Interacting functions of snail, twist and huckebein during the early development of germ layers in Drosophila

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SUMMARY

Two zygotic genes, snail (sna) and twist (twi), are required for mesoderm development, which begins with the formation of the ventral furrow. Both twi and sna are expressed ventrally in the blastoderm, encode transcription factors and promote the invagination of the ventral furrow by activating or repressing appropriate target genes. However, sna and twi alone do not define the position of the ventral furrow, since they are also expressed in ventral cells that do not invaginate. We show that huckebein (hkb) sets the anterior and the posterior borders of the ventral furrow, but acts by different modes of regulation. In the posterior part of the blastoderm, hkb represses the expression of sna in the endodermal primordium (which we suggest to be adjacent to the mesodermal primordium). In the anterior part, hkb antagonizes the activation of target genes by twi and sna. Here, bicoid permits the co-expression of hkb, sna and twi, which are all required for the development of the anterior digestive tract. We suggest that mesodermal fate is determined where sna and twi but not hkb are expressed. Anteriorly hkb together with sna determines endodermal fate, and hkb together with sna and twi are required for foregut development.

Key words: Drosophila, germ layer, gastrulation, mesoderm, endoderm, fate map, gene regulation

INTRODUCTION

The germ layers in Drosophila are formed by two morphogenetic movements during gastrulation. The mesoderm and the anterior endoderm are formed by the invagination of the ventral furrow and the posterior endoderm is formed by the invagination of the amnioprotocoele. The cells remaining at the surface of the embryo constitute the ectoderm. They later will form mainly epidermis and neural tissue, while the endodermal cells will form most of the midgut and the mesodermal cells form muscles, heart, gonads and fatbody.

The primordia of the germ layers in the blastoderm have been mapped by various techniques (Poulson, 1950; Underwood et al., 1980; Hartenstein et al., 1985; Foe, 1989). The most ventral cells between 10 and 80% egg length contribute to the mesoderm while the posterior cap of the embryo constitutes the posterior endoderm primordium, also called the posterior endodermal primitive (Fig. 1A). Anterior to the PMG primordium resides the primordium of the proctodeum. It is thought to consist of a ring of cells around the entire circumference of the embryo which separate the PMG from the mesoderm primordium on the ventral side (Technau and Campos-Ortega, 1985). The proctodeal primordium invaginates together with the prospective PMG (Fig. 1B) and gives rise to anal pads, hindgut and Malpighian tubules, all considered to be ectodermal derivatives.

The anterior end of the ventral furrow is thought to be the primordium of the anterior endoderm, which forms part of the anterior midgut (AMG) (Poulson, 1950; Technau and Campos-Ortega, 1985). We will refer to it as endodermal AMG primordium to distinguish it from the stomodeal AMG primordium. The stomodeum, considered as ectodermal, begins to invaginate during stage 10 (Fig. 1B) and is the precursor of the foregut (which consists of pharynx, esophagus and proventriculus). In addition, the stomodeum provides the cells of the anterior part of the AMG (Technau and Campos-Ortega, 1985).

Several zygotic genes, under control of different maternal morphogens, set up the primordia of the germ layers. The nuclear gradient of the maternal gene product bicoid directs the ventral expression of two zygotic transcription factors, twist (twi) and snail (sna) (Boulay et al., 1987; Thise et al., 1988, 1991; Ip et al., 1992). Both twi and sna are required for the formation of the ventral furrow and the mesodermal germ layer (Simpson, 1983; Grau et al., 1984; Nüsslein-Volhard et al., 1984). They are thought to activate the genes responsible for ventral furrow formation and for mesodermal differentiation (Leptin and Grunewald, 1990; Leptin, 1991). In addition, sna represses genes in the mesodermal primordium that are involved in ectodermal development (Nambu et al., 1990; Leptin, 1991; Arora and Nüsslein-Volhard, 1992; Kasai et al., 1992).

The primordium of the posterior endoderm is set up by the maternal genes of the terminal system (Nüsslein-Volhard et al., 1987; Klingler et al., 1988). One of these genes, torso (tor), indirectly activates the expression of two zygotic transcription factors, huckebein (hkb) and tailless (tl), and thereby patterns the posterior region of the embryo (Weigel et al., 1990a; Pignoni et al., 1990; Casanova, 1990). hkb is expressed in the
posterior cap of the blastoderm (Brönner and Jäckle, 1991) and is required for the invagination of the PMG primordium as well as for its specification (Weigel et al., 1990a). The invagination of the proctodeum proceeds normally in hkb embryos: this aspect of the amnioserosal invagination is dependent on tll (Strecker et al., 1988). tll is expressed in the same domain as hkb posteriorly (Pignoni et al., 1990; Brönner and Jäckle, 1991) and, in addition, in the primordia of the proctodeum and of the posterior segmented region of the embryo (Pignoni et al., 1990). tll is required for the formation of all structures posterior to abdominal segment 7 including anal pads, hindgut and Malpighian tubules (all derived from the proctodeum), but it is not required for the formation of the posterior midgut (Jürgens et al., 1984; Strecker et al., 1988).

