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Drosophila brachyenteron regulates gene activity and morphogenesis in the gut

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SUMMARY

Chromosomal region 68D/E is required for various aspects of *Drosophila* gut development; within this region maps the *Brachyury* homolog *T-related gene* (*Trg*), DNA of which rescues the hindgut defects of deficiency 68D/E. From a screen of 13,000 mutagenized chromosomes we identified six non-complementing alleles that are lethal over deficiencies of 68D/E and show a hindgut phenotype. These mutations constitute an allelic series and are all rescued to viability by a *Trg* transgene. We have named the mutant alleles and the genetic locus they define *brachyenteron* (*byn*); phenotypic characterization of the strongest alleles allows determination of the role of *byn* in embryogenesis. *byn* expression is activated by *tailless*, but *byn* does not regulate itself. *byn* expression in the hindgut and anal pad primordia is required for the regulation of genes encoding transcription factors (*even-skipped*, *engrailed*, *caudal*, *AbdominalB* and *orthopedia*) and cell signaling molecules (*wingless* and *decapentaplegic*). In *byn* mutant embryos, the defective program of gene activity in these primordia is followed by apoptosis (initiated by *reaper* expression and completed by macrophage engulfment), resulting in severely reduced hindgut and anal pads. Although *byn* is not expressed in the midgut or the Malpighian tubules, it is required for the formation of midgut constrictions and for the elongation of the Malpighian tubules.

Key words: *Brachyury*, *brachyenteron* (*byn*), *Trg*, gut development, *Drosophila*, embryogenesis

INTRODUCTION

In the posterior terminus of the *Drosophila* embryo we have an opportunity to understand the genetic pathways controlling development of a relatively simple organ system, the posterior gut, starting from zygotic gene activity that begins at the syncytial blastoderm stage. The posterior gut of the mature *Drosophila* embryo consists of hindgut, Malpighian tubules and posterior midgut, which develop from a primordium that is established at the posterior 0 to 15% egg length (EL) of the blastoderm stage embryo (reviewed by Skaer, 1993). Activation of the maternally encoded Torso receptor at the poles of the embryos leads, via activation of a phosphorylation cascade (Duffy and Perrimon, 1994), to relief of repression of the *tailless* (*tll*) gene, and hence to its transcriptional expression in a cap occupying 0-20% EL at the posterior of the embryo (Liaw et al., 1995). The *huckebein* (*hkb*) gene is activated in a slightly smaller posterior domain as a result of Torso activation (Brönner and Jäckle, 1991).

Both *tll* and *hkb* act at the blastoderm stage to pattern the embryo; in the absence of activity of one or both of these genes the most posterior portion of the anlage plan is deleted and the central segmental domain of the fate map is expanded (Mahoney and Lengyel, 1987; Weigel et al., 1990; Diaz et al., 1996). Analysis of mutant phenotypes reveals that *tll* is required to establish the primordia for the eighth abdominal segment, anal pads, hindgut, Malpighian tubules and part of the posterior midgut, while *hkb* is required to establish the hindgut primordium and is also necessary for Malpighian tubule elongation (Strecker et al., 1986; Pignoni et al., 1990; Brönner et al., 1994; Diaz et al., 1996).

As *tll* and *hkb* encode transcription factors (Pignoni et al., 1990; Brönner et al., 1994), both must act by regulating the expression of other genes. A specific role for *tll* in gene regulation has been demonstrated by its DNA binding in vitro and by its repression of *Krüppel* (*Kr*) and *knirps* (*kni*) and activation of *hunchback* (*hb*) in vivo (Steingrimsson et al., 1991; Pankratz et al., 1992; Hoch et al., 1992; Margolis et al., 1995).

Although no direct regulation has been demonstrated, a number of genes whose expression requires *tll* and *hkb* and which are required for various aspects of posterior gut development have been identified. One of these genes is *forkhead* (*fkh*), which requires both *tll* and *hkb* activity for its expression; in the absence of *fkh* the posterior gut forms, but begins degenerating around stage 12 (Weigel et al., 1989). Several genes have been identified that are required for Malpighian tubule development: in the absence of *Kr* activity the Malpighian tubule primordium does not bud out from the hindgut (although it does display properties characteristic of differentiated Malpighian tubules; Harbecke and Janning, 1989; Skaer, 1993); in the absence of *cut* (*ct*) activity the Malpighian tubule buds do not elongate (Liu and Jack, 1992); in the absence of *wingless* (*wg*), cell division in the tubules is reduced (Skaer and Martinez Arias, 1992). There are also genes that are expressed specifi-

cally in the hindgut, such as *orthopedia* (*otp*) (Simeone et al., 1994), for which no function has yet been ascertained.

These genes, both on the basis of their total number and their mutant phenotypes, do not seem sufficient to carry out the complex process of posterior gut formation. Beginning at the cellular blastoderm stage, based on *tll* and *hkb* activity, the primordia of hindgut, Malpighian tubules and posterior midgut are established. Following the blastoderm stage, there are three post-blastoderm cycles of cell division and by stage 17, many different types of cells have differentiated (Skaer, 1993). Formation of the posterior gut also involves major cellular rearrangements: invagination during gastrulation and germ band elongation, epithelial-mesenchymal transitions in the midgut, evagination of the Malpighian tubule primordia, formation of three constrictions in the midgut, and elongation of the Malpighian tubules, hindgut and midgut.

To identify additional targets of *tll* and *hkb*, as well as genes playing a role in posterior gut development generally, a set of deficiencies uncovering most of the *Drosophila* genome was screened for effects on hindgut and Malpighian tubule formation. From this screen, region 68D/E was identified as having several specific effects on gut development (Harbecke and Lengyel, 1995): embryos homozygous for 68D/E deletions have a reduction in hindgut and anal pads, a defect in midgut constrictions and a defect in Malpighian tubules (Kispert et al., 1994; Harbecke and Lengyel, 1995).

Concomitant with this genetic analysis, molecular screening identified a gene mapping within the same 68D/E region that shows high sequence similarity to the vertebrate *Brachyury* (also known as *T*) gene. This sequence was designated *Trg* (for *T*-related gene). *Trg* was found to be expressed as a posterior cap in early embryogenesis (stage 5) under control of *tll* and then refined to a stripe by repression by *hkb* (Kispert et al., 1994). *Trg* expression persists in the hindgut and anal pads through the end of embryogenesis. Genomic DNA containing the *Trg* transcription unit rescued the defective hindgut phenotype of 68D/E deficient embryos (Kispert et al., 1994). The sequence of *Drosophila Trg* suggests that, like vertebrate *Brachyury* (Kispert and Herrmann, 1993), it encodes a T-domain transcription factor.

To determine if the phenotype of the 68D/E deficiency is due to the absence of a single locus, and to correlate this locus with the *Trg* transcription unit, we have screened for lethal alleles in the 68D/E region. We describe here the complementation characteristics of six mutant alleles, which we have named *brachyenteron* (*byn*); rescue of all alleles to viability by a transgene demonstrates that the *Trg* transcription unit corresponds to the *byn* genetic locus. Phenotypic analysis places the *byn* mutations in an allelic series and shows that *byn* is required to regulate specific gene activity in the primordia of the anal pads and hindgut. In the absence of *byn* activity, programmed cell death occurs in these primordia, resulting in reduced and abnormal anal pads and hindgut. *byn* is also required for specific morphogenetic processes in the gut, namely formation of the midgut constrictions, and the elongation of the Malpighian tubules.

MATERIALS AND METHODS

Screens for lethals in 68D/E

Two different screens were carried out to induce point mutations in the locus affecting posterior gut development in 68D/E. In both cases, 3- to 5-day old adult males were mutagenized either with 25 mM (first screen) or 35 mM (second screen) ethyl methane sulfonate (EMS) as previously described (Grigliatti, 1986).

In the first screen, absence of Tb^+ in individual cultures was used to detect lethals in the 68D/E region, and 10,000 mutagenized chromosomes were screened. Mutagenized *ru h st ry e* (isogenized) males were mated to *M(3)/TM6B, Tb* females. F1 r*u h st ry e*/TM6B, Tb* (* indicates a mutagenized chromosome) females were collected and mass-mated to *Df(3L)vin5* (or *vin7)/TM6B, Tb* males; after 3 days, each female was used to establish a single culture. Cultures giving rise to Tb^+ as well as Tb^- offspring were discarded. From cultures containing only Tb^- offspring, *ru h st ry e*/TM6B, Tb* flies were collected and tested for complementation with the flanking deficiencies *Df(3L)Bk9* and *Df(3L)vin2* and for non-complementation with the deficiencies *Df(3L)vin3* and *Df(3L)vin4*. Embryonic lethal lines mapping within 68D/E were tested for complementation with a mutant allele of *cyclinA* (the only described complementation group within 68D/E for which alleles are still extant; Lehner and O'Farrell, 1989). Three embryonic lethal alleles of *cyclinA* were found. All other lethals in 68D/E were stained as hemizygotes (over a deficiency) with anti-Crumbs antibody and examined for their gut phenotype. The alleles byn^1, byn^2, byn^5 and byn^6 were isolated from this screen.

