Functions for *Drosophila brachyenteron* and *forkhead* in mesoderm specification and cell signalling

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Accepted 1 July; published on WWW 23 August 1999

SUMMARY

The visceral musculature of the larval midgut of *Drosophila* has a lattice-type structure and consists of an inner stratum of circular fibers and an outer stratum of longitudinal fibers. The longitudinal fibers originate from the posterior tip of the mesoderm anlage, which has been termed the caudal visceral mesoderm (CVM). In this study, we investigate the specification of the CVM and particularly the role of the *Drosophila* Brachyury-homologue *brachyenteron*. Supported by *fork head*, *brachyenteron* mediates the early specification of the CVM along with *zinc-finger homeodomain protein-1*. This is the first function described for *brachyenteron* or *fork head* in the mesoderm of *Drosophila*. The mode of cooperation resembles the interaction of the *Xenopus* homologues *Xbra* and *Pintallavis*. Another function of *brachyenteron* is to establish the surface properties of the CVM cells, which are essential for their orderly migration along the trunk-derived visceral mesoderm. During this movement, the CVM cells, under the control of *brachyenteron*, induce the formation of one muscle/pericardial precursor cell in each parasegment. We propose that the functions of *brachyenteron* in mesodermal development of *Drosophila* are comparable to the roles of the vertebrate *Brachyury* genes during gastrulation.

INTRODUCTION

Many of the factors that play important roles in the ontogenesis of different taxa share high structural similarities in functionally relevant domains, such as DNA-binding or protein-protein interaction domains. These similarities allow factors to be grouped into families such as homeodomain proteins or Wnt proteins (for reviews see e.g. McGinnis and Krumlauf, 1992; McMahon et al., 1992). In some exceptional cases, members of these families are associated with the regulation of a particular developmental process. For instance, the Pax-6 subfamily of the Pax proteins are possibly involved in the regulation of photoreceptor development in all bilaterians, suggesting that this function was adopted early during evolution (for a review see Gehring, 1996). Another case are the gene products of the Hox complex, a subfamily of homeodomain proteins, which are involved in patterning the anteroposterior axis in most – if not all – higher metazoans (for a review see Slack et al., 1993). Often, a common function in development is far less obvious, in spite of high structural similarities of the factors within one particular subfamily, making it difficult to distinguish between original and secondarily acquired functions. Such factors might have been assigned to the regulation of multiple developmental processes during the evolution of different taxa and some of the original functional aspects might have been lost. In addition, the complexity of an organism might mask common functions, making detailed functional analyses necessary.

Such a dynamic functional diversification has recently been proposed for the *Brachyury* genes. The Brachyury proteins are a subfamily of the T-domain transcription factors, which are classified by a highly conserved DNA-binding domain (for reviews, see Smith, 1997; Papaioannou and Silver, 1998). Brachyury relatives have been found in the diploblastic Cnidarian as well as triploblasts like chordates, protocordates, echinoderms, hemichordates and insects, suggesting that evolutionarily conserved mechanisms are regulated by these factors (Peterson et al., 1999; Technau and Bode, 1999). However, initial comparisons of the expression and function of *Brachyury* genes between deuterostomes and protostomes indicated diverse rather than common functions. In deuterostomes, *Brachyury* seemed to play a critical role in mesoderm development, whereas the protostome Brachyury proteins have been shown to be involved in hindgut development. For instance, in vertebrates, *Brachyury* is transiently expressed in all nascent mesendodermal cells. As development proceeds, the expression becomes restricted to the notochord and tailbud. In cephalochordates and urochordates,
the distinct aspects of mesodermal expression have been divided between two paralogues, one expressed in the posterior mesoderm and the other expressed in the notochord, whereas no expression is detectable in the gut (Yasu et al., 1996). In echinoderms, Brachyury has only been reported to be expressed in the migrating secondary mesenchyme cells (Yasu et al., 1995). However, it has recently been shown that the hemichordate Brachyury homologue is not only expressed in the mesoderm but also in the oral and anal region of the gut (Tagawa et al., 1998; Peterson et al., 1999). Since Brachyury is transiently expressed in vertebrates in the developing hindgut epithelium of the tailbud region, it has been proposed that the expression of Brachyury in the posterior gut reflects its original function in development. Intriguingly, the Hydra Brachyury gene is expressed in the cells of the gut opening (Technau and Bode, 1999).

The Drosophila Brachyury homologue brachycenteron (byn) is essential for the development of hindgut, anal pads and Malpighian tubules (Kispert et al., 1994; Singer et al., 1996). byn is activated by the terminal gap gene tailless (tll; Pignoni et al., 1990) in a region of 0-20% egg length of the syncytium (0% = posterior tip). With completion of cellularization, the byn expression becomes downregulated in the posteriormost cap of the embryo, which will later form the posterior midgut, by the terminal gap gene huckebein (hkb, Weigel et al., 1990). Thus, the expression of byn is confined to a ring of cells from about 10-20% egg length. The dorsal and the lateral aspects of that ring correspond to the proctodeum from which the hindgut, the anal pads and the Malpighian tubules later develop (for details see Skaer, 1993). Intriguingly, hkb also determines the posterior extent of the ventral mesoderm primordium by repressing the mesodermal determinant snail (sna; Brönner et al., 1994; Reuter and Leptin, 1994). This suggests that the ventralmost aspect of byn expression might comprise the posterior tip of the mesoderm primordium. Recently, it has been reported that a population of cells develops from the posterior region of the mesoderm, which migrate in an orderly movement anteriorly and eventually form an outer layer of longitudinal muscle fibers surrounding the midgut (Georgias et al., 1997; Campos-Ortega and Hartenstein, 1997). The cells have been termed caudal visceral mesoderm (CVM; Nguyen and Xu, 1998) in order to distinguish them from the progenitors of the inner sheet of circular muscles of the midgut (for an overview see Bate, 1993). The latter are recruited from 11 parasegmentally arranged clusters of dorsal mesoderm in the trunk region and were therefore referred to as trunk visceral mesoderm (TVM).