Both genes, hkb and tll, are also expressed in the anterior region of the embryo (Brönner and Jäckle, 1991; Pignoni et al., 1990). There, tll is required for the formation of parts of the brain and head skeleton (Strecker et al., 1988), and hkb for the formation of the anterior midgut (Weigel et al., 1990a). In summary, the establishment of both anterior and posterior midgut primordia depend on hkb activity.

In this paper, we show that hkb sets the anterior and the posterior limits of the mesoderm primordium, acting in two different modes. hkb not only determines endodermal fate in both terminal regions of the embryo, but also prevents mesodermal development there. In addition, we show that hkb, sna and twi cooperate in the determination of anterior gut structures.

MATERIALS AND METHODS

Fly stocks

(1) hkb\textsuperscript{8.1}: a small deletion of the hkb locus (Berleth et al., 1988). (2) hkb\textsuperscript{10.2} ts\textsuperscript{10.2}: double mutant for hkb and the strong torsolike allele ts\textsuperscript{385} (Nüsslein-Volhard et al., 1987; Frohnhöfer, 1987). (3) hkb\textsuperscript{t76}: small deficiency of the fkh locus (Weigel et al., 1989b). (4) hkb\textsuperscript{b} and hkb\textsuperscript{2}: a small deficiency of the hkb locus and a strong hypomorphic allele (Weigel et al., 1990a). The ventral furrow in embryos homozygous for hkb\textsuperscript{b} (for example Fig. 4B) extends to the anterior tip while, in embryos homozygous for the hypomorphic hkb\textsuperscript{2} allele, the ventral furrow extends only about half the way to the anterior tip of the embryo, similar to embryos from tor mothers (Fig. 7F). In hkb\textsuperscript{b} hkb\textsuperscript{2} embryos, the extent of the phenotype varies between complete extension like in hkb\textsuperscript{b} or partial extension like in hkb\textsuperscript{2}. This indicates that no additional locus is removed by the deficiency hkb\textsuperscript{b} that would prevent ventral furrow formation in the anterior part of the embryo. (5) sna\textsuperscript{2w5} and sna\textsuperscript{B3}: based on the cuticular phenotype amorphic sna alleles (Grau et al., 1984; Nüsslein-Volhard et al., 1984; Boulay et al., 1987); both alleles code for sna protein (data not shown), which seems to be defective in its C-terminal half (Boulay et al., 1987). (6) Df(2L)TE116(R)GW11: used as sna deficiency (Ashburner et al., 1990) and as a control for the specificity of our anti-sna antibody. (7) tll\textsuperscript{b} and tll\textsuperscript{1210}: a small deficiency of the tll locus and a strong hypomorphic allele (Jürgens et al., 1984; Strecker et al., 1988). Often we used the overlapping deficiencies tll\textsuperscript{b} and tll\textsuperscript{1210} which give a small synthetic deficiency (Strecker et al., 1988). (8) hkb\textsuperscript{b} tll\textsuperscript{b}: double mutant as described (Weigel et al., 1990a). (9) twi\textsuperscript{E58} and twi\textsuperscript{D10}: amorphic twi alleles (Nüsslein-Volhard et al., 1984; Thissen et al., 1987b). (10) sna\textsuperscript{R1} and sna\textsuperscript{R6}: Df(2R)S60: double mutants as described (Arora and Nüsslein-Volhard, 1992). (11) tor\textsuperscript{121} tor\textsuperscript{D12}: trans-heterozygous combination of strong alleles which do not express detectable tor protein (Sprenger et al., 1991). (12) tor\textsuperscript{E1ts} tor\textsuperscript{E1ts}: a deletion of the tor locus (Sprenger et al., 1989) (13) tor\textsuperscript{R1}: gain-of-function allele of tor (Klinger et al., 1988). (14) A490.2M3: enhancer trap insertion conferring ß-gal expression in midgut and midgut primordia (Bellen et al., 1989). Often we inserted the enhancer trap insertion into the tor locus and a strong hypomorphic allele (Bellen et al., 1989). The insertion (third chromosome) was crossed into the Oregon R flies used as wild type.

Probes and probe detection

The anti-sna antibody was raised in rabbits against the full-length sna protein expressed in E. coli in the PET3c vector. It was affinity-purified using the sna protein coupled to CNBr-activated Sepharose. Anti-twist antibody was a gift from S. Roth (Roth et al., 1989), anti-muscle myosin heavy chain antibody (R722) from D. Kiehart (Kiehart and Feghali, 1986) and anti-fkh antibody from D. Weigel (Weigel et al., 1989a). Murine monoclonal anti-ß-galactosidase (α-ß-gal) antibodies were purchased from Sigma (St. Louis, USA) and biotinylated goat anti-mouse IgG antibodies or biotinylated goat anti-rabbit IgG antibodies from Jackson (Bar-Harbor, USA).

Embryos were fixed and stained following standard protocols, and the antibodies were detected histochemically using the Vectastain ABC kit (Vector Labs, USA). Sectioning of the embryos was performed as described (Leptin and Grunewald, 1990). Embryos were
either mounted individually in Araldite or en masse in methyl salicylate and photographed with a Zeiss Axiophot equipped with Nomarski optics using either Kodak Ektachrome 160T or Agfapan APX 100 film.