In the second screen, 3,000 third chromosomes were screened by using white eye color for screening: mutagenized *bw*; *st* males were crossed to *bw; Df(3L)vin2/TM3* females. Individual F_1 *bw; st*/Df(3L)vin2* males were crossed to *bw; Df(3L)vin4, h st e*; cultures in which there were no progeny with white eye color (homozygous for *bw* and for *st*) were further characterized on the assumption that they carried a chromosome with a lethal mutation in the region uncovered by *Df(3L)vin4* but not *Df(3L)vin2*. Lethals were tested for their ability to be rescued by the 20 kb *Trg* rescue construct (Kispert et al., 1994). The alleles byn^3 and byn^4, as well as an additional *cyclinA* allele, were isolated from this screen.

Detection of specific mRNAs and proteins in embryos

mRNAs were detected by in situ hybridization using digoxigenin-labeled DNA probes prepared according to the manufacturer's instructions (Genius kit, Boehringer Mannheim). In situ hybridization to whole-mount embryos was performed as described by Tautz and Pfeifle (1989). Embryos were mounted whole in glycerol/gelatin (Ashburner, 1989).

Antibody staining was performed using standard techniques (Ashburner, 1989). The primary antibodies were: anti-Crumbs antibody Cq4 (Tepass et al., 1990), anti-Cut antibody (Liu and Jack, 1992), anti-Engrailed/Invected antibody 4D9 (Patel et al., 1989), anti-AbdominalB antibody (Celniker et al., 1989), anti-Twist antibody (Roth et al., 1989) and anti-Decapentaplegic antibody (Panganiban et al., 1990). Biotinylated secondary antibodies and streptavidin conjugated to horseradish peroxidase (Jackson laboratories) were both used at 1:2000. Horseradish peroxidase activity was detected by a diaminobenzidine reaction, leading to a brown or, by adding $NiCl_2$ to 0.07%, a black precipitate. Embryos were dehydrated in ethanol and mounted whole in Epon/Araldite.

Embryos and sections of embryos were photographed using Kodak Ektachrome 160T film on a Zeiss Axiophot microscope equipped with differential interference contrast optics. For preparation of figures, photographic slides were digitized by a SprintScan slide scanner and assembled using Adobe Photoshop; magnifications were adjusted to give images of the same size. Embryo staging is according to Campos-Ortega and Hartenstein (1985).

Histology

Embryos were dechorionated and fixed in heptane/glutaraldehyde as described by Tepass et al. (1990). After manual devitellinization, embryos were postfixed in 1% OsO_4, dehydrated in ethanol, embedded and sectioned at 2 μm with a LKB 2088 Ultratome V,

stained with toluidine blue (Ashburner, 1989), and mounted in Epon under coverslips.

Ectopic expression of *tll*

tll was expressed ectopically in early embryos by heat shocking a stock carrying the *tll* gene under the control of the *hsp70* promoter (construct HST2.1 on the second chromosome; Steingrimsson et al., 1991). Embryos were collected for four hours, given a 20 or 60 minute heat shock at 37˚C and then allowed to develop for another 30 minutes prior to processing for in situ hybridization.

RESULTS

Generation of six *brachyenteron* alleles

To identify point mutants in the gene responsible for the hindgut phenotype of the 68D/E deficiencies, we screened for lethal point mutations in this region. In two separate screens of over 13,000 chromosomes using the mutagen EMS, we identified a complementation group containing six alleles that as hemizygotes (in *trans* to a 68D/E deficiency) show a phenotype similar to that of a homozygous deficiency of the region. It was shown previously that a single copy of a 20 kb genomic fragment containing the 8 kb *Trg* transcription unit, as well as 9 kb of 5′ and 3 kb of 3′ flanking DNA, rescued the phenotype of the hindgut and anal pads in embryos homozygous deficient for the 68D/E region (although rescue to viability was not achieved) (Kispert et al. 1994). We used this same genomic fragment to rescue the six EMS induced *byn* alleles. A single copy of the transgene containing the *Trg* transcription unit can rescue embryos hemizygous for *Df(3L)vin4* and a *byn* mutation; each of the six *byn* alleles can be rescued to complete viability. These results demonstrate that the *Trg* transcription unit (which is the only known coding region in the genomic DNA of the rescue construct; Kispert et al., 1994), corresponds to the gene identified by the point mutations isolated in our screen. Because of its mutant phenotype and the similarity of the encoded gene product to the vertebrate Brachyury protein, we have named the *Drosophila* gene *brachyenteron* (*byn*), or 'short gut.'

Table 1. Intra-allelic complementation among *byn* alleles

Alleles	byn^2	byn^3	byn^4	byn^5	byn^6
byn^1	25% (43/387)	5.8% (17/604)	9.5% 16/352	<0.3% (0/645)	4.9% (10/421)
byn^2		6.9% (20/603)	19.2% (57/652)	1.3% (3/466)	12.2% (15/261)
byn^3			<0.3% (0/769)	<0.3% (0/661)	<0.6% (0/359)
byn^4				<0.3% (0/648)	<0.7% (0/279)
byn^5					<0.5% (0/400)

The various *byn* alleles were balanced over either *TM3, Sb e* or *Tm6B, Tb*; for each individual cross, the same balancer was used and the cross was performed in both directions to check for maternal effects on the outcome (none were found). The number of *byn* heterozygous adults obtained is given over the total number of progeny (in parentheses). By dividing the total number of balancer heterozygous adults obtained in the cross by two, the approximate number of *byn* transheterozygous embryos presumed to have been produced in each cross was estimated.This number was then divided into the total number of *byn* transheterozygotes that survived to adulthood to obtain fraction of *byn* transheterozygous escapers, or survivors, given as a percentage.

Both complementation studies and phenotypic analysis (described below) consistently place the six *byn* alleles in a series of varying strength. The strongest allele is byn^5, as embryos homozygous mutant for this allele have hindgut defects equal in severity (detected by staining with anti-Crumbs (Crb) antibody) to that seen in *byn* deficient embryos (Fig. 1A; Harbecke and Lengyel, 1995). All *byn* alleles fail to complement the strong allele byn^5 and show a reduced hindgut in transheterozygous combination with byn^5.

Certain allelic combinations produce escapers that are viable as flies (Table 1). Crosses between *byn* alleles defined as weak on the basis of their hindgut phenotype (see below) produce more escaper offspring than crosses between strong alleles. The weakest allele, byn^2, when transheterozygous with all five other alleles, allows some (1-25%) survival to adulthood. The next weakest allele, byn^1, also allows escapers (1-25%) in combination with all alleles except the strongest allele, byn^5, which does not give escapers with any allele except byn^2. The other three alleles each give escapers only with byn^1 and byn^2. The order of allelic strength suggested by the fraction of transheterozygotes surviving to adulthood in intra-allelic complementation is therefore: $byn^2 < byn^1 < byn^4 < byn^6 < byn^3 < byn^5$.

Recently, a P-element insertion near the *Trg* gene has been shown to have a phenotype of reduced hindgut and anal pads (Murakami et al., 1995). This insertion, termed *aproctous* (*apro*), fails to complement the *byn* alleles and thus is an additional *byn* allele which we designate byn^{apro}.

The various *byn* transheterozygous escaper adults appear morphologically normal externally; the females, however, are sterile. To further characterize the basis for this sterility, the females were dissected and their ovaries examined. No egg chambers in these ovaries had progressed beyond stage 7 of ovarian development, i.e. none appeared to have proceeded to the point of yolk uptake (data not shown), which begins during stage 8 (King, 1970). The hindgut of these *byn* escaper females is relatively normal in length, but is narrower than that seen in wild-type, apparently because it does not contain digestive products. The escapers usually die within 1-2 days of eclosion.

These experiments demonstrate that the hindgut phenotype described for deletion of the 68D/E region is due to lack of *byn*, a single genetic locus, and that the *Trg* transcription unit corresponds to the *byn* gene.

