df(3R)A177der20*, *Df(3R)A177der25* (Justice et al., 1995) and *Df(3R)A177der22* (generated and kindly provided by P. Bryant) below. While deleted regions are indicated by the dark blue segments of the arrows, their light blue portions delimit the region that includes the breakpoint. The proximal breakpoint of *Df(3R)A177der22* is located between the insertion site of the P element *P[lArB]A177* (Justice et al., 1995) and the *tailless* (*tll*) gene indicated by a vertical arrow.

(Mhox/Phox1-related), or lysine (Bicoid subgroup) at position 9 of the third α-helix of the homeodomain (Frigerio et al., 1986; Bürglin, 1993), which is critical for specificity in DNA sequence recognition (Hanes and Brent, 1989; Treisman et al., 1989). Thus, D-Ptx1, Ptx1 and Unc-30 define a small subfamily of highly conserved Bicoid-related homeodomain proteins, which may indicate that they are homologs of each other. In *Drosophila* and mice, Bicoid-related homeoproteins are known to be involved in the formation of anterior structures (Cohen and Jürgens, 1990; Simeone et al., 1993), or exhibit an expression domain that is consistent with a patterning function in the anterior region, as in the case of Ptx1 (Lamonerie et al., 1996). However, D-Ptx1 is an exception because its posterior expression pattern seems to preclude such a function (see below).

2.3. *D-Ptx1 transcript and protein expression patterns*

Analysis of the RNA and protein expression patterns indicated that *D-Ptx1* gene activity is restricted to a small number of tissues and developing organs in the embryo (Fig. 3). As expected for a homeodomain transcription factor, the D-Ptx1 protein is localized in the nucleus (Fig. 3P).

While no expression of *D-Ptx1* was observed before cellular blastoderm (Fig. 3A), its transcripts were first detectable at this stage in the most posterior region of the embryo (Fig. 3B) that corresponds to the anlagen of the posterior midgut and presumably of the Malpighian tubules (Skaer, 1993). Before embryonic stage 7 (staging according to Campos-Ortega and Hartenstein, 1985), no antibody staining was detectable, which might reflect the long transcrip-

(A)

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  1 MDRSSAVGLR RGRRSGSGRG HQLGHGLGTG WPDQRGRRSG QWRTQRIGAM SAESTGLCLQ DLVSAGTANG
 71 AGSAGSAESA TTTSTALSSG STGSSTVNGG GSSTSGAEHL HSHHSLHDSS SSVSISPAIS SLMPISSLSH
141 LHHSAGQDLV GGYSQHPHHT VVPPHTPKHE PLEKLRSLFF SVWAETGDFR DSHSSMTAVA NSLDSTHLNN
211 FQTSSTSSIS NRSRDRKDGN RSVNETTIKT ENISSSGHDE PMTTSGEEPK NDKKNKRQRR QRTHFTSQQL
281 QELEHTSSRN RYPDMSTREE IAMWTNLTEA RVRVWFKNRR AKWRKRERNA MNAAVAAADF KSGFGTQFMQ
351 PFADDSLYSS YPYNNWTKVP SPLGTKPFPW PVNPLGSMVA GNHHQNSVNC FNTGASGVAV SMNNASMLPG
421 SMGSSLSNTS NVGAVGAPCP YTTPANPYMY RSAAEPCMSS SMSSSIATLR LKAKQHASAG FGSPYSAPSP
491 VSRSNSAGLS ACQYTGVGVT DVV
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(B)

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                                        helix 1          helix 2          helix 3

D-Ptx1   (D.m.)   QRRQRTHFTS QQLQELEHTS SRNRYPDMST REEIAMWTNL TEARVRVWFK NRRAKWRKRE
Ptx1     (M.m.)   ---------- -------A-F Q--------M -----V---- --P------- ---------- 90%
Unc-30   (C.e.)   P--------- H--T---NWF --------AC -----V-IS- --P------- ---------- 80%