The hkb cDNA used for the in situ detection of hkb mRNA was a gift from G. Brönnner (Brönnner and Jäckle, 1991). zfh-1 has been described by Fortini and coworkers (Fortini et al., 1991) and DFR1, one of the Drosophila homologues of the FGF receptor, by Shishido and coworkers (Shishido et al., 1993). The zfh-1 and the DFR1 probes used were a gift from J. Casal and originated from a molecular screen for ventrally expressed genes (J. Casal and M. Leptin, unpublished data). Probes were labelled with digoxigenin-dUTP (Boehringer) and used for in situ hybridization as described (Tautz and Pfeifle, 1989). For the simultaneous detection of sna and hkb gene products, embryos were first processed for in situ hybridization, subsequently hkb mRNA was detected using digoxigenin-dUTP-labelled cDNA probe and finally sna protein using the anti-sna antibody.

RESULTS

The expression domains of sna and twi do not coincide with the limits of the ventral furrow

sna and twi are required for ventral furrow formation but their patterns of expression do not define the anterior and posterior borders of the ventral furrow. They both are expressed on the ventral side of the blastoderm embryo (Thissee et al., 1987a, 1988; Alberga et al., 1991), including the anterior 20% of the egg where no ventral furrow is formed (Fig. 2A-C). In the posterior part of the embryo, the border of sna expression sharpens after the onset of cellularization. It then coincides with the posterior border of the ventral furrow (see Fig. 7), while twi continues to be expressed beyond the border around the posterior pole. In a simple model (Fig. 2D) for how the anterior and posterior limits of the ventral furrow are set, a gene X active in the anterior region of the embryo counter-acts the effect of sna and twi on their target genes which otherwise would promote ventral furrow formation. In the posterior region, a postulated gene Y, presumably under the control of the terminal system, represses sna expression and thereby delimits the ventral furrow posteriorly.

hkb sets the anterior and the posterior border of the mesoderm primordium by two different modes

(1) hkb delimits the ventral furrow

The zygotic terminal gap gene hkb is expressed at both termini of the embryo at blastoderm stage (Brönnner and Jäckle, 1991; see also Fig. 3A). At early gastrulation the spatial relationship of hkb expression to the ventral furrow is striking: anteriorly and posteriorly it lies directly adjacent to the ventral furrow (Fig. 3B,C). Therefore, hkb appeared as a good candidate for both the factor X and the factor Y (Fig. 2D). The phenotype of hkb null embryos indicates that hkb does indeed have these functions. In hkb null mutant embryos, the ventral furrow extends all the way to the anterior tip of the embryo (Fig. 4B), now encompassing the entire sna- and twi-expressing domain (see also below).

(2) hkb represses mesodermal differentiation anteriorly

Mesodermal differentiation normally appears to be repressed by hkb, as the expression patterns of mesodermal genes are expanded in hkb embryos. An example for one such gene is...
**zfH-1**, which is normally expressed under the control of **sna** and **twi** only in those cells that form the ventral furrow (Lai et al., 1991 and Fig. 5A,E). In **hkb** embryos, the domain of **zfH-1** expression is expanded anteriorly (Fig. 5B,F). Thus, **hkb** normally represses **zfH-1** expression and therefore appears to antagonize the effect of the **zfH-1** activators **sna** and **twi**. At later stages, cells expressing **zfH-1** (Fig. 5G) fill the head of the **hkb** embryo (Fig. 5I) and seem to form disorganized muscle cells at the tip of the embryo (Fig. 5K,L). We take this as an indication for the expansion of the mesoderm at the expense of head structures in **hkb** embryos.

(3) **hkb** represses **sna** expression and mesodermal differentiation posteriorly

Since cellularization the posterior border of **sna** expression coincides with the anterior border of **hkb** expression (Fig. 6), **hkb** also appeared as a good candidate for the zygotic gene **Y** that sets this border of **sna** expression. The phenotypic analysis of **hkb** mutants shows that indeed this function is fulfilled by **hkb**. In **hkb** embryos, the posterior boundary of the ventral furrow is shifted to the posterior (Fig. 5C,D) and **sna** protein is expressed up to the posterior pole (Fig. 7G,H). This shows that **hkb** also acts as the proposed factor **Y** that represses **sna** expression at the posterior terminal region and presumably thereby delimits the ventral furrow. Concomitantly with setting the border of the ventral furrow, **hkb** sets the border of the mesoderm primordium. The expression domain of **zfH-1** (again serving as a mesodermal marker) extends in **hkb** embryos to the posterior pole during the blastoderm and gastrulation stage (Fig. 5B,F). Later, the posterior expression domain of **zfH-1** within the mesoderm is enlarged indicating an expansion of the mesodermal germ layer at the expense of the endoderm (Fig. 5G,H).