Structures expressing *Trg* are abnormal in *byn* mutants

Trg (*byn*) expression begins as a posterior cap which resolves into a posterior stripe and is then maintained in later stages in the hindgut and anal pad primordia (Kispert et al., 1994). We carried out immunological staining of whole-mount embryos to characterize morphological defects in the structures arising from these primordia in *byn* mutants. The results described in this and in subsequent sections are consistent with the allelic series deduced from the complementation studies above. Staining with anti-Crb antibody reveals that in embryos homozygous for the strongest allele byn^5, the hindgut is almost entirely missing (Fig. 1A). In embryos homozygous for alleles of moderate strength, such as byn^3 and byn^6, there is a less severe reduction in hindgut size (Fig. 1B,C). Embryos

homozygous for the weakest allele, *byn*^{2}, have a hindgut that is only slightly shorter than that of a wild-type embryo (cf. Fig. 1D,E).

The expression of *byn* itself in the anal pads and hindgut provides a useful marker for characterizing the defects in *byn* mutant embryos (Fig. 2A). In situ hybridization shows that *byn*^{5} mutants continue to express *byn* in reduced anal pads and hindgut (Fig. 2B). Thus, in the absence of *byn* activity, there are still remnants of structures that normally express *byn*.

Although, as described previously (Kispert et al., 1994) and below, many genes are expressed abnormally in the absence of *byn* activity, the hindgut remnant in *byn* mutant embryos shares a number of properties with the normally differentiated hindgut. The *twist* gene, encoding a bHLH transcriptional factor (Thisse et al., 1988), is expressed normally in the visceral mesoderm that surrounds the foregut and hindgut (Fig. 2C). In *byn*^{5} embryos and deficiency homozygotes, staining for Twist protein is seen in cells around the hindgut remnant, suggesting that it is surrounded by visceral mesoderm (Fig. 2D). The A4.1M2 enhancer trap line, which expresses *lacZ* in the hindgut (Fig. 2E; Bellen et al., 1989) was crossed to *byn*^{5} (Fig. 2G). In the A4.1M2-bearing, *byn* mutant embryos, *lacZ* expression is observed in the hindgut rudiment. Thus the gene that can be inferred from the A4.1M2 enhancer continues to be expressed in the hindgut remnant in the absence of *byn* activity. At the morphological level, examination of sectioned embryos reveals that the hindgut remnant consists of cells with the same columnar shape as that seen in the normal hindgut (see Fig. 8D,F).

The above experiments show that, in embryos lacking *byn* activity, structures that normally express *byn* (hindgut and anal

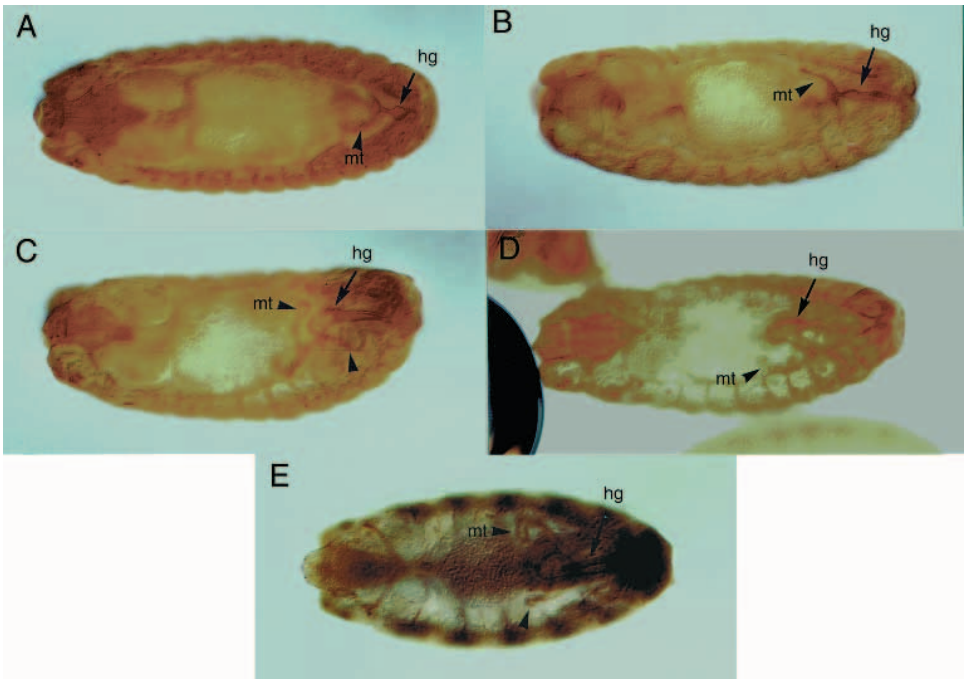

Fig. 1. Range of defects in the *byn* allelic series. The severity of the mutant phenotype of the different *byn* alleles defines an allelic series consistent with that deduced from complementation analysis (Table 1). (A) Embryos homozygous for the strong *byn*^{5} allele have a greatly reduced hindgut (arrow, hg) and short, unextended Malpighian tubules (arrowheads, mt), as detected by staining with antibody to the Crb protein. The severity of this mutant phenotype decreases progressively along the range of *byn* alleles: (B) *byn*^{3}, (C) *byn*^{6} and (D) *byn*^{2}. (E) Homozygous mutant embryos were distinguished from their phenotypically wild-type siblings by maintaining *byn* alleles over a TM3 balancer chromosome carrying a *lacZ* gene driven by the *fushi tarazu* promoter. Double staining with antibody to β-galactosidase allows phenotypically wild-type embryos to be recognized by the presence of dark stripes.

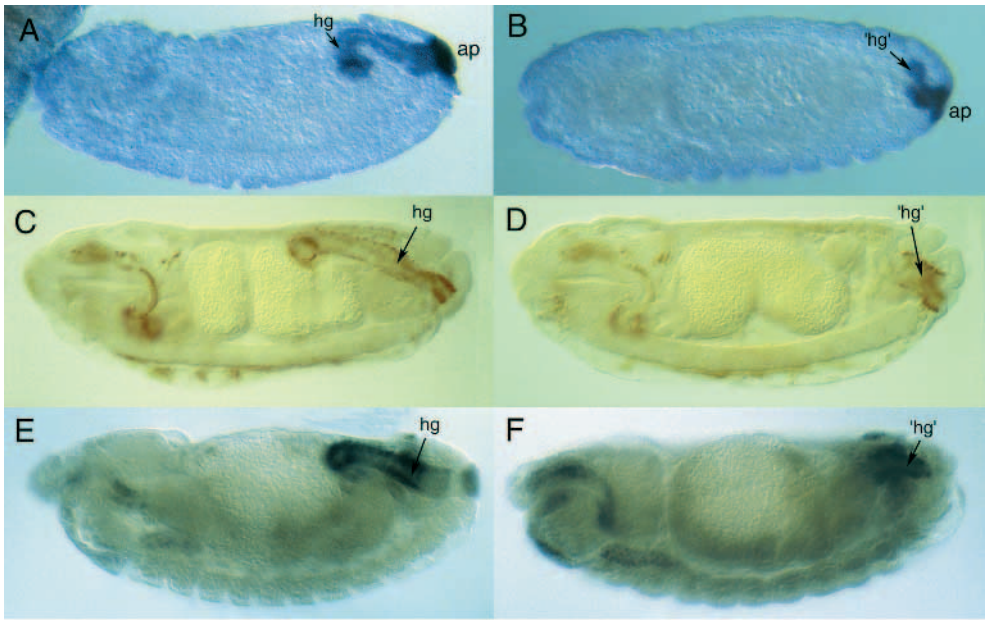

Fig. 2. Differentiated characteristics of the hindgut remnant in *byn* mutant embryos. (A) In situ hybridization of a *byn* probe to a wild-type embryo stains the hindgut (hg) and anal pads (ap). (B) *byn* expression in a *byn*^{5} embryo is reduced, but some *byn*-expressing hindgut (hg) and anal pad tissue remain. (C) Twi protein is expressed in the visceral mesoderm surrounding the hindgut of a wild-type embryo and (D) in the mesoderm surrounding the hindgut rudiment of a *byn*^{3} mutant. (E) *lacZ* expression in a normal embryo directed by the enhancer trap A4.1M2; note strong staining of the hindgut. (F) *lacZ* expression is seen in the hindgut rudiment of a *byn*^{5} embryo carrying the same enhancer trap as in E.