In this study, we wished to elucidate the role of byn and other genes in the specification of the CVM during early embryogenesis. In addition, we addressed the byn-mediated functions of the CVM during later stages of development. The CVM is specified through the interaction of byn and fork head (fkh; Weigel et al., 1989a,b) and high levels of the gene product of zinc-finger homeodomain protein 1 (zf1-1; Fortini et al., 1991; Lai et al., 1991; Broihier et al., 1998). Additionally, byn confers specific adhesive and signalling properties on the CVM which are prerequisite for the inductive and migratory behaviour of the CVM. In the light of these findings, the role of byn in the mesoderm of Drosophila is reminiscent of proposed mesodermal functions of vertebrate Brachury genes.

MATERIALS AND METHODS

Fly stocks

byn<sup>1</sup> and byn<sup>5</sup>, intermediate and strong hypomorphic brachycenteron alleles, respectively (Singer et al., 1996). Df(2L)JE116(R)GW11: used as snail deficiency (Asshburner et al., 1990). twi<sup>F55a</sup>; amorphic twist allele (Simpson, 1983; Thissen et al., 1987); zfh-1<sup>2</sup>: allele of zinc-finger homeodomain gene 1 (Lai et al., 1993). hkb<sup>W5</sup>; amorphic huckebein allele (Brönner et al., 1994). tll<sup>10</sup> and Df(3R)N10: strong hypomorphic tailless allele and deficiency of the tll locus, respectively (Sprecker et al., 1988; Jürgens et al., 1984; Pignoni et al., 1990). fkh<sup>XT6</sup>; small deficiency of the fork head locus (Weigel et al., 1989a). byn fkh: double mutant, generated by meiotic recombination of byn<sup>2</sup> and fkh<sup>XT6</sup>. croc-lacZ: transgene that directs lacZ reporter gene expression in the CVM under the control of a 7 kb BynII fragment from the 3'-region of the croc transcription unit (Häcker et al., 1995). cpo-lacZ: P<sub>fry</sub>; cpo10kb2A, enhancer trap in the couch potato (cpo) locus that confers β-galactosidase (β-gal) expression in the developing longitudinal muscle fibers (Bellen et al., 1992; Campos-Ortega and Hartenstein, 1997). hkb<sup>DD26</sup>: amorphic heartless allele (Gisselbrecht et al., 1996). dpp<sup>57</sup>; amorphic dpp allele (Hoffmann and Goodman, 1987). G4472: GAL4 enhancer trap that directs expression in the CVM from stage 10 onward (Georgias et al., 1997). twi-GAL4: transgene on the second chromosome for mesodermal GAL4 expression (Giebel et al., 1997). UAS-lacZ: transgene for the detection of GAL4 activity in the embryo (Brand and Perrimon, 1993).

For the generation of UAS-byn transgenes, the byn cDNA (Kispert et al., 1994) was isolated from the pNB40 vector (Brown and Kafatos, 1988) as a HindIII-EcoRI fragment, cloned into pBluescript KS II (+), isolated again as a driver line were crossed into the hemichordate allele (Simpson, 1983; Thisse et al., 1987). The transgene comprises a 2.2 kb genomic subclone of the byn gene (Kispert et al., 1994) in a region of 0-20% egg length of the syncytium (0% = posterior tip). With completion of cellularization, the byn expression becomes downregulated in the posteriormost cell cap of the embryo, which will later form the posterior midgut, by the terminal gap gene huckebein (hkb, Weigel et al., 1990). Thus, the expression of byn is confined to a ring of cells from about 10-20% egg length. The dorsal and the lateral aspects of that ring correspond to the proctodeum from which the hindgut, the anal pads and the Malpighian tubules later develop (for details see Skaer, 1993). Intriguingly, hkb also determines the posterior extent of the ventral mesoderm primordium by repressing the mesodermal determinant snail (sna; Brönner et al., 1994; Reuter and Leptin, 1994). This suggests that the ventralmost aspect of byn expression might comprise the posterior tip of the mesoderm primordium. Recently, it has been reported that a population of cells develops from the posterior region of the mesoderm, which migrate in an orderly movement anteriorly and eventually form an outer layer of longitudinal muscle fibers surrounding the midgut (Georgias et al., 1997; Campos-Ortega and Hartenstein, 1997). The cells have been termed caudal visceral mesoderm (CVM; Nguyen and Xu, 1998) in order to distinguish them from the progenitors of the inner sheet of circular muscles of the midgut (for an overview see Bate, 1993). The latter are recruited from 11 parasegmentally arranged clusters of dorsal mesoderm in the trunk region and were therefore referred to as trunk visceral mesoderm (TVM).

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**RESULTS**

**Development of the longitudinal muscle fibers surrounding the midgut**

The visceral musculature surrounding the midgut consists of two layers: the inner layer of circular fibers and the outer, longitudinally oriented fibers. During germ band retraction and midgut closure, the progenitors of these latter fibers, the so-called caudal visceral mesoderm (CVM), perform an ordered movement that can be subdivided into three phases. The first migratory phase starts at early germ band retraction when the cells begin to move anteriorly from their position at the posterior tip of the mesodermal germ layer and split into two tightly packed, bilaterally symmetrical clusters on each side of the posterior midgut primordium (Fig. 1A,B). When these clusters have reached the anterior tip of the posterior midgut primordium, the cells detach from each other and disperse anteriorly as two rows along the germ band, the second phase of the migration. During this movement, the cells are arranged along the dorsal and ventral edge of the midgut primordia and are in close contact with the band of progenitors of the circular muscle fibers. The band seems to serve as a migration substratum (Georgias et al., 1997; Fig. 1C,D). During the last phase of the migration, which takes place as the midgut encloses the yolk, the progenitors of the longitudinal muscle fibers spread regularly over the underlying circular muscle fibers (Fig. 1E-G). The cells acquire a spindle shape, then stretch in an anteroposterior direction and form about 16-20 regularly spaced longitudinal muscle fibers. These fibers reach from the proventriculus to the midgut-hindgut transition where the ureters of the Malpighian tubules insert (Fig. 1G-I). The foregut and the hindgut lack any longitudinal muscles and are solely covered by the inner layer of circular muscles (Fig. 1H,I).