(4) The limits of the mesoderm primordium do not depend on **tll** or **fkh**

**tll**, the other terminal gap gene, and **fork head** (**fkh**), which mediates some of the functions of **hkb** and **tll** (Jürgens and Weigel, 1988; Weigel et al., 1990a; Gaul and Weigel, 1991), are not involved in setting the borders of the mesoderm primordium. Neither the blastoderm expression of **zfH-1** (Fig. 5C,D) and of **sna** (Fig. 8A,B) nor the position and form of the ventral furrow (data not shown) are changed in **tll** or **fkh** mutant embryos. The maternal terminal system of positional information appears to set the posterior border of the mesoderm primordium exclusively through the zygotic action of **hkb**. This is particularly obvious in embryos derived from **tor** gain-of-function mothers whose posterior border of the mesoderm primordium is shifted to about 30% egg length due to the hyperactivity of **tor** (Fig. 8C,E). **hkb** embryos derived from such **tor** mutant mothers express **sna** from pole to pole (Fig. 8F) and correspondingly form an extended ventral furrow (data not shown) similar to **hkb** embryos from **tor** mothers. The lack of **tll** function, however, has no consequence for the expression of **sna**, even in the background of the hypermorphic **tor** allele (Fig. 8D). Although **tll** mediates many effects of **tor**, **tll** is not responsible for delimiting the mesoderm primordium.
Fig. 5. *hkb*, but not *tll* delimits the mesoderm primordium. *zfh-1* mRNA expression in wild-type (A,E,G), *hkb* null mutant embryos (B,F,H), *tll* embryos (C) or *fkh* embryos (D). (A-D, late blastoderm; E, F, early gastrulation; G, H, extended germ band stage). In the wild-type embryo as well as in *tll* and *fkh* embryos, *zfh-1* expression is confined to the cells that form the ventral furrow. This makes *zfh-1* an excellent marker for the mesoderm primordium, unlike other genes, which are not expressed throughout the entire mesoderm primordium, e.g. *tinman* (Bodmer et al., 1990). In *hkb* embryos, *zfh-1* expression is expanded towards the anterior and posterior poles as is the ventral furrow (B,F). Later, *zfh-1* is expressed in anterior and posterior mesoderm, and these domains are significantly enlarged in *hkb* embryos (E,F). (I-L) Differentiated muscle in the head of wild-type (I) and *hkb* embryos (K,L) detected by immunostaining of the muscle myosin heavy chain. In wild-type embryos, all muscles are internal and located in the pharynx (ph) or around the esophagus (es), while in *hkb* embryos many muscle cells are at the surface of the embryo and not associated with any particular organ. L shows an optical section through the rudimentary pharynx (ph') which is frequently found in old *hkb* embryos.
We now examined the expression in the various mutant
sna
appear to act in a synergistic fashion. Rather, they
does not act in a simple epistatic pathway. Rather, they
hkb
determination of the anterior mode of
bcd
(2)
Embryos from bicoid (bcd) mothers. (E,F) Embryos from
torso (tor) mothers. (G,H) hkb embryos. (A,C,E,G, late blastoderm; B,D,F,H, early gastrulation). At the stages shown in B and D, the sna expression (arrowhead) extends slightly beyond the posterior end of the ventral furrow (arrow). The cells between arrow and arrowhead invaginate a few minutes later. They behave like the peripheral cells within the mesodermal primordium, which do not constrict their apices and which are internalized after the midventral cells (Sweeton et al., 1991; Kam et al., 1991). There is some variability in the extent to which sna is expressed around the anterior pole in hkb embryos (compare G and H). The arrowheads indicate the sharp edges of sna expression, the open arrows the cephalic furrow, and the small arrows the posterior end of the ventral furrow.

Fig. 7. Comparison between sna expression and the form of the ventral furrow in wild-type and mutant embryos. (A,B) Wild-type. (C,D) Embryos from bicoid (bcd) mothers. (E,F) Embryos from torso (tor) mothers. (G,H) hkb embryos. (A,C,E,G, late blastoderm; B,D,F,H, early gastrulation). At the stages shown in B and D, the sna expression (arrowhead) extends slightly beyond the posterior end of the ventral furrow (arrow). The cells between arrow and arrowhead invaginate a few minutes later. They behave like the peripheral cells within the mesodermal primordium, which do not constrict their apices and which are internalized after the midventral cells (Sweeton et al., 1991; Kam et al., 1991). There is some variability in the extent to which sna is expressed around the anterior pole in hkb embryos (compare G and H). The arrowheads indicate the sharp edges of sna expression, the open arrows the cephalic furrow, and the small arrows the posterior end of the ventral furrow.

Fig. 6. The posterior border of sna expression coincides with the anterior border of hkb expression during cellularization. Wild-type embryos were probed for hkb mRNA (blue), and subsequently immunostained for the sna protein (brown). There is no gap between sna and hkb expression.