Otx1,2   (M.m.)   ---E--T--R S--DV--ALF AKT----IFM ---V-LKI-- P-S--Q---- -----C-QQQ 57%
Gsc      (D.m.)   K--H--I--E E--EQ--A-F DKTH---VVL --QL-LKVD- K-E--E---- --------QK 55%
Bcd      (D.m.)   P--T--T--- S-IA---QHF LQG--LTAPR LADLSAKLA- GT-Q-KI---- ---RRHKIQS 37%

Prd      (D.m.)   ---C--T-SA S--D---RAF E-TQ---IY- ---L-QR--- ----IQ---S ----RL--QH 60%
Gsb      (D.m.)   ---S--T-SN D-IDA--RIF A-TQ---VY- ---L-QS-G- -----Q---S ----RL--QL 57%

Al       (D.m.)   ---Y--T--- F--E---KAF --TH---VF- ---L--KIG- ----IQ---Q --------Q- 68%
Otp      (D.m.)   -K-H--R--P A--N---RCF -KTH---IFM ------RIG- --S--Q---Q ------K--K 62%
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Fig. 2. Amino acid sequence of D-Ptx1 protein and classification of its homeodomain. (A) Amino acid sequence of the D-Ptx1 protein derived from a nearly full-length cDNA. The homeodomain is underlined. (B) Comparison of the D-Ptx1 homeodomain with the homeodomains of Ptx1 (Lamonerie et al., 1996) and Otx1, Otx2 of the mouse (Simeone et al., 1993), Unc-30 of *C. elegans* (Jin et al., 1994) and Gsc (Goriely et al., 1996; Hahn and Jäckle, 1996) and Bcd of *Drosophila* (Frigerio et al., 1986). These homeodomains belong to the Bicoid-related subgroup of the much larger group of Paired-related homeodomains, which includes, among others, the homeodomains of Paired (Prd; Frigerio et al., 1986), Gooseberry (Gsb; Bopp et al., 1986), Aristaless (Al; Schneitz et al., 1993) and Orthopedia (Otp; Simeone et al., 1994). The extent of the three α-helices are indicated by horizontal bars, the lysine (K) at position nine of the third α-helix, characteristic for Bicoid-related homeodomains, is boxed, and the percentage of amino acid identities are indicated in the right margin. The cDNA sequence of *D-Ptx1* is deposited in the EMBL data base (accession No. AJ001519).

tional delay before the processed transcript is translated. However, beginning with stage 7, transcript and protein patterns were identical throughout embryogenesis. Expression of D-Ptx1 is maintained in the invaginating posterior midgut, which extends posteriorly during germ band elongation and the extended germ band stage (Fig. 3C,D). D-

Fig. 3. *D-Ptx1* expression during *Drosophila* embryogenesis as revealed by in situ hybridization (A–C), anti-D-Ptx1 (D–I, L–P) or anti-LacZ antibody staining (J, K) to whole mount embryos. No expression is detectable prior to and during syncytial blastoderm (A). Initial zygotic expression is restricted to the anlage of the posterior midgut at the posterior end of the embryo (B) and subsequently to the posterior midgut invagination of early stage 8 embryos (C). Note that the neighboring proctodeum, the anlage of the hindgut (Campos-Ortega and Hartenstein, 1985), does not stain. Embryos at the extended germ band stage (stage 11; D, E) and during germ band retraction (stage 12; F) show expression in the developing CNS in a metameric pattern and in the posterior part of the outgrowing posterior portion of the developing midgut. Stage 13 embryos (G, H) indicate strong expression in the posterior midgut before its fusion with the anterior midgut. During stage 15, the expression in the midgut is restricted to the second midgut constriction (I; see also close up in P). (J–O) Expression during stage 16. (J, K) Expression in ventral muscles. The enlargement (K) of panel (J) shows expression in the thoracic and first abdominal segments of a transgenic line, *D-Ptx1:lacZ*, expressing the *lacZ* gene under the control of a 12-kb *Eco*RI fragment including the *D-Ptx1* promoter and enhancers. Note that anti-lacZ staining is cytoplasmic rather than nuclear, which allows a better identification of the muscle cells that express it, whereas the D-Ptx1 protein is localized in the nucleus, an observation that is consistent with its function as a transcription factor. In the first thoracic segment, expression is restricted to ventral inter segmental muscles 1 and 3 (VIS1 and VIS3; Bate, 1993), in the second thoracic segment to ventral oblique muscle 2 (VO2) and ventral longitudinal muscle 3 (VL3), in the third thoracic segment to VO2, VO3, VL3, and VL4, in the first abdominal segment to VO5, VL3, and VL4, in the second to seventh abdominal segment to VO6 and VL4, and in the eighth abdominal segment probably to ventral acute muscle 1 (VA1) and VO1. (L) Enlarged ventral view of D-Ptx1 expression in the CNS. Lateral (M), dorsal (N) and ventral views (O) of the head region, demonstrating D-Ptx1 expression in various portions of the developing brain and nervous system. (P) Restricted expression of D-Ptx1 in the second midgut constriction. All embryos (A–J) are shown as lateral views, except (E) and (H) which are ventral views. Anterior is to the left.