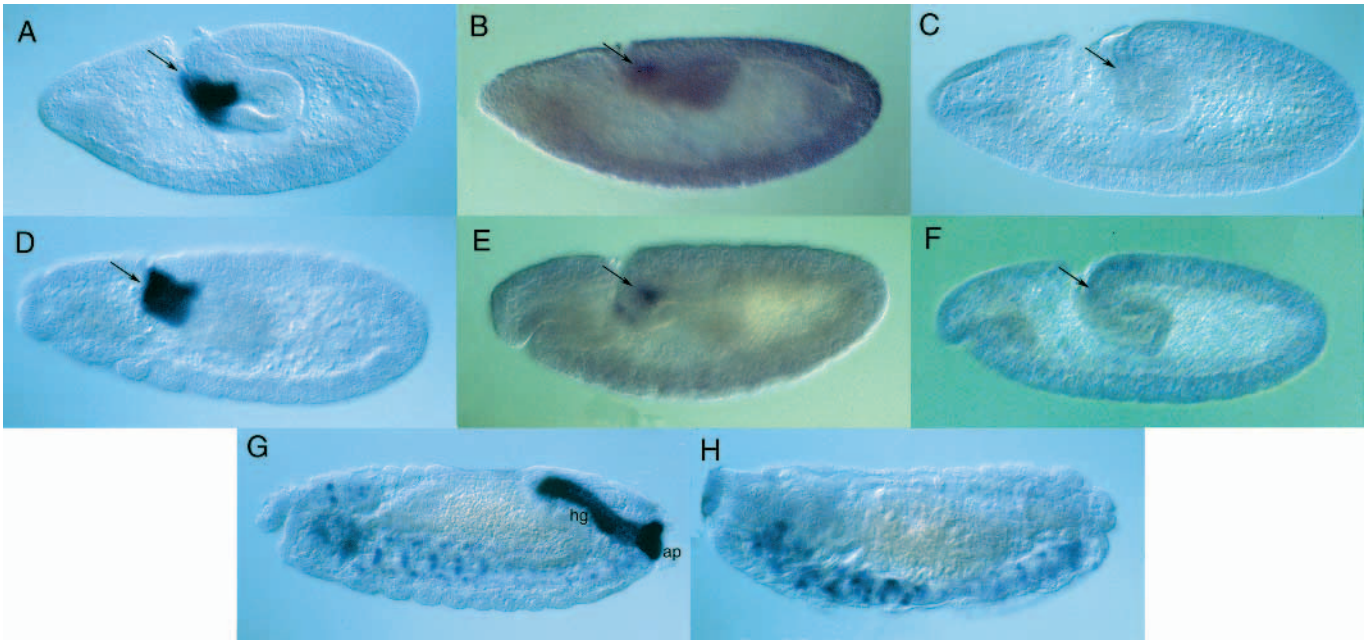

Fig. 3. Absence of *otp* expression in the proctodeum of *byn* embryos. (A,D) *otp* is first expressed in the hindgut and anal pad primordia (arrow) during germ band extension. (B,E) This expression is reduced in embryos homozygous for the weak allele byn^1 and is absent (C,F) in embryos homozygous for the strong allele byn^5. (G,H) *otp* expression persists in the differentiated hindgut (hg) and anal pads (ap) of wild-type embryos. Expression of *otp* in the central nervous system is seen in both (G) wild-type and (H) byn^5 embryos.

pads) are reduced; the hindgut remnant, however, displays several molecular and morphological features that are characteristic of the normal differentiated hindgut.

byn regulates *orthopedia*, *even-skipped*, *caudal*, *wingless* and *decapentaplegic* in the anal pad and hindgut primordia

As *byn* encodes a transcription factor, it likely regulates genes that specify the hindgut and anal pad anlagen. Approximately 4 hours after the initiation of *byn* expression, deviation from the normal expression of *engrailed* (*en*) and *caudal* (*cad*) in the hindgut and anal pad primordia of 68D/E deficient embryos is observed (Kispert et al., 1994). To identify candidates for direct regulation by *byn*, we determined, for a number of genes, the earliest developmental stage at which their expression is altered in *byn* point mutant embryos.

The *otp* gene encodes a homeodomain protein that is specifically expressed in hindgut and anal pad primordia; *otp* expression begins during stage 7 and persists into late embryonic development (Fig. 3A,D,G; Simeone et al., 1994). Embryos hemizygous for the weak byn^1

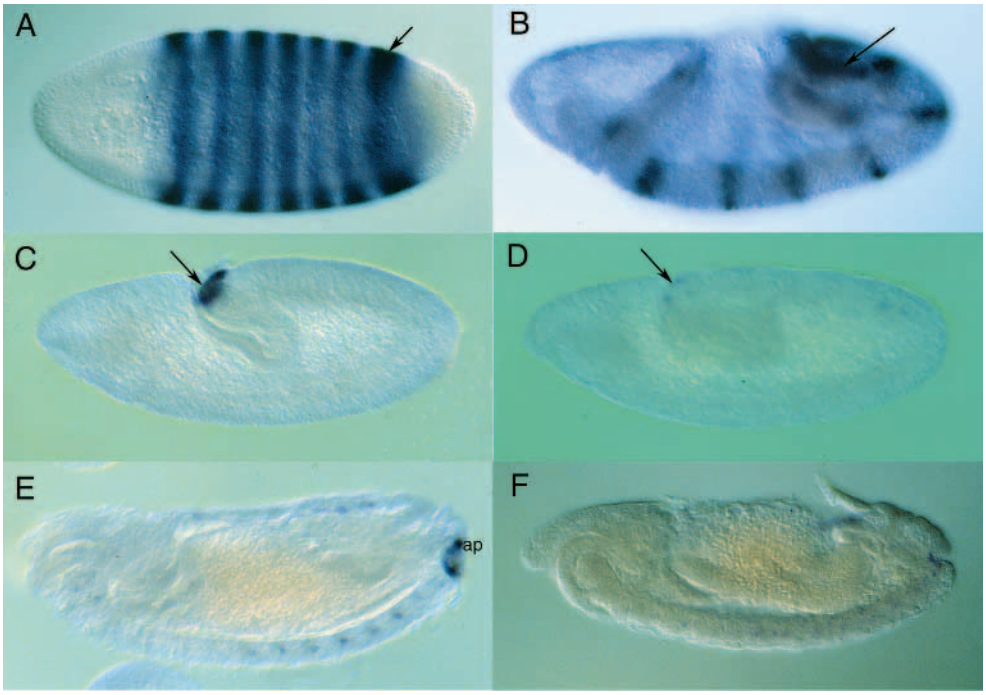

Fig. 4. *eve* expression is not maintained in *byn* mutant embryos. (A) *eve* is expressed in seven stripes in stage 5 and (B) stage 6 embryos. After gastrulation, the anal pad primordia expressing the seventh *eve* stripe (arrow) move anteriorly during germ band extension (C) and continue to express *eve*. (D) byn^5 embryos fail to maintain *eve* expression in that tissue. (E) The rim of the differentiated anal pads (ap) continue to express *eve* in wild-type embryos at stage 14, while (F) this expression is absent in the reduced anal pad tissue of byn^5 embryos.

allele show dramatically reduced expression of *otp* in the proctodeum at stages 7 and 8 (Fig. 3B,E), while individuals homozygous for the strong allele byn^5 completely fail to express *otp* in the proctodeum at any stage (Fig. 3C,F). A useful internal control is that a later appearing expression domain of *otp*, in the embryonic nervous system beginning at stage 12, is the same in both wild-type and byn^5 homozygous embryos (Fig. 3G,H).

The pair-rule gene *even-skipped* (*eve*) encodes a homeodomain protein that is first expressed in seven, and then in fourteen stripes (Fig. 4A,B; Frasch et al., 1987). During gastrulation, all of these stripes disappear, except the posteriormost. The anal pad primordium arises from among the cells expressing the most posterior stripe (Fig. 4C,E). Early *eve* expression, in the seven and fourteen stripe pattern, is unaffected in *byn* mutants. By stage 8, however, *eve* expression in the anal pad primordium is almost entirely absent from byn^5 homozygous embryos (Fig. 4D,F).