Regulatory interactions between bcd, tor, hkb and sna
(1) The regulation of hkb by tor and bcd
If hkb represses sna in the posterior region of the blastoderm why does it not have the same effect in the anterior? Apparently, bcd counteracts the inhibitory effect of hkb on sna expression. hkb itself is expressed anteriorly under the control of both bcd and tor. In embryos derived from tor lack-of-function mutant mothers (for simplicity called ‘tor embryos’) there is still some anterior expression of hkb (Brönner and Jäckle, 1991 and Fig. 9C). This expression is controlled by bcd as no blastodermal expression of hkb is detectable in embryos lacking both bcd and tor function (e.g. bcd tsl double mutants, Fig. 9D). In bcd embryos, hkb is expressed at both poles, but the anterior domain is smaller than in wild-type (Fig. 9B). Posteriorly, hkb is dependent only on tor (Brönner and Jäckle, 1991 and Fig. 9C). Thus, hkb is activated by both tor and bcd at the anterior pole and by tor alone at the posterior pole. However, tor and bcd direct hkb expression in specific subdomains in the anterior part of the embryo (compare Fig. 9B and C). Apparently, bcd and tor act maximally at slightly different points within the anterior pole, bcd at the apex and tor slightly dorsal to the apex. The tor-dependent (Fig. 9B) and the bcd-dependent (Fig. 9C) subdomain partially overlap, and their sum seems to give the distribution of hkb in the wild-type domain (Fig. 9A).

(2) bcd determines the anterior mode of hkb action
We now examined the sna expression in the various mutant embryos. In tor embryos (Fig. 7E) and in embryos lacking both bcd and tor function (data not shown), sna is expressed from pole to pole as in hkb embryos (Fig. 7G). In contrast, in bcd embryos (Fig. 7C,D) sna expression is not expanded at the posterior or the anterior pole and instead shows a sharp border of expression at a clear distance from the anterior pole. Not unexpectedly, this anterior border looks very similar to the posterior border, since in bcd mutant embryos the posterior terminal region is duplicated at the anterior pole (Frohnhöfer and Nüsslein-Volhard, 1986). In bcd embryos, the residual anterior hkb seems to repress sna, and we conclude that bcd normally inhibits hkb from repressing sna at the anterior pole. The ventral furrow in the mutant embryos follows the expression patterns of hkb and sna in agreement with the model depicted in Fig. 2D. It invaginates where sna and twi but not hkb are expressed. In tor embryos the ventral furrow extends towards the posterior pole of the embryo (Fig. 7F) as in hkb mutants (Fig. 7H), but it only partially extends towards the anterior pole of the embryo (Fig. 7F), unlike in hkb null mutants (Fig. 7H). hkb still represses ventral furrow formation in its anterior, tor-independent expression domain.

The functions of hkb, sna and twi in the blastoderm anterior to the mesoderm primordium
(1) Stomodeal development in sna, twi and in hkb embryos
Is there any function of hkb anteriorly besides antagonizing the formation of the ventral furrow? Can any function be attributed to the expression of sna and twi anterior to the region which contributes to the ventral furrow? hkb embryos do not form an anterior midgut (Weigel et al., 1990a and Fig. 10C). Fig. 10 shows that they also have no stomodeal invagination, and therefore most of the ectodermal foregut of the embryo does not develop. Often, a rudimentary pharynx forms late during the aberrant head involution (Fig. 5L). Interestingly, embryos mutant for both sna and twi also do not form an AMG or a stomodeum (Fig. 11D), and usually they lack all anterior gut structures (Fig. 11F). This phenotype cannot be attributed to the lack of anterior hkb expression: in sna twi double mutant embryos, hkb expression is not changed (data not shown). Similarly, the expression of sna and twi does not require hkb function (Fig. 7F and data not shown), and thus sna, twi and hkb do not act in a simple epistatic pathway. Rather, they appear to act in a synergistic fashion.
Interaction between *hkb*, *sna* and *twi*
(2) *twi* and *sna* do not act through *fkh*

*fkh*, one of the genes acting downstream of *hkb*, has been reported to be essential for a proper stomodeal invagination (Weigel et al., 1990b). *fkh* therefore might also be a target for *sna* and *twi*, and the phenotype of *sna twi* double mutants might be attributable to the dependence of *fkh* on *sna* and *twi*. This, however, is not the case. In wild-type embryos, *fkh* is expressed within the primordium of the stomodeum, in the invaginating stomodeum and later in the esophagus (Weigel et al., 1989a,b). In *sna twi* mutant embryos, early *fkh*-expression is normal (Fig. 11A-D). The group of *fkh*-expressing cells on the anterior surface of older mutant embryos (Fig. 11E) are probably the cells that would have invaginated and formed the esophagus in a normal embryo (Fig. 11F). Thus, at least some of the aspects of gene expression that depend on *hkb*, like the expression of *fkh*, are independent of *sna* and *twi*, and we conclude that the failure to form a stomodeum cannot be explained by an effect of *sna* and *twi* on *fkh* expression. Rather, *sna* and *twi* might act in parallel to *hkb* on other genes that are needed for the stomodeal invagination to occur. *sna* and *twi* appear to act in a redundant fashion since, in embryos lacking either *sna* or *twi*, we observe no defects in the formation of the stomodeum (Fig. 12B,C). This is in contrast to the formation of the ventral furrow where *sna* and *twi* exert different functions.