**byn and ifkh are essential for the specification of the caudal visceral mesoderm**

Previous studies have shown that byn is expressed at the blastoderm stage in a ring of cells at the posterior terminus of fusion protein was sent to Eurogentec (Seraing, Belgium) for the immunization of rabbits.

**In situ detection of mRNA, proteins and of apoptotic cells**

For detection of zfh-1 transcripts a 0.4 kb EcoRI fragment of the zfh-1 transcription unit was used (kindly provided by Dr Z. Lai). The 412 retrotransposon was used to detect gonadal mesoderm (Brookman et al., 1992). The probes were labelled with digoxigenin-dUTP (Boehringer, Mannheim, Germany) and used for in situ hybridization as described by Tautz and Pfeifle (1989). Protein was detected in situ using standard immunohistochemical techniques with avidin-biotinylated peroxidase complexes. For the dissection of guts, devitelinated embryos were filleted with a glass needle under PBS. The gut was then laid out on the glass, fixed with 4% formaldehyde for 15 minutes at room temperature and processed for immunostaining as usual. The following antibodies were used in the study: anti-FasIII (Snow et al., 1989), anti-Mef2 (rabbit; Lilly et al., 1996), anti-muscle MHC (rabbit 722; Kiehart and Feghali, 1986), anti-Eve (rabbit, Frisch et al., 1987), anti-α-Tubulin (mouse monoclonal 3A5; Wolf et al., 1988), anti-Vasa (rabbit, Brohier et al., 1998), anti-Srp (Riechmann et al., 1997), anti-Kr (Gaul et al., 1987) and anti-β-galactosidase (mouse polyclonal; Sigma, St. Louis, USA).

For sectioning, immunostained embryos were embedded in Araldite and cut on a Leica microtome RM 2065 in slices of 10 mm thickness. The sections were then transferred to a glass slide and covered with Araldite and a coverslip.

Apoptotic cells were detected by end-labelling the DNA of fragmented chromatin by the TUNEL assay (see White et al., 1994).

**Microscopy**

Embryos were mounted and oriented in polymerizing Araldite, the dissected guts in glycerol. Pictures were either taken on a Zeiss Axioshot on Kodak Ektachrome 64T film and then digitized by transfer to Kodak PhotoCDs or recorded by a Kontron ProgRes 3008 digital camera on a Zeiss Axioplan. The figures were assembled in Adobe Photoshop.
the embryo, which is posteriorly delimited by hkb (Kispert et al., 1994, see also Fig. 2A). The posterior border of the mesoderm anlage is also set by the repressor function of hkb, suggesting that the ventral aspect of the byn expression domain comprises the posteriormost region of the mesoderm anlage. In fact, this aspect is internalized by the ventral furrow and forms the posterior end of the mesodermal germ layer shortly after the onset of gastrulation (Fig. 2B). The byn RNA expression ceases in these cells when the germband extends (data not shown; see also Kispert et al., 1994), but an anti-Byn antiserum can detect the Byn protein far longer. We were able to follow the Byn-expressing cells until they migrate anteriorly as progenitors of the longitudinal muscle fibers at late stage 11 (Fig. 2C,D). This observation confirms the proposed caudal origin of the progenitors of the longitudinal visceral muscle fibers (Georgias et al., 1997), and thus it seems suitable to adopt the suggested name caudal visceral mesoderm (CVM) for this mesodermal cell population (Nguyen and Xu, 1998).

We investigated the specification of the CVM and monitored its fate by the detection of Byn protein or the expression of CVM-specific markers like croc-lacZ and cpo-lacZ (Häcker et al., 1995; Campos-Ortega and Hartenstein, 1997). The initial byn expression at the posterior pole is regulated by tll and hkb (Kispert et al., 1994). Thus it was likely that the CVM cells are specified under the control of the same genes. In fact, in hkb embryos, the size of the CVM primordium is enlarged and comprises more cells than normal (Fig. 3A,B). This corroborates the notion that the CVM primordium constitutes the most posteriorly located mesoderm primordium. tll expression reaches more anteriorly than the hkb domain and encompasses the primordia of the proctodeum and of the CVM. One would therefore expect that the formation of the CVM is entirely dependent on tll. Indeed, this is the case: the CVM is missing in tll mutant embryos (Fig. 3C). Part of the function of tll seems to be mediated by byn. In byn mutants, a significantly reduced number of CVM cells is seen, and these few cells form clusters that are less compact and migrate significantly slower than in wild type (Fig. 4B,D). Later, they fail to contact the TVM and do not distribute along the germband (Fig. 4F). During stage 11, most of the cells acquire a condensed appearance resembling apoptotic bodies (Fig. 4D,F). We detect a high level of apoptosis in the proctodeum of byn embryos as well as in the posteriormost mesoderm (Fig. 4H). By stage 13, cells with the properties of the CVM are not detectable any longer in the mutants (data not shown) and, as expected from that, the dissected midguts of byn embryos lack the outer, longitudinal muscle fibers (Fig. 4I).

byn embryos show morphological aberrations at a time before the CVM begins to migrate anteriorly (Singer et al., 1996; Fig. 4H). The severely shortened hindgut causes a significant shift in the spatial relationship of the various primordia at the posterior region of the embryo and thereby might indirectly affect the migration of the CVM. In order to exclude such an indirect influence, we generated byn embryos expressing byn in the CVM precursors, but not in the hindgut (Fig. 4L). In such embryos, the CVM survives and disperses virtually the same as in wild type along the TVM, whereas the proctodeum remains rudimentary as in ordinary byn mutants (compare Fig. 4L,N). These results demonstrate that the defective migration and the death of the CVM cannot be attributed to the disordered
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brachyenteron function in the mesoderm