Zygotic expression of *cad*, which also encodes a homeodomain protein, begins with a posterior stripe in the blastoderm stage (Fig. 5A; Fjose et al., 1985); this early expression pattern is unaffected in embryos deficient for *byn* (not shown). After gastrulation and germ band extension (Fig. 5B,C), cells within the *cad* stripe, which approximately overlaps the posterior *eve* stripe, become the anal pads. In *byn* embryos there is a substantial reduction of *cad* expression in the anal pad primordium around stage 10 (Fig. 5D). Additional domains of *cad* expression that appear in the Malpighian tubules and posterior midgut are not affected in *byn* mutant embryos, even though the Malpighian tubules are quite misshapen (Kispert et al., 1994). Thus, of the several regions of the *cad* expression pattern, only that in the anal pads requires *byn* for its maintenance.

wg, which encodes a cell signaling molecule (Rijsewijk et al., 1987), is also expressed in a pattern that includes a strong posterior stripe (Fig. 6A,B; Baker, 1988). Like the *eve* and *cad* stripes with which it overlaps, this posterior *wg* stripe contains the primordium of the anal pads (Fig. 6C). In byn^5 mutant embryos, this posterior domain of *wg* expression pattern has largely disappeared by stage 11 (Fig. 6D). The expression of *dpp*, which encodes a signaling molecule of the TGF-β family, also requires *byn* function. *dpp* is normally expressed in the central part (along the antero-posterior axis) of the hindgut primordium after stage 9 (Fig. 6E). In embryos homozygous or hemizygous for one of the stronger *byn* alleles, *dpp* expression in the hindgut is not detected (Fig. 6F).

We conclude that *byn* activity regulates expression of *otp*, *cad*, *eve*, *wg* and *dpp* in specific primordia. The hindgut expression of *otp* and *dpp* require *byn* for their initial transcriptional activation. *eve*, *cad* and *wg*, in contrast, are initially expressed normally in the anal pad anlage in the absence of *byn*, but require *byn* for proper maintenance of their expression. The brief time that elapses between the onset of *byn* expression and visible failure of expression of *otp* (<1 hour) and *eve* (approx. 1 hour) suggests that those genes are direct targets of *byn*.

An important point is that even though they fail to express certain genes correctly in *byn* embryos, the hindgut and anal pad primordia are still formed initially. This is deduced from the fact that, in *byn* mutant embryos, the proctodeum is seen even though it does not hybridize *otp* probe (Fig. 3C,F), and that there is a normal domain of *cad* expression in the anal pad primordia until stage 10.

Programmed cell death as the cause of reduced hindgut and anal pads

While the hindgut and anal pad primordia appear normal in *byn* mutant embryos up to stage 9, by stage 10 the first morphological defect can be distinguished. In wild-type embryos the hindgut primordium projects downward from its dorsally located opening, makes a distinct bend into a horizontal orientation, and then connects to the posterior midgut primordium (Fig. 7A). This bend is not observed in stage 10 *byn* embryos, and the hindgut primordium appears slightly shorter (Fig. 7B). By late stage 11/early 12, there appears to be a reduction in the anal pad primordium, which is located at the dorsal side of the embryo. At this stage, the primordium in wild-type embryos does not express AbdB and is delimited by the terminal (a9) En stripe in the epidermis, while in *byn* embryos AbdB expression and the terminal En stripe are shifted to the very end of the germ band (Fig. 7C-H). By stage 13, both hindgut and anal pads are further reduced (Fig. 4F and data not shown). In the stage 14 *byn* embryo, the AbdB-expressing epidermis, which normally ends outside the anal pads (Fig. 7I), can be seen to extend into the interior of the embryo and border the hindgut rudiment (Fig. 7J).

The most likely explanation for this reduction in the size of these structures is cell death. As the products of the *reaper* (*rpr*) and *head involution defective* (*hid*) genes are capable of inducing apoptotic cell death in *Drosophila* (White et al., 1994; Grether et al., 1995), we examined the expression of those genes in *byn* mutants. Expression of *hid* did not deviate from the wild-type pattern in *byn* mutants (not shown). However, we observed a substantial patch of ectopic *rpr* expression in the primordia of the hindgut and anal pads of byn^5 mutant embryos early in stage 10, indicating that cell death will soon occur in those areas (Fig. 8A,B).

To directly visualize dead or dying cells, embryos were sectioned and stained with toluidine blue. Wild-type embryos of stage 14 contain a few, scattered apoptotic cells in the posterior region (Fig. 8C,E). Embryos homozygous for byn^5, recognizable by their short hindgut remnant, have a significantly larger number of darkly stained cells, including large clusters of macrophages, which have ingested pycnotic (apoptotic) cells (Fig. 8D,F). The location of these cells around the hindgut and anal pad remnants is consistent with their origination in and subsequent extrusion from those structures as they undergo apoptosis.

These results show that the primary cause of the reduced hindgut and anal pads of *byn* mutants is ectopic cell death occurring in the primordia. This apoptosis is likely triggered by *rpr* expression in the proctodeum. Presumably ectopic *rpr* activity is the result of failure to properly express genes normally regulated by *byn*, such as *otp*, *eve*, *cad*, *dpp* and *wg*.

Defects in gut morphogenesis in *byn* mutants

Our previous analysis of the 68D/E deficiency phenotype (Harbecke and Lengyel, 1995) described a reduction in size of Malpighian tubules and a failure of midgut constrictions to form. As *Trg* is expressed in hindgut and anal pads, but not in Malpighian tubules or midgut, the defects observed in these latter organs in 68D/E deficiency embryos might be due to the deletion of genes other than *byn*. The availability of the *byn*

point mutants allows us to address this issue directly. Surprisingly, the null *byn* phenotype resembles the deficiency phenotype not only in the hindgut and anal pads, but also in the Malpighian tubules and the midgut.

The midgut phenotype in *byn*^{5}*/Df* embryos is as extreme as in the homozygous deficiency embryos. This phenotype is variable and affects all three constrictions, although the first constriction is more commonly the most severely affected; the constrictions do not form completely and the dorsal part of the constriction appears most affected or most delayed (see Fig. 2C,D).

To analyze the basis for the shorter Malpighian tubule phenotype, we stained *byn* point mutant embryos with antibody to the homeodomain protein Cut, which is expressed in all Malpighian tubule nuclei (Blochlinger et al., 1993). This staining shows that the tubule rudiments in the *byn* mutants, although shorter than wild-type, have a larger diameter than in wild-type embryos of the same stage, and do not appear to contain a dramatically reduced number of cells. This suggests that the major reason for the shorter Malpighian tubule phenotype observed in *byn* mutant embryos is a defect in elongation. The severity of disruption of tubule elongation varies across the *byn* allelic series. In wild-type embryos, the Malpighian tubules are fully extended in stage 16 embryos (Fig. 9A). The tubules of *byn*^{3} embryos at the same stage are much shorter and largely unextended (Fig. 9B), and *byn*^{5} Malpighian tubules are almost entirely unextended (Fig. 9C).

These results demonstrate that the loss of *byn* function is responsible for the described embryonic gut phenotype of the 68D/E deficiency. *byn* is therefore required not only for the differentiation of the tissues in which it is expressed (hindgut and anal pads), but also for the morphogenetic processes of elongation and constriction that occur in the Malpighian tubules and midgut, respectively.

tll is necessary and sufficient to activate *byn*

Previous studies of *byn* expression in *tll* mutant embryos showed that *tll* activity is required for *byn* transcription, and that the amount of *byn* expression is proportional to the level of *tll* activity (Kispert et al., 1994; Diaz et al., 1996). To ascertain whether *tll* is also sufficient to activate *byn* transcription, ectopic *tll* expression was induced in various ways. In wild-type embryos, *byn* is expressed in a posterior cap at the blastoderm stage; this rapidly resolves into a broad posterior stripe (Fig. 10A; Kispert et al., 1994). In embryos derived from *torso* (*tor*) gain-of-function mutant mothers, in which *tll* expression is dramatically expanded (Steingrimsson et al., 1991), the area in which *byn* is expressed greatly increases (Fig. 10E,F). Embryos carrying a construct expressing *tll* under the control of a heat shock promoter also show an enlarged *byn* expression domain after a brief (20 minutes) heat shock (Fig. 10B); increasing the duration of the heat shock (to one hour) results in a further expansion of the domain of *byn* expression (Fig. 10C,D) similar to that seen in embryos from *tor* mutant mothers. Ectopic *tll* activity can thus induce ectopic *byn* expression.

DISCUSSION

byn alleles are mutations affecting the *Trg* transcription unit

We have generated six lethal EMS induced alleles that define a new complementation group within chromosomal region 68D/E; we have named this locus *byn*. The *apro* P-element insertion described by Murakami et al. (1995) is an additional allele of *byn*. The mutant phenotype of *byn* alleles includes reduced hindgut and anal pads, incompletely extended and misshapen Malpighian tubules and defective midgut constrictions. As the phenotype of the strongest *byn* allele is similar in severity to that described for embryos deficient for the 68D/E region (Kispert et al., 1994; Harbecke and Lengyel, 1995), we conclude that absence of *byn* function is the cause of the gut defects in that deficiency phenotype.