(3) Anterior midgut development in *twi* embryos

*twi* and *sna* have specific functions for the development of the endodermal AMG primordium. We followed the development of the AMG by the enhancer trap A490.2M3 which from the formation of the stomodeal plate onwards (stage 9) confers uniform β-gal expression in the primordia of AMG and PMG. In particular, β-gal is expressed both in the epithelium of the stomodeal AMG primordium and in the mesenchymal cells of the endodermal AMG primordium. These two primordia are morphologically well distinguishable at stage 10 (Fig. 12A,E) and are differentially affected by the lack of *sna* or *twi* function. In *twi* mutants, both parts express genes typical for the midgut as visualized by A490.2M3. However, early in gastrulation the endodermal primordium fails to invaginate (Fig. 12B,I), like the ventral furrow. This finding corroborates the notion that the endodermal midgut primordium is derived from the tip of the ventral furrow (Poulsom, 1950; Technau and Campos-Ortega, 1985). Interestingly, during the course of germ band extension the cells ingress into the interior of the

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**Fig. 8.** *hkb*, but not *tll* represses terminal *sna* expression. *sna* protein in *fkh* (A) and *tll* (B) embryos and in zygotically normal (C,E), *tll* (D) and *hkb* (F) embryos derived from *tor*⁰⁰²¹ gain-of-function mothers (C-F). (A-D,F, late blastoderm; E, early gastrulation. The posterior border of *sna* expression in *fkh* or *tll* embryos is positioned as in wild-type embryos (see Fig. 7). In embryos from *tor* gain-of-function mothers, the posterior border of *sna* expression is shifted towards 25 to 35% egg length (C) and the ventral furrow is reduced in size (E). The difference between the embryos in C and D reflects the variability of expressivity in the *tor*⁰⁰²¹ stock and is not due to the lack of *tll* in D. In *hkb* embryos derived from *tor* gain-of-function mothers, *sna* expression is not restricted by the terminal system (F).
appears to sna or of the bound-
sna gene, is not involved in the regulation of at the place where the anterior tip of the ventral furrow would have been located (Fig. 12C,G). Therefore, the other terminal gap tll expression.

There is no midgut-specific gene activity of ingressing cells in embryos, delimits the posterior border of the ventral hkb furrow and of the mesoderm primordium by setting the mutants are distinguishable (data not shown). In addition, there is no midgut-specific gene activity of the anterior tip of the ventral furrow is not formed and thus the early morphogenesis of the AMG is abnormal.

Fig. 9. Anterior hkb expression depends on both the anterior and the terminal system. hkb RNA expression is shown in wild-type embryos (A) or in embryos from bcd (B), tor (C), and bcd tsl (D) mothers. We used bcd tsl rather than tor; bcd double mutants for practical reasons. tsl acts upstream of tor (Casanova and Struhl, 1989).

DISCUSSION

The blastoderm expression of sna and hkb

The expression of sna and twi is initiated in the ventral region of the blastoderm embryo by high doses of nuclear dl (Roth et al., 1989; Thisse et al., 1991; Ip et al., 1992). While there is no apparent restriction of twi expression along the anteroposterior axis (twi protein and RNA are found from the anterior to the posterior pole of the blastoderm embryo), sna RNA and protein become excluded from the posterior pole at the onset of cellularization (Kosman et al., 1991 and Fig. 7A). We have shown that hkb is responsible for this repression (Fig. 7G) and propose that hkb acts as a direct negative regulator of sna transcription (Fig. 13). Initially, before the end of nuclear cycle 13, sna is weakly expressed around the posterior pole (Alberga et al., 1991; Ray et al., 1991; data not shown). We hypothesize that at this time hkb has not yet been translated and therefore cannot be active as a transcriptional repressor.

Interestingly, sna and hkb gene products coexist during cellularization and early gastrulation in the anterior part of the embryo. Here, unlike at the posterior pole, hkb does not act as a transcriptional repressor of sna. We have shown that bcd is responsible for the inhibition of the repression (Fig. 7C), but we do not know the mechanism by which bcd modulates the action of hkb on sna. However, the inhibition by bcd of the repressive effect of hkb is apparently not complete. If it were complete, then wild-type embryos should express the same high level of sna at the anterior pole as is observed in hkb mutant embryos. Instead, in wild-type embryos, sna expression fades out in graded fashion around the anterior pole (Fig. 7A), while it is strongly expressed in this region in hkb mutants (Fig. 7G). There seems to be a delicate balance between the effects of bcd, tor and hkb on sna expression. sna is strongly expressed in the anterior pole of tor embryos (Fig. 7E), although there is still some weak hkb expression anteriorly (Fig. 9C). We suggest that, in tor embryos, bcd is able to inhibit completely the repressing effect of the low dose of hkb.

Implications for the fate map of the posterior part of the embryo

hkb determines the PMG primordium (Weigel et al., 1990a) and is expressed in the region of the embryo (Brömmner and Jäckle, 1991) to which this primordium has been mapped. We propose that hkb delimits the posterior border of the ventral furrow and of the mesoderm primordium by setting the posterior border of sna expression. tll, the other terminal gap gene, is not involved in the regulation of sna or of the bound-

embryo and attach to the posterior surface of the stomodeum (Fig. 12F,J,K). Thus, the lack of twi function appears to be without consequence for the ultimate fate of the AMG, although the ventral furrow is not formed and thus the early morphogenesis of the AMG is abnormal.