We therefore conclude that byn in the mesoderm is essential for the adhesive and migratory properties of the CVM precursors. byn cannot be the only gene that mediates the function of tll in the specification and further development of the CVM since the lack of tll causes a far stronger phenotype than the lack of byn. In addition to byn, the gene fkh is known to act downstream of tll in the posterior gut (Weigel et al., 1990; Casanova, 1990). fkh is expressed in a large domain at the posterior pole (Weigel et al., 1989) that encompasses the byn expression domain including the ventral, mesodermal aspect. In fkh mutants, the CVM specification seems less impaired than in byn mutants: the number of CVM cells is initially quite normal. However, as in byn mutants, the cells fail to migrate along the germ band (Fig. 3E) although differentiation of the migration substratum, the TVM, is not affected (data not shown). By stage 14, most of the CVM cells have been eliminated by apoptosis. On this level of analysis, fkh mutants resemble embryos homozygous for weak byn alleles. However, the phenotype of byn fkh double mutants shows that byn and fkh either have distinct functions in the specification of the CVM or act synergistically. In double mutants, no CVM cells are distinguishable (Fig. 3F), just as in tll mutants (Fig. 3C). Therefore, the function of tll in the specification of the CVM appears to be mediated by byn and fkh.

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Fig. 4. byn is essential for migration and survival of the CVM. CVM development in (A,C,E,G,I) wild type and (B,D,F,G,J) byn mutants. (A,B) The CVM cells (arrowheads) of a (B) hypomorphic byn4 embryo form less-condensed clusters and migrate significantly slower than in (A) wild type. Both embryos are at the same developmental stage and are immunostained for Byn protein (dorsal view). (C,D) Embryos carrying croc-lacZ stained for β-gal (dorsal view). The number of CVM cells is strongly reduced in (D) byn2 mutants. (E,F) Magnification of the tail region of stage 12 embryos double-labelled for FasIII (brown) and β-gal (blue). (E) In the wild type, the CVM cells (blue, white arrowheads) disperse along the TVM (brown, black arrowheads) whereas they fail to contact the TVM in (F) a byn2 embryo. (G,H) Apoptotic cells detected by the TUNEL assay. (H) Massive apoptosis is seen in the proctodeum (arrow) and in the CVM (open arrowhead) of byn2 embryos. (I,J) Dissected guts at stage 16 immunostained for MHC protein. (I) In wild-type midguts, the cell bodies of the longitudinal muscle fibers are regularly spaced (arrowheads). (J) No longitudinal muscle fibers can be detected in the byn2 mutant. The circular muscle layer shows sporadic ruptures (grey arrowheads). No midgut constrictions are formed. (K-N) Rescue of CVM development of byn embryos by the forced expression of byn in the CVM primordium. (K) An embryo double heterozygous for the GAL4 driver G447 and UAS-byn. Endogenous Byn protein marks the hindgut (arrow; also see insert), whereas the G447-controlled expression of Byn from the UAS-byn transgene is seen in the CVM (white arrowheads). (L) A byn mutant carrying G447 and UAS-byn and stained for Byn protein. The CVM cells have survived and migrate along the TVM almost as in wild type, while the hindgut is still rudimentary (arrow; see insert). (M) Expression of the G447 driver detected by β-gal. (N) Non-transgenic byn5 mutant stained for Byn protein.
**byn and fkh act synergistically in the specification of the caudal visceral mesoderm**

We asked whether byn and/or fkh would be sufficient in the mesoderm to specify the development of the CVM. Therefore, we expressed either byn, fkh, or a combination of both genes throughout the mesoderm shortly after gastrulation. In the first case, after unrestricted mesodermal byn expression, a few CVM-like cells emerge from the mesoderm of the head region (Fig. 5A). We did not observe any gross changes in the specification of other mesodermal derivatives such as somatic mesoderm, fat body or TVM (data not shown, but also see below). In contrast, the ectopic expression of fkh within the mesoderm does not lead to the formation of additional CVM-like cells (Fig. 5E). Rather, the differentiation of other mesodermal derivatives like somatic mesoderm and the TVM is severely affected (K.-P. Rehorn and R. R., unpublished). This might explain why in these embryos the CVM cells only distribute over the posterior midgut primordium where the cells later undergo apoptosis (data not shown). Intriguingly, the combined overexpression of byn and fkh leads to a drastic increase in the number of anteriorly derived CVM-like cells (Fig. 5F). Hence, fkh strongly enhances the effects of byn in the specification of mesodermal cells as CVM.

Only the anterior and the posterior mesoderm are competent to be specified by byn in conjunction with fkh as CVM (Fig. 5A,F). Therefore, at least one other gene must exist that confines the competence to form CVM to these two regions. A good candidate for this gene is zinc finger homeodomain protein-1 (zfh-1; Lai et al., 1991). At the blastoderm stage, zfh-1 is expressed in high levels in the terminal regions of the mesoderm including the primordium of the CVM (Fig. 5I) and zfh-1 is essential for the migration of the CVM (Broihier et al., 1998): in zfh-1 mutant embryos, CVM-specific gene expression such as croc-lacZ is deleted (Fig. 5H). From the restricted effects of ectopic byn/fkh, we propose that the two genes are capable of specifying CVM development only in the region of high zfh-1 expression. zfh-1, byn and fkh act in parallel downstream of tll. High levels of caudal zfh-1, as for byn and fkh, are dependent on tll (Fig. 5J), and there is no crossregulation between zfh-1, byn and fkh (Fig. 5L; data not shown; Singer et al., 1996; Kispert et al., 1994).

**The caudal visceral mesoderm is specified independent of twist function**

The overlapping expressions of the two zygotic genes twist (twi) and snail (sna) are essential for gastrulation and specification of...
the mesoderm (for a review see Leptin, 1995). twi is an activator of mesodermal gene expression, whereas sna mainly functions as a repressor of neuroectodermal gene expression in the mesoderm. Deviating from this rule, mesodermal zfh-1 expression is missing in sna mutants (Hemavathy et al., 1997), whereas high zfh-1 expression is unaffected in the termini of the mesoderm in twi mutants (Lai et al., 1991; see also Fig. 6G). The expression of byn and posterior fhk does not depend on sna or twi function (data not shown), raising the question whether the CVM might be at least partially specified in twi or sna mutants.