The mapping of the *Trg* transcription unit to the vicinity of *byn* and the effects of *byn* mutations on tissues (hindgut, anal pads) expressing *Trg* suggest that *byn* is the genetic locus corresponding to *Trg*. This is confirmed by the fact that a *Trg* transgene rescues all *byn* alleles (as hemizygotes) to viability.

Together these results show that absence of *byn* is responsible for the defects in posterior gut development previously reported for deletion of the 68D/E region, and that the *byn* genetic locus corresponds to the *Trg* transcription unit.

Genetic hierarchy in the posterior pole of the embryo

Regulation of byn

Transcription of *byn* requires *tll* function (Kispert et al., 1994; Diaz et al., 1996). While *tll* control of *byn* transcription might conceivably be mediated through another transcription factor, the lack of any likely candidate for that intermediary as well as the brief period (approximately 1 hour) between the production of the first *tll* mRNA and the onset of *byn* transcription suggests that the Tll protein is a direct regulator of *byn*. As homozygous null embryos continue to express *byn* during late embryogenesis, *byn* activity is not required for its own maintenance.

The ability of ectopically expressed *tll* to induce *byn* expression indicates that *tll* is not only necessary, but also sufficient to activate *byn* transcription. However, in certain regions of the embryo (0-10, 35-55, and 80-100% EL), ectopic *tll* results in little or no *byn* expression. In the anterior, *byn* is likely repressed by Bicoid (Kispert et al., 1994), which similarly represses the most anterior *tll* expression (Pignoni et al., 1992). In the central region, repression could be due to action of one or more of the gap genes *Kr*, *kni* and *hb*. That expression of *byn* is not altered in embryos singly mutant for any one of these genes, however, would be consistent with redundancy in their effect on *byn* expression. In the posterior, the *hkb* activity that normally represses *byn* (Kispert et al., 1994) is presumably sufficiently robust to function even in the presence of additional *tll* activity.

Genes regulated by *byn*

We have identified several downstream targets of *byn*. Activation of expression of *otp* and *dpp* in the hindgut primordium requires *byn*. The time elapsed between the earliest detection of *byn* mRNA and the onset of *otp* transcription (less than one hour) suggests that *otp* is a direct target of the Byn protein. The later initiation of expression of *dpp* in the hindgut makes it unlikely to constitute a direct target for Byn.

The genes *eve*, *cad* and *wg*, although not requiring *byn* for expression in a posterior stripe at the blastoderm stage, do require *byn* for maintenance of expression in the anal pad

primordia at later stages. The short time between the initiation of *byn* expression and the time at which maintenance of its expression becomes sensitive to loss of *byn* activity suggests that *eve*, like *otp*, is also a direct target of Byn.

On the basis of these and other studies on gene expression in the gut primordia (reviewed by Skaer, 1993), we propose a hierarchy of gene activity that is required to establish the hindgut and anal pads (Fig. 11). During stage 4 the terminal system activates nested caps of expression of *tll* and *hkb* at the posterior pole. During stage 5 *tll* activates a cap of *byn* expression; *byn* expression is then repressed (but not completely eliminated) at the most posterior tip by *hkb*. Since the posterior cap of *fkh* expression, although also regulated by a combination of *tll* and *hkb* activity, is unaffected in *byn* mutants and *byn* expression is not affected in *fkh* mutants (data not shown), *fkh* and *byn* must function in parallel pathways. It is conceivable that those features of differentiation and morphology of the hindgut primordium that are independent of *byn*, for instance the attachment of visceral mesoderm, depend on *fkh*.

During stage 7, *byn* maintains expression of *eve* in the anal pad primordia and activates *otp* expression. At later stages *byn* is required for maintenance of expression of domains of *cad* and *wg* in the anal pad primordia and for the initiation of *dpp* expression in the hindgut primordium. When this constellation of *byn*-regulated gene activity is not established, the gene *rpr*, an initiator of programmed cell death, is expressed ectopically in the improperly specified regions. Consistent with descriptions of ectopic cell death in embryos mutant for various gap genes (Lehmann and Nüsslein-Volhard, 1987), cell death then ensues in the hindgut and anal pad primordia.

Role of *byn* in morphogenesis

In *byn* mutants, the midgut constrictions do not occur normally, and the Malpighian tubules do not elongate. Neither *byn* mRNA nor Byn protein, however, is detected in these tissues (Kispert et al., 1994; Murakami et al., 1995; Fig. 2A). Although β-gal expressed from the *apro* P-element can be detected at stage 15 in the Malpighian tubules and posterior midgut (Murakami et al., 1995), the mRNA results suggest that this is due to the high

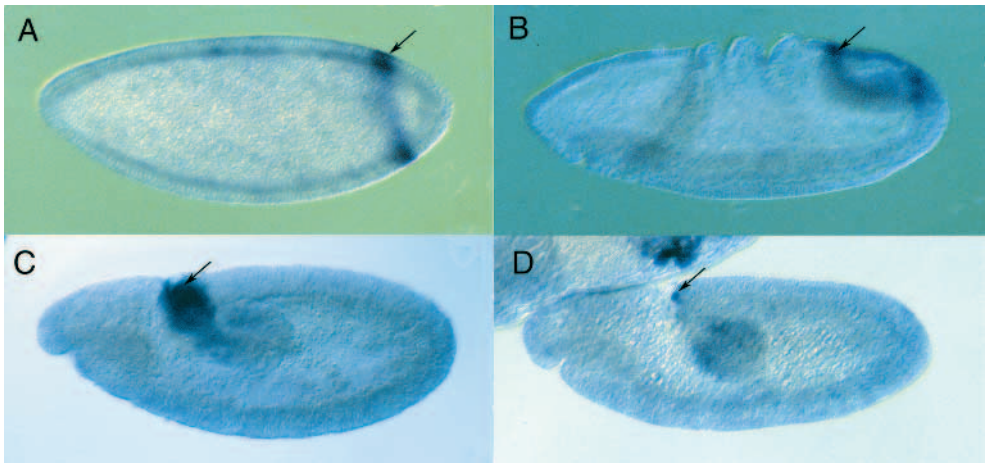

Fig. 5. Loss of *cad* expression in the anal pad primordia in *byn* mutant embryos. (A) Zygotic *cad* expression begins as a posterior stripe (arrow) in the blastoderm stage embryo. (B) The cells expressing *cad* remain on the embryo surface during gastrulation. (C) During germ band extension, *cad* is expressed in the anal pad primordium (arrow). (D) This expression is not maintained in byn^5 embryos.

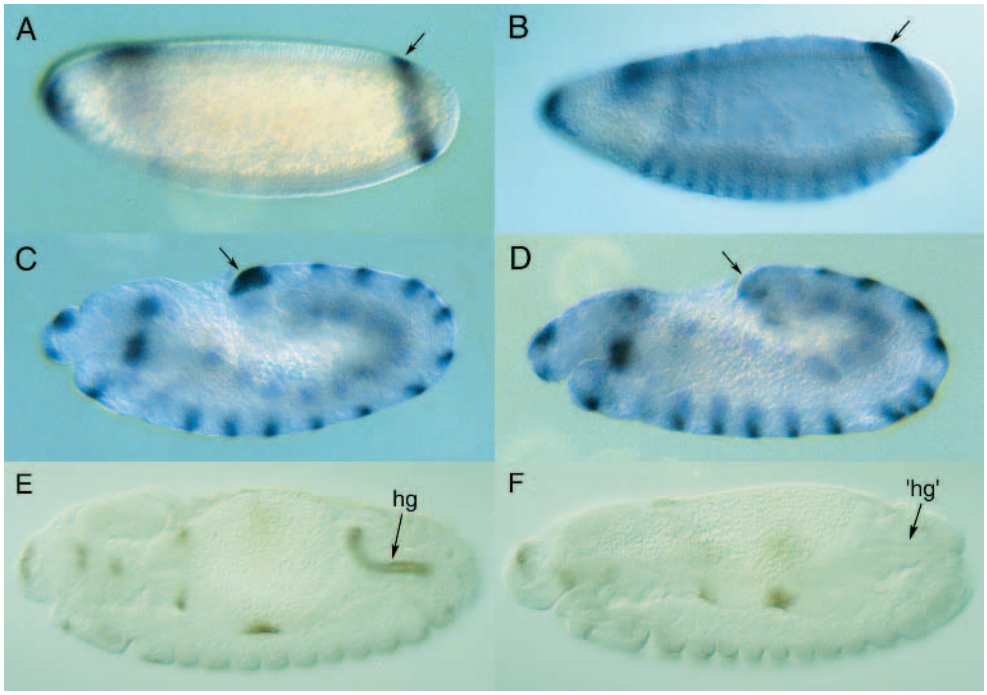

Fig. 6. Change in *wg* and Dpp expression in *byn* mutant embryos. (A,B) Early *wg* expression includes a posterior stripe (arrow), which is maintained through gastrulation and germ band extension. (C) *wg* expression in the anal pad primordium of a stage 12 embryo (arrow). (D) This domain of expression is not maintained in byn^5 embryos at the same stage. (E) A major part of the hindgut primordium expresses Dpp protein during and after germ band retraction (stage 13 shown). (F) In *byn* embryos of the same stage there is no Dpp expression in the hindgut rudiment while other aspects such as expression in the visceral mesoderm are not affected.