(4) Anterior midgut development in sna embryos

In sna mutants the endodermal part of the AMG primordium does not become specified. No cells corresponding to the group of ingressing cells in twi mutants are distinguishable (data not shown). In addition, there is no midgut-specific gene activity at the place where the anterior tip of the ventral furrow would have been located (Fig. 12C,G). Therefore, sna appears to function as an activator in the specification of the endodermal AMG primordium. This is in contrast to the proposed role of sna in the mesodermal primordium where it acts as a suppres-
sor of lateral, neuroectodermal genes. As mentioned above, the stomodeum invaginates almost normally and expresses midgut-specific genes. However, no cells attach to its posterior surface, and the stomodeum stays epithelial for a long period of time (data not shown). Thus, sna and twi, previously considered as specifically required for mesodermal differentiation, are also involved in the specification of endodermal and ecto-
dermal structures that are derived from the ventral side of the embryo anterior to the mesoderm primordium.
Fig. 10. *hkb* is not only required for formation of anterior and posterior midgut but also for the invagination of the stomodeum. β-gal expression under the control of the enhancer trap 1A121 is shown in wild-type embryos (A,B) or in *hkb* embryos (C,D). (A,C, stage 9; B,D, stage 10. The arrowheads mark the stomodeal opening in the wild-type and the corresponding position in the head of a *hkb* embryo. st, stomodeum; amg, anterior midgut; pmg, posterior midgut.

Fig. 11. In *sna twi* double mutant embryos, no stomodeum is formed. The expression of *fkh* protein is shown in wild-type (A,C,E) and *sna twi* double mutant embryos (B,D,E). (A,B, late blastoderm stage; C,D, late stage 9, early stage 10; E,F, stage 12). The arrowheads indicate the ‘anterior’ boundary of the stomodeal *fkh* expression. In *sna twi* double mutant embryos an abnormally deep cephalic furrow is formed.
Interaction between hkb, sna and twi

Fig. 12. Requirement for sna in the development of anterior gut structures and twi-independent ingression of anterior midgut cells. The more posterior stomodeal cells and the AMG and PMG cells express β-gal protein under the control of the enhancer trap A490.2M3. (A,E) Wild-type embryos. (B,F) twi embryos. (C,G) sna embryos. (D) sna twi double mutant embryo. (H) Cross-section through the anterior midgut of a wild-type embryo at the position indicated in E. (I,J,K) Cross-sections through the anterior midgut of twi embryos at the positions indicated in B or F. All embryos shown are at about stage 10. The twi embryo shown in I is slightly younger than the one shown in J and K.
aries of the ventral furrow (Figs 5, 8). This is surprising when we consider the blastoderm fate map for the posterior primordia and compare it with the expression domains of the genes that have been suggested to set up these primordia. The map places the primordium of the proctodeum, which is set up by *tll*, as a ring between the primordium of the PMG and the primordium of the mesoderm (Hartenstein et al., 1985). In contrast, *hkb* and *sna* are expressed adjacent to each other (Fig. 6) and are not required for proctodeal development. If the region expressing *sna* constitutes the mesoderm primordium one has to conclude that mesoderm and PMG primordium are juxtaposed without an intervening proctodeal primordium. This view is consistent with the expansion of the mesoderm at the expense of the posterior midgut in *hkb* embryos (Fig. 5) and with the finding that *tll* function is irrelevant for the posterior border of the mesoderm primordium. Thus, all these data suggest that mesodermal and PMG primordium are not separated by the proctodeal primordium. This view does not necessarily contradict the data that were used to compile the fate map (Technau and Campos-Ortega, 1985), which suggests that there is an intervening proctodeum. These authors transplanted HRP-labelled cells after the formation of the ventral furrow, so that the resulting fate map was actually a fate map of the early gastrula. Therefore, the deduction of the blastoderm fate map required the assumption that the ventral furrow forms anterior to the cells contributing to the proctodeum. (An exact judgment in live embryos is very difficult, since with the extension of the germ band the ventral furrow joins the amnioserosal invagination). However, the results are equally consistent with mesoderm forming ventrally at the same position along the anteroposterior axis where more laterally and dorsally located cells form the proctodeum.

The fate map of the anterior ventral blastoderm

*sna*, *twi*, and *hkb* function in the development of structures that are derived from the anterior pole of the embryo. We relate these functions to the anterior expression domains of the genes and the anterior aspect of the fate map.