In sna embryos, no byn-expressing cells can be detected in a mesoderm-specific position, i.e. between the epidermis and the midgut epithelium. Such embryos lack CVM-specific gene expression (data not shown), which is in accordance with the findings that zfh-1 depends on sna (Hemavathy et al., 1997) and CVM development on zfh-1 (Broihier et al., 1998). Strikingly, in twi mutants, byn-expressing cells can be found at an internal, mesoderm-typical position in the tail region (Fig. 6B,D). It is not clear how the cells find their way into the twi embryos, which are characterized by the failure to form a ventral furrow. The cells probably immigrate after they have been internalized together with the adjoining posterior gut anlagen. Later the cells begin to express CVM-specific marker genes and undergo the first phase of the normal migration movement: they arrange as two clusters ventrolaterally to the posterior midgut (Fig. 6F; data not shown). The cells initiate the second phase of migration as well; they become migratory, disperse and acquire the typical spindle shape of normal CVM cells. However, most likely because of the absence of their putative migration substratum, the TVM, they merely spread over the posterior midgut in twi embryos. Thus, the CVM is not only internalized during gastrulation independent of twi function, but also acquires at least some of its adhesive and migratory properties. This view is consistent with the finding that the expressions of byn, fhk and zfh-1, which are required for the specification of the CVM, are not affected in twi mutants.

**byn specifies surface properties of the caudal visceral mesodermal cells**

The defects in the CVM of a byn mutant suggest that byn is not only involved in the early specification of the CVM, but also plays an essential role in establishing the adhesive and migratory properties of the CVM cells. These do not properly form the two bilaterally symmetrical clusters and later fail to disperse along the TVM in byn mutants (Fig. 4B,D,F). Moreover, the ubiquitous mesodermal byn expression strongly affects the normal migratory behaviour of the CVM (Fig. 5A-D). The cells do not exclusively migrate along the TVM towards the anterior (Fig. 5D), but attach to any other cell in the mesoderm. As a consequence, migration is not restricted dorsoventrally and the cells do not reach the anterior half of the midgut (Fig. 5B). In these experimental embryos, it is impossible to physically separate splanchnopleura and somatopleura, since they are firmly attached to each other (data not shown). Normally this is not the case. We conclude from these observations that ectopic byn expression changes the adhesive properties of the mesoderm.

The specific effects of ectopic byn on the surface properties of other mesodermal cells also include the rescue of the germ cell migration defect of byn mutants. Normally, the germ cells pass through the posterior midgut epithelium, as it becomes mesenchymal prior to germ band retraction. From the basal side of the endoderm, the germ cells migrate to the adjoining mesoderm of the body wall. Later, they migrate anteriorly and intermingle with the somatic gonadal mesoderm (Fig. 7A). It has been suggested that the CVM plays an important role in directing or facilitating this transition of the germ cells to the mesoderm (Broihier et al., 1998). During the first phase of migration, the CVM cell clusters are located at a position where the germ cells pass through the epithelium of the posterior midgut before they migrate to the gonadal mesoderm. In byn mutants, the germ cells pass through the midgut epithelium as in wild type, but virtually all cells distribute over the ventral surface of the posterior midgut rather than contacting the mesoderm (Fig. 7B). byn is neither expressed in the germ cells nor in the gonadal mesoderm and the latter develops normally in byn mutants (data not shown).

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**Fig. 6.** twist is dispensable for the internalization and specification of the CVM. CVM development is shown for (A,C,E) wild-type and (B,D,F,G) byn embryos by the detection of (A-D) Byn protein, (E,F) croc-lacZ expression or (G) zfh-1 RNA. (A) In the wild-type embryo, the CVM migrates along the germ band while Byn protein expression diminishes (arrowhead). (B) In a byn embryo, Byn-expressing cells are distinguishable in the position from which the CVM originates. (C,D) Transversal sections through the posterior region of the germ band. In (C) wild type and (D) byn mutants, the CVM (arrowheads) is located between hindgut epithelium and epidermis. (E,F) Embryos carrying the croc-lacZ reporter were double-labelled for β-gal (black) and for FasIII (brown) protein in order to follow the specification of the CVM (arrowhead) and the TVM, respectively. (E) In wild type, the migrating CVM is closely attached to the TVM. (F) In byn mutants, the CVM expresses croc-lacZ and spreads over the midgut. (G) zfh-1 expression is detectable in the CVM of a byn embryo (arrowhead). All embryos are at stage 11, except for C (stage 10).
Therefore, the observed phenotype is most likely due to defects in a byn-dependent signalling or adhesive properties of the CVM. This idea is supported by our finding that ubiquitous expression of byn in the mesoderm rescues the defective germ cell migration in byn mutants almost completely (Fig. 7C): only a few germ cells do not coalesce with the somatic gonadal precursors. Thus, either the few rescued CVM cells of byn embryos at the normal position close to the migrating germ cells, or the adhesive or signalling properties that ectopic byn confers to other mesodermal cells, are sufficient for the transition of the germ cells from the midgut to the gonadal mesoderm.

byn-dependent signalling from the caudal visceral mesoderm

There are a number of mesodermal tissues that do not properly develop in embryos lacking the CVM, as in byn, fkh or till embryos. For instance, the TVM develops aberrantly in byn mutants during late stages of embryogenesis. Although the inner layer of circular muscles differentiates in the absence of the CVM as in wild type, the morphogenesis of this layer does not proceed properly. The nuclei of the TVM are normally arranged as one broad band on each side of the midgut during germband retraction and subsequently split into two bands when the midgut primordia meet at stage 13. During this movement, the nuclei pass the rows of CVM cells, which are located at the dorsal and the ventral edge of the midgut primordium, respectively (Fig. 4K, and data not shown). In a byn mutant, however, the movement of the TVM nuclei is irregular, so that their organization into bands is lost and they become distributed over the entire gut circumference (Fig. 7E,F). Since byn is never expressed in the TVM, we conclude that the proper arrangement and integrity of the circular muscle fibers requires the presence of the CVM. The irregular dorsoventral extension of the fibers results in an incomplete closure of the layer and the circular muscle layer of the midgut in byn embryos shows sporadic ruptures (Fig. 4J). These defects might be the reason why the three constrictions that normally subdivide the midgut tube into four gastric chambers are not normally subdivided by the midgut tube. Thus, either the few rescued CVM cells of byn embryos at the normal position close to the migrating germ cells, or the adhesive or signalling properties that ectopic byn confers to other mesodermal cells, are sufficient for the transition of the germ cells from the midgut to the gonadal mesoderm.