Fig. 4. *D-Ptx1* expression at cellular blastoderm requires *tailless*-dependent *fork head* activity. *D-Ptx1* expression in wild-type (A, C), homozygous fkh^{XT6} (B), and tll^{1-49} (D) embryos is revealed by in situ hybridization with a digoxigenin-labeled probe (blue staining) and related to *spalt* (*sal*) expression (C, D) visualized by a biotin-labeled probe (purple). Note that *sal*-expression in the posterior domain (salPD) and in the anterior horse-shoe domain (salHD) is dependent on *tll* activity whereas the *sal* anterior expression domain (salAD) is not (D; Kühnlein et al., 1997). *D-Ptx1* expression at the posterior pole is absent in embryos lacking either *fkh* or *tll* activity.

Ptx1 protein continues to be expressed during germ band retraction in the most anterior portion of the developing posterior midgut as it is pushed anteriorly to fuse with its anterior counterpart (Fig. 3F). Before and during fusion at stage 13, the protein accumulates in the most anterior portion of the posterior midgut (Fig. 3G,H), which gives rise to the second midgut constriction during stage 15 (Fig. 3I,P). The protein is also expressed in all four Malpighian tubules, a kidney-like organ that forms at the midgut/hindgut boundary (Skaer, 1993; Hoch et al., 1994), when they begin to form during stage 11 (not shown). D-Ptx1 remains expressed at the second midgut constriction and in the Malpighian tubules until the end of embryogenesis.