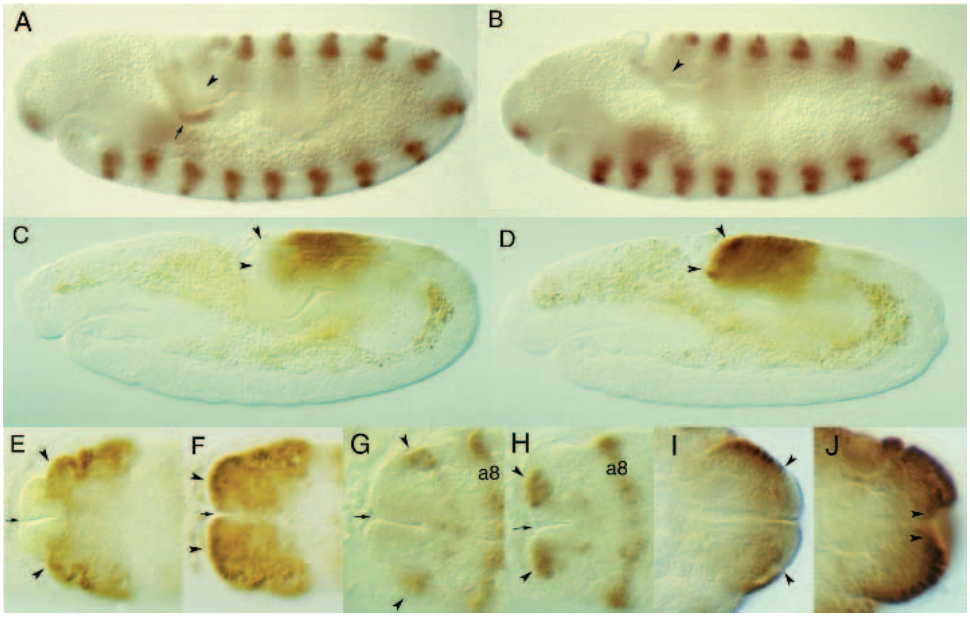

Fig. 7. Altered AbdB and En expression in *byn* embryos. (A) Wild-type En expression in a stage 10 embryo; En expression in the hindgut is indicated by an arrow, the bend in the hindgut is marked with an arrowhead. (B) The bend of the hindgut does not form in a *byn* embryo of the same stage; in addition, no En protein is detected in the hindgut. (C) AbdB protein is expressed in the posteriormost segmented and in the terminal region of the embryo (early stage 12), but excluded from the anal pad primordium (arrowheads). (D) In *byn* embryos, AbdB expression reaches the opening of the proctodeum. Enlargement of the posterior end of the germ band of (E) a wild-type embryo and (F) a *byn* embryo as in C and D in superficial horizontal view; arrow indicates proctodeal opening and arrowheads the posterior limit of AbdB expression. (G) The posteriormost En stripe, a9 (arrowheads), is located anterior to the anal pad primordium in a wild-type embryo at stage 11; the eighth abdominal segment, a8, is also indicated. (H) In *byn* embryos of the same stage, these En-expressing cells are located at the posterior tip of the germ band at the opening of the proctodeum (arrow). (I) AbdB expression at the posterior end of a wild-type embryo is delimited by the anal pads (arrowheads), while (J) in a *byn* embryo of the same age (stage 14) the expression reaches the hindgut rudiment. The embryos are hemizygous byn^3 mutants.

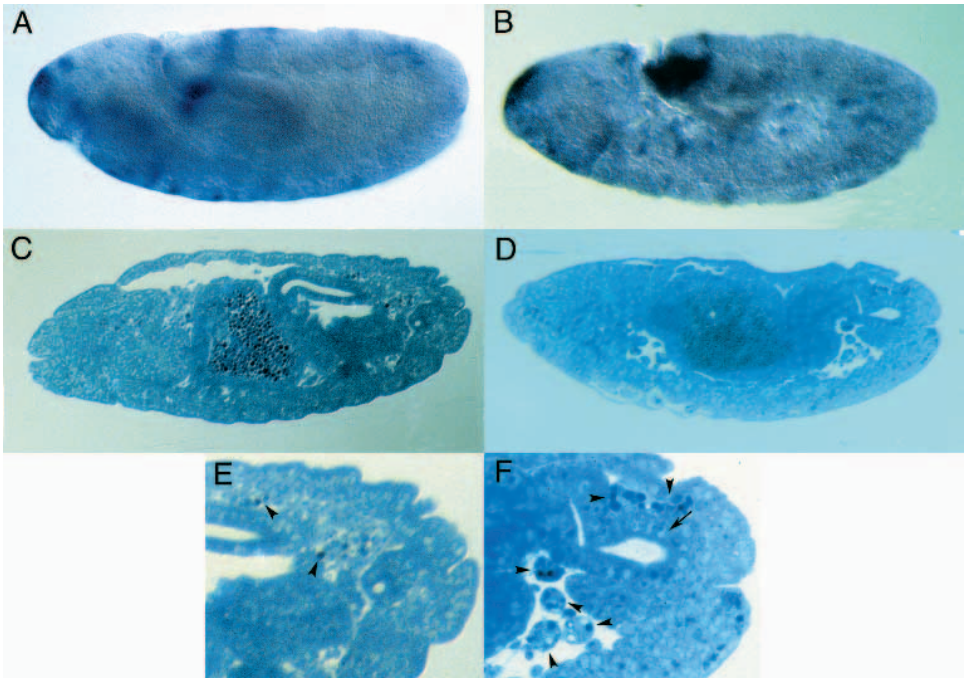

Fig. 8. Apoptotic cell death in *byn* mutant embryos. (A) Wild-type *reaper* expression at stage 10. (B) *rpr* is ectopically expressed in the hindgut and anal pad primordia of a stage 10 *byn* embryo. Segmented expression in the dosal ectoderm is part of the wild-type expression pattern, although it is not visible in A. (C) Section of wild-type and (D) *byn* embryo at stage 13, stained with toluidine blue. (E) Higher magnification of the wild-type embryo in C, showing a few pycnotic cells engulfed by macrophages (arrowheads). (F) Higher magnification of the *byn* embryo in D; note the large number of pycnotic cells that have been engulfed by macrophages, which are very large (arrowheads). The short hindgut remnant characteristic of *byn* embryos is indicated by an arrow.

stability of *lacZ* mRNA and of β-gal, rather than continued *byn* expression.

There are several possible explanations for how *byn* might affect Malpighian tubule and midgut morphogenesis, even though its expression is not detected in these tissues. The amount of *byn* activity required for normal development of these tissues might be below detectable levels. Since mutants defective in visceral mesoderm affect tubule elongation (Reuter et al., 1993), tubule elongation and midgut constriction might require *byn* activity in the visceral mesoderm arising from the ventral portion of the *byn* blastoderm expression domain. Finally, effects of *byn* function might be propagated from the hindgut, where *byn* is expressed, into the contiguous Malpighian tubules and midgut.