Fig. 7. The CVM is required for germ cell migration, midgut morphogenesis, and dorsal mesoderm patterning. (A-C) Gonad development in (A) wild type, (B) byn mutants or (C) byn mutants after enforced mesodermal byn expression. Magnifications of the tail region are shown after immunostaining for Vasa protein to visualize the primordial germ cells (PGC, embryos in dorsal view at stage 14). (A) In the wild type, the PGC (arrowheads) have reached the gonadal mesoderm. (B) Most of the PGC fail to do so in the byn embryo and are distributed over the basal side of the posterior midgut epithelium. (C) In byn mutants expressing byn under the control of twi-GAL4, most of the PGC have reached the gonads. Dissected guts of (D) a wild-type embryo and (E,F) byn mutants at stage 15 (D,E) and 16 (F) immunostained for Mef-2 protein. (D) In the wild-type midgut, nuclei of the longitudinal muscle fibers are regularly spaced and organized in rows (arrowheads). The nuclei of the TVM form four bands, one of which is visible (bracket). (E,F) The nuclei of the longitudinal muscle fibers are absent in the byn mutant, while the nuclei of the TVM become irregularly distributed. (G-I) Somatic muscles and heart of (G) wild type, (H) byn mutants or (I) byn mutants after enforced expression of byn in the CVM primordium (embryos at stage 16 stained for MHC, dorsal view). (G) The split arrowhead indicates the heart and the outlines of the dorsal muscle DA1 are marked with white dots. (H) In the mutant, the two rows of cardiac cells do not meet and DA1 is missing in numerous hemisegments. (I) These defects can be considerably restored by the rescue of the CVM by byn expression under the control of G447.
that can be followed from stage 10 on by their *even-skipped* (*eve*) expression (Frasch et al., 1987). Three cells per hemisegment begin to express *eve* in each of 11 dorsal clusters in the mesoderm (Fig. 8A). By stage 12, the number of mesodermal *eve* cells increases by one in each cluster. We found that this additional *eve* cell appears in succession from posterior to anterior clusters (Fig. 8A, B). Furthermore, we noted that the cells of the CVM pass the mesodermal *eve* clusters at a distance of about one cell diameter as they migrate anteriorly along the TVM (Fig. 8C). Shortly after the time when the leading edge of the CVM had passed, the fourth *eve* cell is added to the cluster. This addition occurs towards the CVM and by recruitment from neighbouring cells rather than by cell division (data not shown). Most importantly, the temporal and spatial correlation between the appearance of the fourth *eve* cell and the migration of the CVM is not a mere coincidence. In *byn*, *til* or *zfh-1* mutants in which the CVM fails to migrate anteriorly or is absent, the number of *eve* cells does not increase during germband retraction (Fig. 8E, G; data not shown). We propose that this is the primary defect in the dorsal mesoderm that causes the defects in heart that this is the primary defect in the dorsal mesoderm that causes the defects in heart that this is the primary defect in the dorsal mesoderm that causes the defects in heart.

**Fig. 8.** *byn* is responsible for a signal coming from the CVM that is essential for dorsal mesoderm development. (A-C) Embryos carrying *croc-lacZ* were double-labelled for β-gal (brown) and Eve protein (blue). (A) The CVM has passed *eve* cluster No. IX in which a 4th cell becomes visible (grey arrow). All *eve* clusters anterior to No. IX consist of 3 cells. (Dorsolateral view, early stage 11; the panel is generated from two different focal planes about 1 cell apart.) (B) All *eve* clusters anterior to No. VI (black arrow) comprise 3 cells. The leading edge of the CVM has almost reached cluster No. III (stage 12 embryo). (C) A ventral view reveals that the CVM cells pass the *eve* clusters in a distance of about 1 cell (bracket). (D-J) Magnification of the mesodermal *eve* cells in (D,F,H) wild-type, (E,G,I) *byn* or (J) *byn* embryos with specific rescue of the CVM. (D,E) At stage 12, there are 4 *eve* cells (arrow) in wild type (D) and 3 *eve* cells in the (E) *byn* mutant. (F) At stage 14, the DA1 precursors (grey arrowhead) separate ventrally from the pericardioblasts. (G) In a *byn* mutant, *eve* expression appears weaker and the number of the *eve* cells is reduced. (H) The heart of a stage 17 embryo in a dorsal view. By then, Eve protein is only visible in pericardial cells. (I) The heart of a *byn* mutant has not formed properly and less pericardial cells are detectable. (J) The rescue of the CVM of a *byn* mutant restores morphogenesis of the dorsal vessel and the number of pericardial cells has increased. (K,L) *eve* cells (black) and CVM (brown) in embryos ectopically expressing *byn* throughout the mesoderm. (K) Early stage 11. In proximity of the CVM (white arrowhead) a dorsal band of the *eve* cells (arrow) has formed. (L) Late stage 11. The strip of *eve* cells has further thickened in proximity of the CVM and extended to the entire dorsal trunk mesoderm. (M,N) *eve* expression in (M) *htl* embryos and (N) *htl* embryos with ubiquitous *byn* in the mesoderm. (M) No *eve* cells are seen in the *htl* mutant. (N) *htl* is epistatic to the formation of additional *eve* cells by ectopic mesodermal expression of *byn* (grey arrowheads). Dorsal views of early stage 11 embryos.
Fig. 9. Model for the early specification of the CVM. The CVM is specified by byn, fkh and high levels of zfh-1. The expression of zfh-1 generally requires sna, whereas high levels of zfh-1 in the CVM are depending on tll function. tll is also required to activate the expressions of byn and fkh in the CVM. byn and fkh act synergistically in the specification of the CVM, i.e. fkh enhances the action of byn. hkb sets the posterior border of the CVM anlage by repressing sna and byn.