D-Ptx1 expression is also found in a segmentally repeated

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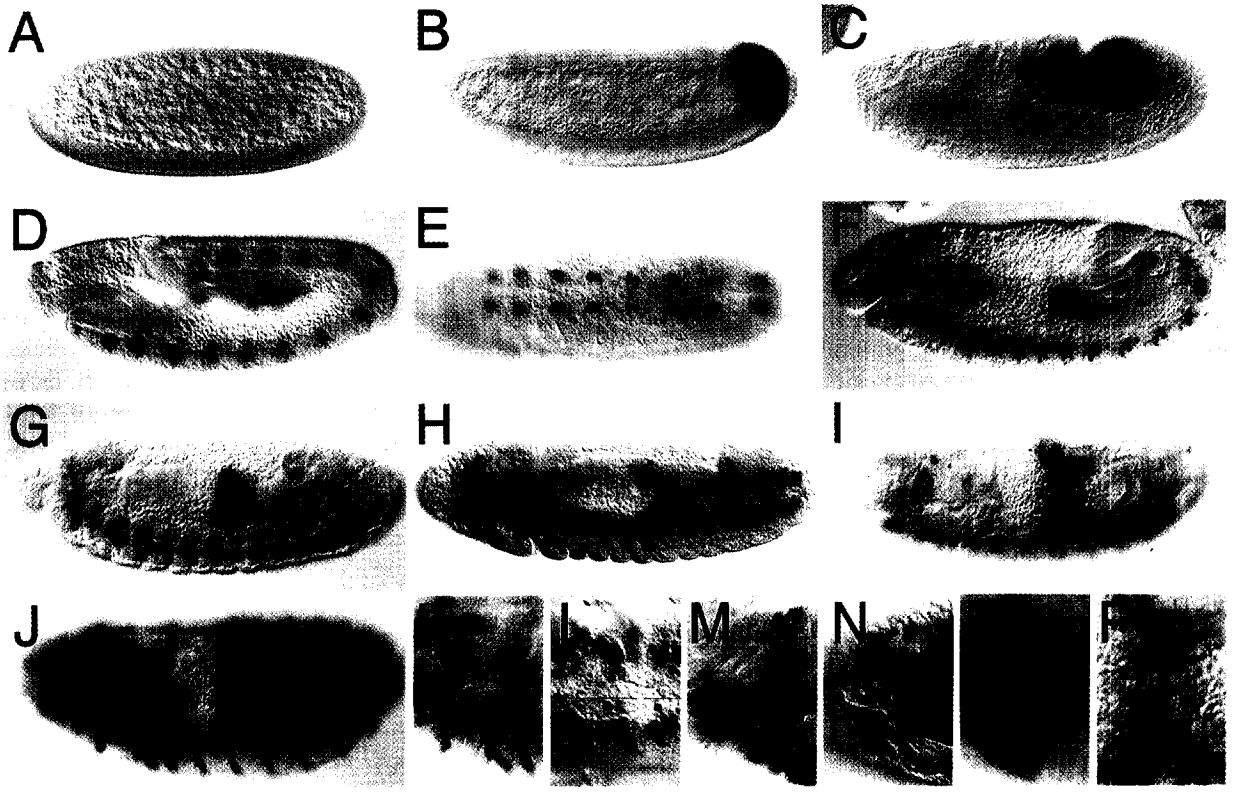

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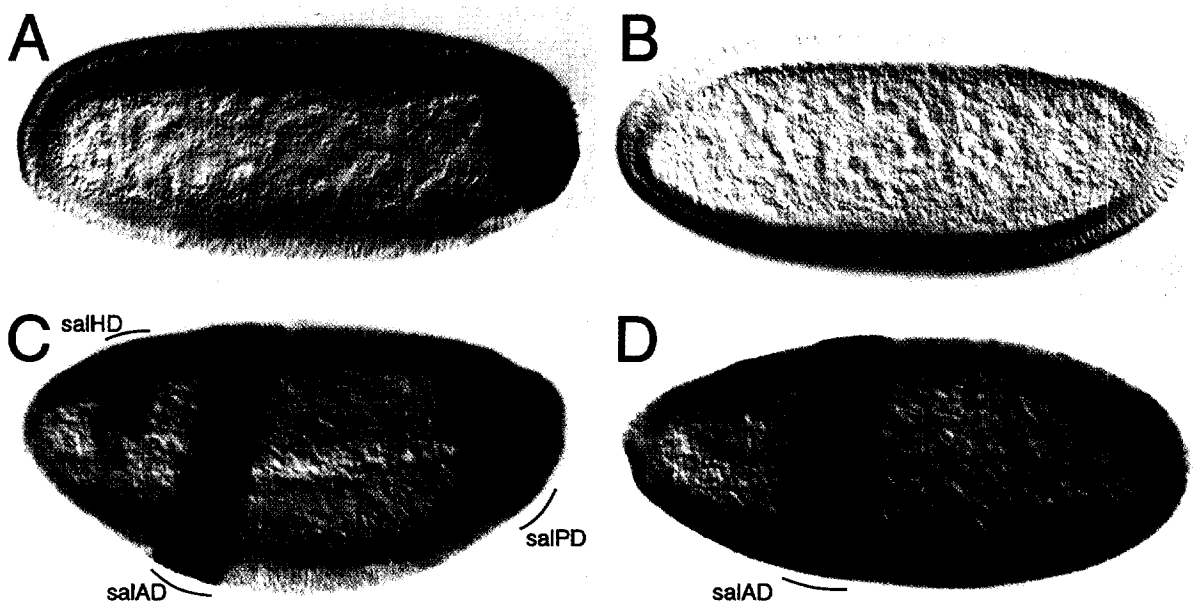

pattern in the developing CNS beginning at stage 10 (Fig. 3D–G,L). In addition, expression occurs in the brain in a position anterior to the anlage of the optic lobe as well as in a number of sensory organs of the head region, including the light sensory organ (Fig. 3M-O), termed Bolwig's organ (Schmucker et al., 1992). Finally, D-Ptx1 expression is found in a subset of somatic muscle precursor cells that give rise to ventral larval muscles (Fig. 3J,K; for a detailed description of the muscle pattern, see Bate, 1993).