1. The anterior boundary of the ventral furrow and the mesoderm primordium

The ventral furrow ends anteriorly in a Y-shape, which is visible when viewed from the ventral side. This structure is presumably formed for topological reasons when a long rectangular array of cells invaginates. Without *hkb* function the ventral furrow extends to the tip of the anterior pole (Figs 4, 5). We observe this phenotype in *hkb* null mutants as well as in embryos from *bcd* *tsl* mothers (data not shown). We propose that *hkb* sets the anterior border of the ventral furrow by antagonizing the action of *sna* and *twi* on those target genes that actually drive the cell shape changes leading to the ventral furrow (Fig. 13). The expression of *hkb* is compatible with this proposed function (Fig. 3B,C). *hkb* is expressed within the cells that form the anterior rim of the Y-shape, invaginate with the ventral furrow, but do not constrict apically (Fig. 3C). Presumably these cells are pulled in passively by the action of the immediately neighbouring ventral furrow cells. Two mesoderm-specific genes, which are expressed under the control of *sna* and *twi* within the entire mesoderm primordium, are indeed repressed by *hkb*: *zfh-1* (Fig. 5) and *DFR1* (data not shown). They are repressed by *hkb* within the anterior ventral region of the embryo including the anterior rim of the Y-shape (Fig. 5 and data not
shown). This part of the rim presumably does not belong to the mesoderm primordium, but constitutes the primordium of the endodermal AMG primordium (see below). In addition, in the head of hkb mutants, we find supernumerary muscle cells at the surface of the embryo (Fig. 5K). These findings indicate that hkb not only sets the anterior limit of the ventral furrow but also of the mesoderm primordium.

We suggest that the expression domains of the three genes hkb, sna and twi are sufficient to position ventral furrow and mesoderm primordium along the anteroposterior axis (Fig. 13). The mesoderm primordium is limited to the region where sna and twi but not hkb are expressed. Of the two genes, sna and twi, the expression of sna appears to be more indicative for mesodermal fate. The expression domain of sna is contained within the twi domain, and its lateral boundaries define the precise lateral limits of the ventral furrow (Kosman et al., 1991; Leptin, 1991) as is also the case for the posterior limit. In contrast, twi expression is initially significantly wider than sna both laterally and at the posterior border of the ventral furrow (Kosman et al., 1991; Leptin, 1991) and becomes restricted to the mesodermal germ layer when the germ band has extended about half-way.

(2) Specification of the endodermal AMG primordium and the stomodeum

hkb is required for the development of the entire AMG (Weigel et al., 1990a, see also Fig. 9) and sna for the specification of the endodermal AMG primordium (Fig. 12C,G). Are the expression patterns of hkb and sna compatible with the location of the endodermal AMG primordium at the tip of the ventral furrow? sna is expressed in the entire anterior ventral region of the embryo and hkb in all the cells of the anterior cap including those which form the anterior rim of the Y-shaped end of the ventral furrow (Fig. 3C). These latter cells, which invaginate with the ventral furrow, might therefore constitute the endodermal primordium of the AMG. However, the two genes hkb and sna cannot be sufficient to give the spatial information for the specification of the AMG primordium since their expression domains cover a much larger region. Thus, to determine the extent of the endodermal AMG primordium along the anteroposterior axis, a third factor has to be proposed: either a particular concentration of the morphogen bcd itself or a zygotic gene under the control of bcd. We then would suggest that the positional specification for the endodermal primordium is given by sna, hkb and the unknown third gene in a combinatorial fashion.

The other structure that is formed anteriorly from the ventral side of the embryo is the stomodeum. We have shown that hkb is required for the stomodeal invagination (Fig. 10) and thus for the proper development of stomodeal derivatives like the esophagus. In addition, sna or twi are required for the stomodeal invagination as well, since in sna twi double mutants this morphogenetic movements does not occur (Fig. 11). However, several aspects of the functions of hkb, sna and twi in stomodeal development remain unclear. First, we do not have an explanation for the redundancy shown by sna and twi. Possibly, sna and twi have merely permissive roles in foregut development, for example by repressing more lateral and dorsal fates. One such case, where sna and twi act redundantly, has indeed been observed. The anterolateral mitotic domain 89, which flanks the stomodeal primordium, is expanded around the ventral side of sna twi double mutant embryos, but not of sna or twi embryos (Arora and Nüsslein-Volhard, 1992). Moreover, it is not known whether hkb has an instructive role for the stomodeal invagination or whether hkb is merely required to repress mesodermal development in the stomodeal primordium. This question might be answered by experimental manipulation of the hkb expression. Finally, we do not yet know any genes involved in stomodeal development that are regulated by twi and sna in a redundant fashion and might also be regulated by hkb.

In summary, we propose that the ‘ground’ fate of the ventral cells of the blastoderm embryo is to form a ventral furrow and the mesodermal germ layer (Fig. 13). For this determination, the combination of twi and sna is required. Since sna is expressed only in a subset of the blastoderm cells expressing twi, it is sna, but not twi, that is spatially limiting for mesoderm determination. By the action of the terminal gap gene hkb, the two ends of the embryo follow a different fate. Anteriorly mostly ectodermal and partly endodermal structures of the digestive tract are formed. Here, hkb, sna and twi are involved in the formation of the stomodeum and sna and hkb in the determination of the endodermal AMG primordium. In the posterior region of the embryo, hkb determines endodermal gut structures, represses sna expression and excludes the mesoderm primordium.

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