(Fig. 8K-L). Initially, these additional cells only appear close to the CVM, i.e. in the posterior half of the experimental embryos (Fig. 8K). Later, they also fill the gaps between the anterior eve clusters, to which the CVM fails to migrate upon ubiquitous mesodermal byn expression (compare Fig. 5B), and form a band of cells along the entire dorsal mesoderm (Fig. 8L). Only the dorsal mesoderm appears to be competent to (directly or indirectly) respond to byn. This notion is supported by our finding that, in htl embryos that specifically lack derivatives of the dorsal mesoderm (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997), ubiquitous mesodermal expression of byn does not lead to ectopic eve expression (Fig. 8M,N).

DISCUSSION

The specification of the caudal visceral mesoderm: a cooperation between byn and fkh

The ventral aspect of the byn expression domain comprises the primordium of the caudal visceral mesoderm (CVM), which forms the longitudinal muscle fibers surrounding the midgut and influences dorsal mesodermal development during its migration along the germ band. The specification of the CVM occurs under the control of the genes byn, fkh and zfh-1 (Fig. 9). The CVM primordium is not specified in either zfh-1 mutants or byn fkh double mutants. Moreover, the unrestricted expression of byn and fkh in the mesoderm leads to the formation of ectopic CVM. This ectopic CVM is only found when high levels of zfh-1 are present and we therefore assume that the dose of zfh-1 matters.

The genes tll, hkb and sna act upstream of byn, fkh and zfh-1. sna formally functions as an activator of zfh-1 and probably sets the dorsoventral boundary of the CVM primordium. Astonishingly, the CVM initially develops independently of the tll gene product, which otherwise is essential for early mesoderm development. This tll independence is consistent with the finding that posterior zfh-1 expression is present in tll mutants (compare Figs 5K and 6G). The CVM cells are internalized in tll mutants and even acquire a number of CVM characteristics, such as expression of markers like cpo-lacZ or croc-lacZ, spindle shape and migratory behaviour (Fig. 6F, and data not shown); however, the terminal differentiation to muscles does not occur. The CVM also does not form fibers since proper morphogenesis certainly requires the migration along the TVM, which is missing in twi mutants.

tll has a central role in setting up the CVM primordium. tll is not only required for the strong zfh-1 expression in the posterior mesoderm region (Fig. 5J), but also for the expression of byn. In addition, the expression of fkh is severely reduced in tll mutants and is solely activated by hkb. As a consequence, fkh is not expressed in the posterior part of the mesoderm primordium. hkb in turn limits the CVM primordium towards the posterior in more than one way. It represses sna at the posterior terminus and thereby indirectly zfh-1, and it also represses byn. We suggest that the CVM is derived from the very posterior end of the ventral furrow since these cells express byn and since both the ventral furrow and the CVM primordium are expanded in hkb mutants.

byn and fkh act in parallel in the specification of the CVM. In single mutants, the CVM still partially develops while, in the double mutant, it is entirely absent. The consequences of overexpression of the two genes in the mesoderm suggest that fkh primarily enhances the effect of byn in the specification. The unrestricted mesodermal expression of fkh shows no discernable effect on the formation of CVM (Fig. 5E). This suggests that fkh does not have the capacity to repress the anterior mesoderm since, in this experiment, the situation in the anterior mesoderm (i.e. fkh and high zfh-1) should be created as is present in the posterior mesoderm of byn embryos. Ectopic byn triggers the formation of a few CVM cells in the anterior tip of the mesoderm (Fig. 5A). Only the combined overexpression of the two genes leads to a large number of CVM-like cells in the anterior region of the embryo (Fig. 5F). Unfortunately, it is impossible to show that these cells have the full potential to become longitudinal muscle fibers since other requirements for their development, such as the formation of the CVM, are severely disturbed by ectopic fkh. Nevertheless, the results indicate that fkh enhances the effects of byn in directing mesodermal development. This mode of cooperativity is reminiscent of the functional logic found between the fkh homologue Pintallavis (Ruiz i Altaba and Jessell, 1992) and the byn homologue Xbra (Smith et al., 1991) in mesoderm specification of Xenopus. The two genes are co-expressed in the mesoderm primordium and in the dorsal mesoderm. The misexpression of Pintallavis in animal pole explants does not lead to the formation of ectopic mesodermal derivatives (Ruiz i Altaba and Jessell, 1992; Ruiz i Altaba et al., 1993), while ectopic Xbra only causes the formation of ventral mesoderm (Cunliffe and Smith, 1992). However, when both genes are simultaneously misexpressed in animal caps more ventral and also dorsal mesoderm is formed (O’Reilly et al., 1995). Thus, a similar functional relationship might exist between the two genes in mesoderm development of Drosophila and Xenopus. Both code for members of highly conserved DNA-binding protein families and thus might even act on homologous target genes in insects and vertebrates.

brachyenteron establishes adhesive and migratory properties of the caudal visceral mesoderm

byn is not only relevant for the specification of the CVM in conjunction with fkh. byn also establishes the surface properties of the cells. The primary defect in the mesoderm of byn mutants is a change in cell adhesion of the CVM rudiment as compared to wild type. The CVM cells fail to form properly the two bilateral clusters and later to contact the TVM. Thus, byn is
essential for some form of cell association and we propose that it acts as a transcriptional regulator of the responsible adhesion molecules. All the other defects observed, the failure to distribute along the germband and the apoptosis, might be of secondary nature. Moreover, the unrestricted mesodermal byn expression severely affects the proper migration of the CVM along the TVM (Fig. 5B,D). In such experimental embryos, the cells are able to migrate, but fail to reach the anterior half of the embryo. Instead of distributing along the TVM, the cells attach to splanchnopleura as well as somatopleura without dorsoventral restriction. We propose that ectopic byn expression changes the adhesive properties of other mesodermal cells to the probably homotypic form of adhesion, which might be the basis for the byn-dependent formation of the two clusters during the first phase of the CVM migration.