2.4. Blastoderm D-Ptx1 expression depends on fork head activity

The initial expression of *D-Ptx1* in a posterior cap of the blastoderm embryo suggested that it is regulated by maternal pattern organizing activities, namely by the posterior system acting through *nanos* and/or the terminal system acting through the *torso*-dependent Ras/Raf signal transduction pathway (St Johnston and Nüsslein-Volhard, 1992). In embryos lacking the activity of the posterior maternal organizer system, *D-Ptx1* is normally expressed (not shown). In contrast, when *torso* is removed maternally, *D-Ptx1* expression is absent (not shown). Hence, activation of *D-Ptx1* does not depend on the posterior, but only on the terminal maternal organizer system. To further investigate which of the two terminal gap genes, *huckebein* (*hkb*) and *tll*, zygotically activated by the Torso-signalling pathway (Weigel et al., 1990), is required for *D-Ptx1* activation, its expression was examined in *hkb* and *tll* mutant embryos. While *D-Ptx1* is expressed in *hkb* embryos in a pattern indistinguishable from that of wild-type embryos (Fig. 4A,C), it fails to be activated in *tll* embryos (Fig. 4D). Similarly, no expression is observed when the *fork head* (*fkh*) gene, a target of the terminal gap genes (Weigel et al., 1990), is inactivated (Fig. 4B). It may be noteworthy that *D-Ptx1* expression is observed only at the posterior pole although *fkh* is expressed in both anterior and posterior regions of the embryo (Weigel et al., 1989). Therefore, activation of *D-Ptx1* either requires additional factors absent in the anterior region or its activation is overruled in the anterior region by a repressor.

2.5. D-Ptx1 lacks an obvious morphoregulatory function during embryogenesis

Using two combinations of deficiencies, *Df(3R)A177-der20/Df(3R)A177der22* and *Df(3R)A177der25/Df(3R)-A177der22*, which either express or fail to express the *D-Ptx1* gene (Fig. 1C), we were able to examine the phenotypes caused by the lack of *D-Ptx1* activity. The expression patterns of marker genes, such as those of *tll* (Pignoni et al., 1990) and *caudal* (Mlodzik et al., 1985; Macdonald and Struhl, 1986) in the posterior region, and the striped expression patterns of pair-rule and segment polarity genes, such as those of *fushi tarazu* (Hafen et al., 1984) and *engrailed* (Poole et al., 1985), were all not affected by the absence of *D-Ptx1* activity. Furthermore, the primordia of the hindgut, midgut, and Malpighian tubules developed normally. We also found that the second midgut constriction and the muscle pattern, as revealed by histochemical staining with anti-myosin heavy-chain antibodies (Kiehart et al., 1990), formed properly. In addition, the larvae developed a normal cuticle pattern with the exception of pattern defects posterior to the eighth abdominal segment. However, control experiments with transheterozygous *Df(3R)A177der20/Df(3R)A177der22* embryos revealed that this defect is caused by a gene proximal to *D-Ptx1* (Fig. 1C). Unfortunately, we could not assess whether the loss of *D-Ptx1* activity is lethal because *Df(3R)A177der20/Df(3R)A177-der22* embryos, which served as controls, die during embryogenesis.

Taken together, these results indicate that *D-Ptx1* does not play a role in segmentation or in any other morphological aspects of the development of those tissues and organs where it is normally expressed. A possible exception might be the nervous system, which is difficult to assess on the basis of morphological criteria. However, the lack of *D-Ptx1* did not affect the normal expression of the neural marker Mab22C10 (Zipursky et al., 1984) nor did it cause detectable changes in the overall architecture of the embryonic nervous system (data not shown).