Although the mechanism by which *byn* regulates Malpighian tubule elongation remains to be elucidated, it must

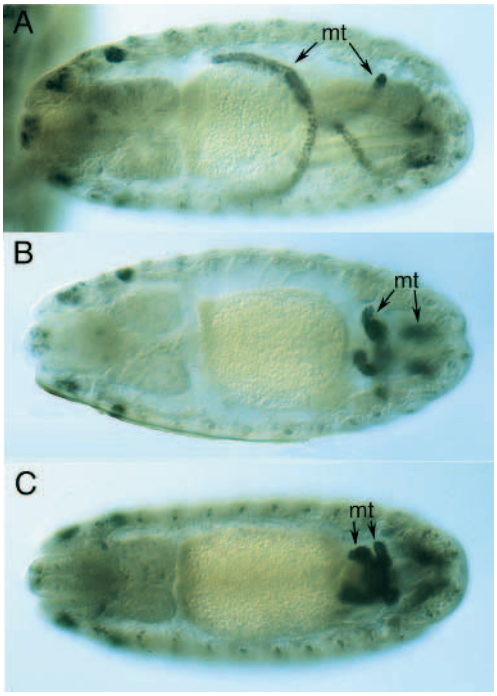

Fig. 9. Defective Malpighian tubule elongation in *byn* embryos. (A) Wild-type embryo at stage 14 stained with anti-Cut antibody, showing normal Malpighian tubule (mt) extension. (B) Reduced Malpighian tubule extension in a byn^3 embryo. (C) Severely reduced Malpighian tubule extension in a byn^5 embryo.

involve a modulation within the tubules themselve of genes that control the elongation process. Three genes known to act in a hierarchy and to control Malpighian tubule development, *fkh*, *ct* and *Kr*, as well as a fourth gene *cad*, which does not have a known function in the tubules (reviewed by Skaer, 1993), are all expressed normally either in the tubule primordia (*Kr*) or the misshapen tubules (*fkh*, *ct* and *cad*) of *byn* mutant embryos (Kispert et al., 1994; Fig. 9 and data not shown). Thus *byn* must affect tubule elongation by a pathway independent of these genes. We conclude that there is a yet to be identified class of genes that, in response to embryonic *byn* activity, acts to promote Malpighian tubule elongation.

Relationship between *byn* and the chordate *Brachyury* genes

The question of whether the insect and vertebrate *T* genes are homologous must be considered at a number of levels (Bolker and Raff, 1996). Homology (orthology) at the gene level means simply that two genes in two different species are most closely related to the ancestral gene (Bolker and Raff, 1996). Analysis of relative levels of sequence identity indicates that by this criterion *byn* is a true homolog (ortholog) of the vertebrate *T* gene (Agulnik et al., 1995). Based on the expression of *byn* in the hindgut and *T* in the notochord, and on an argument that the notochord is phylogenetically derived from a portion of the gut, it has been proposed that *byn* and *T* are also homologs in the sense of being expressed in homologous structures (Kispert et al., 1994). We consider here two further questions: (1) are *byn* and *Brachyury* homologous at the pathway level, i.e., do the mechanisms by which they are

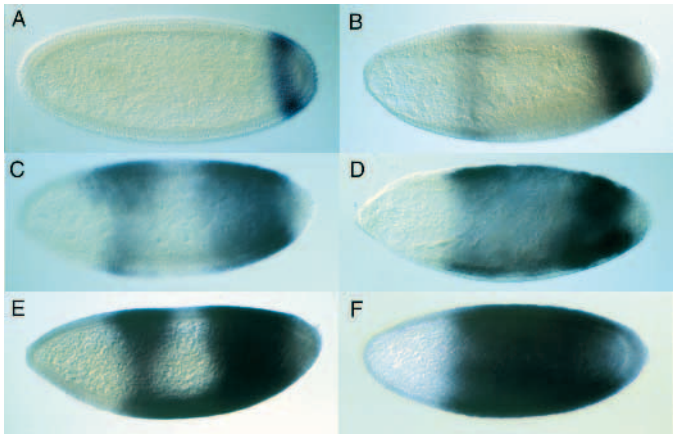

Fig. 10. Control of *byn* expression by the *torso* signaling pathway and *tailless*. (A) Normal *byn* expression in the blastoderm stage embryo. (B) *byn* expression in *hs-tll* embryo after 20 minutes heat shock. (C,D) *byn* expression in *hs-tll* embryos after 60 minutes heat shock. (E,F) *byn* expression in embryos from tor^{D4021} mothers.

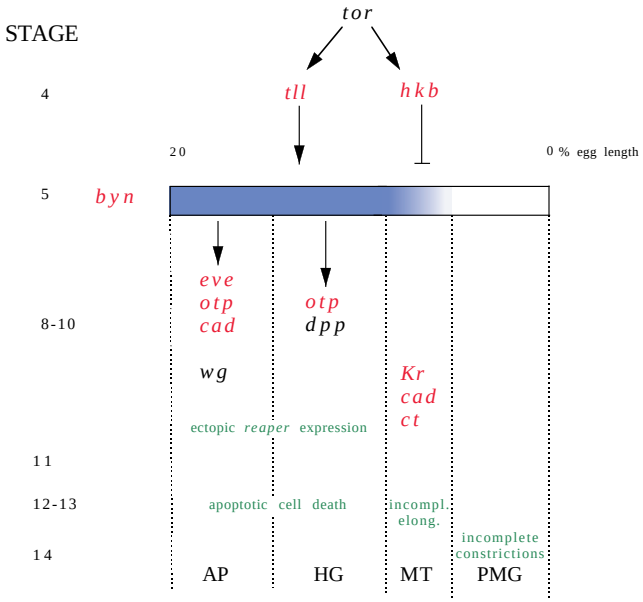

Fig. 11. Summary of *byn* related gene activity and function. Earliest detected expression of *tll*, *hkb* and *byn* is shown. For *eve*, *otp*, *cad*, *dpp* and *wg*, the earliest expression influenced by *byn* is shown. Stages of embryogenesis are indicated on axis at left. Activating functions are indicated by arrows; the repressing function of *hkb* by a bar. Genes encoding transcription factors are indicated in red. The phenotype observed in *byn* mutant embryos is indicated in green at the stage when it is first detected.

regulated, and the target genes they affect suggest that they are imbedded in an evolutionarily conserved pathway? and (2) do *byn* and *Brachyury* have homologous functions in the structures in which they are expressed?

With regard to upstream regulation, activation of the insect and chordate genes must rely on different transcription factors, since the Tll protein activates *byn* in *Drosophila*, while the protein encoded by the vertebrate *tll* homolog, Tlx, is not present when *Brachyury* (for which no inducing transcription

factor is known) is turned on (Yu et al., 1994; Monaghan et al., 1995). Both *byn* and *Brachyury*, however, can be turned on as the result of activation of receptor tyrosine kinases: Torso in *Drosophila* and the FGF receptor in *Xenopus* (Smith et al., 1991; Duffy and Perrimon, 1994).

With regard to regulation of downstream 'targets', two of the genes shown here to be regulated by *byn*, *eve* and *wg*, have vertebrate cognates, *Evx-1* and *Wnt3a* and *5a*, that are regulated by *Brachyury* (*T*) (Rashbass et al., 1994). In both cases, the downstream genes are expressed normally in the absence of *byn* or *T* and only later is posterior expression lost in the anal pad primordium of *Drosophila* (*eve* and *wg*) and in the posterior tail bud of the mouse (*Evx-1* and *Wnt 3a* and *5a*). Unlike the vertebrate *Brachyury* genes in the mouse, frog and zebrafish, all of which have been reported to autoregulate (Herrmann, 1991; Conlon et al., 1996; Schulte-Merker et al., 1994), *byn* activity is not required for its own continued expression. It has been proposed that *Brachyury* autoregulation results from a feedback loop between *Brachyury* and FGF (Schulte-Merker and Smith, 1995). As the *Drosophila* FGF receptor homologs are not expressed in the hindgut epithelium (Shishido et al., 1993), the lack of autoregulation may be due to the absence of a *byn*-FGF feedback loop.

With regard to function, both *byn* and *Brachyury* play required roles in maintenance and morphogenesis of already established primordia. As shown here, the hindgut and anal pad primordia are formed normally in *byn* mutant embryos, but, presumably because of the abnormal program of gene expression that ensues in the absence of *byn* function, they become sites of excessive programmed cell death and partially degenerate. Similarly in *T/T* mutant embryos the tissue in which *Brachyury* is primarily expressed, the notochordal primordium (head process), forms normally but then degenerates (Herrmann, 1991). Both *byn* and *Brachyury* are also required for morphogenesis of a number of structures that do not express the gene. The defects in allantois, somites and neural tube in the T/T mouse embryos have been attributed to the axial inductive properties of the notochordal primordium (reviewed by Beddington et al., 1992); defects in Malpighian tubules and midgut observed in *byn* embryos raise the possibility that the *Drosophila* hindgut might also influence neighboring tissues.

In conclusion, some broad similarities between the *byn* and *Brachyury* orthologs are seen in the pathways leading to their transcriptional activation, their function in maintenance (via regulation of cognate genes) of primordia that arise during gastrulation, and their effects on non-expressing tissues. Further investigation of these issues is required however, before one can conclude that *byn* and *Brachyury* are truly homologous at the level of pathways in which they are imbedded or the function they perform in differentiation. Information regarding whether these genes are homologs at a deeper level, i.e. whether they control development of homologous structures, might be obtained by the identification and characterization of a *Brachyury* ortholog in a species derived from the common primitive ancestor of protostomes and deuterostomes.

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