Regulation of cell adhesion and migration might be a general feature of the Brachyury-type T domain transcription factors. Studies on the function of the mouse Brachyury gene have provided evidence that Brachyury affects the morphogenetic movements of nascent mesodermal cells by influencing their surface properties. Isolated mesodermal cells of Brachyury mice show a decreased aggregation and migration rate (Yanagisawa and Fujimoto, 1977; Hashimoto et al., 1987), and the extracellular matrix of these cells is defective (Jacobs-Cohen et al., 1983). These defects are cell autonomous: Brachyury mutant ES cells, which are introduced into wild-type embryos, accumulate in the primitive streak and later in the tail bud (Beddington et al., 1992; Wilson et al., 1995). Furthermore, Brachyury orthologues are expressed in migrating mesodermal cells in other taxa such as echinoderms and enteropneustes (Yasu et al., 1995; Peterson et al., 1999). Our observations in Drosophila suggest that one original and evolutionary conserved function of Brachyury proteins is to confer particular adhesive and migratory properties to mesodermal cells. Given this evolutionary conservation, the identification of byn target genes coding for cell surface molecules might give insights in the function of Brachyury proteins in early mesodermal development.

**brachyenteron is required for the inductive properties of the caudal visceral mesoderm**

Our data revealed an hitherto unknown inductive process during Drosophila development: the specification of a single cell per hemisegment within the dorsal mesoderm by the passing CVM. This cell is the fourth of a group of eve-expressing cells, of which two cells later form the dorsal muscle DA1 and two become pericardial cells. In mutants lacking the CVM, this fourth eve cell is not added to the original triplet and, as a consequence, the number of pericardial cells and DA1 cells is reduced. We propose that these defects are caused by an impaired induction based on the following observations. (i) The CVM passes the dorsal eve cells prior to the addition of the fourth cell. During this passage, the distance appears close enough for a signal from the CVM to trigger the recruitment of the fourth eve cell (Fig. 8A-C). (ii) A common feature of byn, tll or fkh mutants is that they lack the CVM (Fig. 3) and none of these genes are expressed in the dorsal mesoderm itself. (iii) No matter which marker we used to trace the cells of the CVM, for instance, lacZ under the control of the GAL4-driver G447 (Georgias et al., 1997; compare Fig. 4M), they did not become located to the dorsal mesoderm. (iv) The expression of byn directed by G447 not only restores the defective migration of the CVM, but also rescues the dorsal mesoderm patterning and the heart defects in a byn mutant (Figs 4L, 7I, 8J).

We had one tool to manipulate the induction besides the mutants lacking the CVM, i.e., forced byn expression. Ubiquitous mesodermal byn expression led to a significant increase of eve-expressing cells at the dorsal rim of the mesoderm (Fig. 8K,L). This is even the case in tll mutants, which lack the CVM entirely (data not shown). We conclude that byn in the mesoderm is sufficient to trigger the production of the signal that normally emerges from the CVM. This would constitute the third distinguishable function of byn in the development of the CVM besides participation in the specification and the regulation of the CVMs adhesive properties. We rule out the possibility that byn is directly involved in transcriptionally activating eve in the dorsal mesoderm, since byn is normally never expressed in the eve clusters. Instead, we propose that byn regulates the expression of the ligand in the signalling process. byn can only exert this function on mesodermal cells, since a strictly ectodermal misexpression of byn (for instance, under the control of the Zkg-GAL8-driver; Frasch, 1995) has no effect on mesodermal eve expression (data not shown). In fact, only cells in the neighbourhood of the eve cells begin to express eve upon ubiquitous mesodermal byn expression, indicating that the competence to perceive the byn-mediated signal is dictated by contact to other eve cells (Fig. 8K,L).

The nature of the emerging signal and of its receptor are unknown. The mesoderm-specific Drosophila FGF-receptor HtI is essential for the formation of the eve clusters (Beiman et al., 1996; Gisselbrecht et al., 1996), and also for the byn-mediated formation of supernumerary mesodermal eve cells (Fig. 8N). However, HtI is already required for the early step of the migration of the mesoderm to dorsal positions where the cells become specified as dorsal mesoderm by a Dpp signal (Frasch, 1995). Later, HtI is more directly involved in the specification of heart and dorsal muscle progenitors within the dorsal mesoderm (Michelson et al., 1998). We have not yet separated these two functions of HtI with regard to the signalling from the CVM. Nevertheless, it is tempting to speculate that HtI and a corresponding growth factor are involved in the signalling from the CVM. In Xenopus, the byn homologue Xbra acts as direct activator of the eFGF gene and, in the mouse, FGF-4 seems to be activated by Brachyury (Casey et al., 1998). FGF-4 in turn has been shown to induce cardiogenesis in the so-called precardiac mesoderm, a primordium that is specified by the Dpp homologue BMP-2 (Lough et al., 1996). If indeed byn activated a yet unidentified FGF gene in the CVM that signals via HtI to the dorsal, pericardiac mesoderm, this would represent a considerable degree of conservation of inductive properties mediated by Brachyury homologues in mesoderm development.

We thank H. Thelen for expert technical assistance and Drs. H. Bellen, M. Frasch, M. Fuller, B. Giebel, U. Häcker, U. Hinz, D. Kiehart, R. Lehmann, A. Paululat, K.-P. Rehorm, V. Riechmann and T. Seher for sending fly stocks or reagents. We are indebted to E. Foley, T. Klein, F. Sprenger and R. Wilson for critical reading the manuscript. The work was supported by the Deutsche Forschungsgemeinschaft as part of the SFB 243 and by the Fond der Chemischen Industrie.

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