Finally, we examined whether ectopic *D-Ptx1* expression interferes with proper embryonic development, which might allow us to derive a possible physiological function. To this end, *D-Ptx1* was expressed ubiquitously from a heat-inducible transgene during blastoderm and subsequent stages of embryogenesis (see Section 3). The heat-shocked embryos were again examined in the same manner as were the mutants that lack *D-Ptx1* activity. However, we could not detect any apparent morphological defects of these embryos in segmentation, myogenesis, or gut development (data not shown). Thus, *D-Ptx1* lack-of-function and gain-of-function phenotypes consistently indicated that the newly identified homeodomain protein has no obvious morphoregulatory function during embryogenesis. Therefore, we conclude that *D-Ptx1* is required for morphogenetic functions later during development or for physiological aspects not addressed by our analysis, or that its function is largely redundant.

2.6. Conclusions

The D-Ptx1 homeodomain shares a particularly high homology with the homeodomains encoded by *Ptx1* and *unc-30*. Expression of the mouse gene *Ptx1* is restricted to the pituitary of embryos and adults. It is initially expressed throughout Rathke's pouch at day 10.5 and continues to be expressed in the corresponding cell lineage where it becomes restricted to a subpopulation of POMC-expressing cells in the anterior pituitary lobe (Lamonerie et al., 1996). Functional analyses have suggested that Ptx1 acts as a tran-

scriptional activator of the POMC gene. The early expression pattern of Ptx1 during embryogenesis was further taken as evidence that it may also play a role in pituitary formation (Lamonerie et al., 1996). Despite the high degree of sequence similarity to Ptx1, the function of the *unc-30* gene of *C. elegans* differs in important aspects. While Ptx1 is not expressed in neural tissues, Unc-30 is both expressed in and required for development of the inhibitory GABAergic type D neurons, which control locomotion (Jin et al., 1994). Furthermore, ectopic expression of *unc-30* induced GABA synthesis in cells that are normally not GABAergic (Jin et al., 1994). Hence, the available evidence suggests that *unc-30* controls the terminal differentiation of type D neurons, in which it induces GABA synthesis. Thus, it appears that the only common features of *Ptx1* and *unc-30* consist in their regulation of the synthesis of secreted hormonal or transmitter substances and in their possible requirement for the differentiation of the cells producing these substances. Our lack- and gain-of-function analyses of *D-Ptx1* would have been unable to reveal such functions in *Drosophila* embryos.

An alternative explanation is that the *D-Ptx1* gene carries a developmental function whose absence could be compensated by redundant functions of one or several other genes. One might argue that in this case it would be surprising that ectopic expression of *D-Ptx1* does not interfere with normal development of the embryo. Yet, ectopic expression of *unc-30* does not result in a morphologically discernible phenotype either. Therefore, we favor the proposal that *D-Ptx1* controls physiological functions, such as transmitter or hormone production, whose absence do not cause an altered morphology.

3. Materials and methods

3.1. Drosophila stocks

The three deficiency stocks, *Df(3R)A177der20, ry^{506} e/ TM3, Sb, Df(3R)A177der22/TM6B, Tb ca* and *Df(3R)A177-der25/TM6B, Tb ca*, were generated as described (Justice et al., 1995). The *fkh^{XT6}* and *tll^{L49}* mutant stocks were obtained from the Tübingen stock collection.

3.2. General procedures

Standard procedures such as the isolation of genomic DNA, screening of genomic and cDNA libraries, chromosomal walking, whole genome Southern and Northern blot analysis, and in situ hybridization to salivary gland chromosomes were carried out essentially as described (Frei et al., 1985; Kilchherr et al., 1986).

3.3. Molecular characterization of the D-Ptx1 locus

The genomic walk of the region shown in Fig. 1C was initiated to clone the *discs overgrown* (*dco*) gene (Zillian et al., in prep.) and extends a previously described walk distally (Justice et al., 1995). The cosmid JHD5:32D1 (Hoheisel et al., 1991), which includes the 3′ moiety of the *D-Ptx1* gene, was isolated by serendipity and spans the region between about 32 kb and 73 kb of the genomic walk (Fig. 1C). cDNA clones were isolated from embryonic cDNA-libraries with genomic DNA probes, derived from the cosmid JHD5:32D1 and the chromosomal walk, that revealed the in situ hybridization signal shown in Fig. 1A. DNA fragments isolated from genomic or cDNA clones were subcloned into Bluescript vectors (Stratagene), and cDNAs and all intron/exon boundaries of the genomic DNA were sequenced with a DNA sequencer of Applied Biosystems Inc.

3.4. Preparation of anti-D-Ptx1 antibodies

A 1593 bp *Pst*I-*Nco*I cDNA fragment, encoding the 455 C-terminal amino acids of the putative 513 amino acid D-Ptx1 protein, was cloned into the His-tag expression vector pRSET (Invitrogen) and used to transform *E. coli* BL21(DE3). Upon induction, a novel protein of the predicted size of 62 kDa was expressed and purified on Pro-Bond™ Resin (Invitrogen) according to the manufacturer's protocol and separated from contaminants by SDS-PAGE. Polyacrylamide slices containing 400 μg of recombinant protein were cut out from the gel and used for immunization of rabbits (Eurogentec). The antiserum was pre-absorbed with fixed devitellinized embryos overnight and used at a final dilution of 1:2000.

3.5. Analysis of RNA and protein expression patterns

In situ hybridization to whole-mount embryos using digoxigenin-labeled DNA probes was performed according to Tautz and Pfeifle (1989) with modifications of the labeling reaction. Double labeling in situ hybridization to whole-mount embryos with digoxigenin-labeled and biotinylated probes was performed as described (Hartmann and Jäckle, 1995). Antibody stainings for D-Ptx1 protein or β-galactosidase were performed with the use of a Vectastain ABC Elite horseradish peroxidase kit as described (Macdonald and Struhl, 1986).

3.6. Construction of D-Ptx1-lacZ transgenic fly stocks

A 12 kb genomic *Eco*RI fragment, located upstream and adjacent to the 5′ end of the longest *D-Ptx1* cDNA, was cloned into the pCaSpeR hs43 lacZ vector (Thummel and Pirrotta, 1992) and the recombinant plasmid was introduced into *Drosophila* by P-element-mediated germline transformation. Several independent transformant lines were established. Expression of the *lacZ* gene was the same in all lines and identical to that of the wild-type *D-Ptx1* gene.

3.7. Construction of heat-shock inducible D-Ptx1 transgenic stock and ubiquitous expression of D-Ptx1

The construct for the heat-inducible expression of *D-Ptx1* was prepared by insertion of the longest *D-Ptx1* cDNA into the *Eco*RI site of the pCaSpeR-hs vector, which permits expression of an open reading frame under control of the *hsp70* promoter (Thummel and Pirrotta, 1992). After P-element-mediated germline transformation with this construct (Rubin and Spradling, 1982), five lines with a *hsp70-D-Ptx1* transgene insertion were obtained. For heat shock-induced expression of Ptx1 during blastoderm stages, embryos were collected for 30 min after egg deposition at 18°C and allowed to develop at 18°C for an additional 1 h before they were heat shocked for 10 min at 37°C and returned to 18°C as previously described (Hartmann et al., 1997).

For heat shock experiments at postblastoderm stages, embryos were collected for 1 h after egg deposition at 25°C and subsequently kept at 18°C, heat shocked three times for 15 min at 37°C with 15-min intervals of recovery at 18°C in between. Embryos were then returned to 18°C and examined at various stages after the heat-shock treatment. No phenotype in the nervous system, gut, or somatic muscle pattern was detected after staining with Mab22C10 and α-MHC antibodies. Also no cuticule phenotype was observed, and the heat-shocked embryos developed into hatched first instar larvae with no obvious defects. The experiments were repeated twice with four transgenic lines.

Heat shock-induced *D-Ptx1* expression was confirmed by staining with anti-D-Ptx1 antibodies within 15 min after the heat shock treatment.

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