**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 1-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 orthodentopedia) 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 (Llau 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 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 the Krüppel (Kr) and knirps (kni) genes. Inactivation of kkrpfech (hh) in vivo (Steinrimsos 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 tubes 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 oniltr 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 ofiltr 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 asT) gene. This sequence was designatedTrg (for T-related gene). Trg was found to be expressed as a posterior cap in early embryogenesis (stage 5) under control ofiltr 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 Hermann, 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 ofTrb+ in individual cultures was used to detect lethals in the 68D/E region, and 10,000 mutagenized chromosomes were screened. Mutagenized ruthstery e (isogonized) males were mated to M(3)/TM6B, Tb females. F1 ruthstery 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 Trb+ as well as Trb- offspring were discarded. From cultures containing only Trb- offspring, ruthstery 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 ofcycIina (the only described complementation group within 68D/E for which alleles are still extant; Lehner and O’Farrell, 1989). Three embryonic lethal alleles ofcycIina 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 byn4, byn2, byn3 and byn4 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/TM6B females. Individual F1 bw; st*/Df(3L)vin2 males were crossed to bw; Df(3L)vin4, h st ec; 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 byn4 and byn2, as well as an additional cycIina 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 NiCl2 to the medium, 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% OsO4, 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 till**

*till* was expressed ectopically in early embryos by heat shocking a stock carrying the *till* 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 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.'

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*5, when transheterozygous with all five other alleles, allows some (1-25%) survival to adulthood. The next weakest allele, *byn*4, 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*5. 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*5 < *byn*4 < *byn*3 < *byn*6 < *byn*2 < *byn*1.

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 *aprojectus* (*apro*), fails to complement the *byn* alleles and thus is an additional *byn* allele which we designate *byn*apre.

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.

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

<table>
<thead>
<tr>
<th>Alleles</th>
<th><em>byn</em>1</th>
<th><em>byn</em>2</th>
<th><em>byn</em>3</th>
<th><em>byn</em>4</th>
<th><em>byn</em>5</th>
<th><em>byn</em>6</th>
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</thead>
<tbody>
<tr>
<td><em>byn</em>1</td>
<td>25%</td>
<td>5.8%</td>
<td>&lt;0.3%</td>
<td>4.9%</td>
<td>9.5%</td>
<td>&lt;0.3%</td>
</tr>
<tr>
<td>(43/387)</td>
<td>(17/604)</td>
<td>(16/352)</td>
<td>(0/645)</td>
<td>(10/421)</td>
<td>(0/645)</td>
<td>(0/645)</td>
</tr>
<tr>
<td><em>byn</em>2</td>
<td>6.9%</td>
<td>19.2%</td>
<td>1.3%</td>
<td>12.2%</td>
<td>12.5%</td>
<td>12.5%</td>
</tr>
<tr>
<td>(20/603)</td>
<td>(57/652)</td>
<td>(3/466)</td>
<td>(15/261)</td>
<td>(15/261)</td>
<td>(15/261)</td>
<td>(15/261)</td>
</tr>
<tr>
<td><em>byn</em>3</td>
<td>&lt;0.3%</td>
<td>&lt;0.3%</td>
<td>&lt;0.6%</td>
<td>&lt;0.6%</td>
<td>&lt;0.6%</td>
<td>&lt;0.6%</td>
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<tr>
<td>(&lt;0/769)</td>
<td>(&lt;0/661)</td>
<td>(&lt;0/559)</td>
<td>(&lt;0/559)</td>
<td>(&lt;0/559)</td>
<td>(&lt;0/559)</td>
<td>(&lt;0/559)</td>
</tr>
<tr>
<td><em>byn</em>4</td>
<td>&lt;0.3%</td>
<td>&lt;0.7%</td>
<td>&lt;0.7%</td>
<td>&lt;0.7%</td>
<td>&lt;0.7%</td>
<td>&lt;0.7%</td>
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<tr>
<td>(&lt;0/648)</td>
<td>(&lt;0/279)</td>
<td>(&lt;0/279)</td>
<td>(&lt;0/279)</td>
<td>(&lt;0/279)</td>
<td>(&lt;0/279)</td>
<td>(&lt;0/279)</td>
</tr>
<tr>
<td><em>byn</em>5</td>
<td>&lt;0.5%</td>
<td>&lt;0.5%</td>
<td>&lt;0.5%</td>
<td>&lt;0.5%</td>
<td>&lt;0.5%</td>
<td>&lt;0.5%</td>
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<tr>
<td>(&lt;0/400)</td>
<td>(&lt;0/400)</td>
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<td>(&lt;0/400)</td>
<td>(&lt;0/400)</td>
<td>(&lt;0/400)</td>
</tr>
</tbody>
</table>

The various *byn* alleles were balanced over either *TM3, Sh b* 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.

### 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-Cr Br 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, \( \text{byn}^2 \), have a hindgut that is only slightly shorter than that of a wild-type embryo (cf. Fig. 1D,E).

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

Although, as described previously (Kispert et al., 1994) and below, many genes are expressed abnormally in the absence of \( \text{byn} \) activity, the hindgut remnant in \( \text{byn} \) mutant embryos shares a number of properties with the normally differentiated hindgut. The \( \text{twist} \) 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 \( \text{byn}^2 \) 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 \( \text{byn}^5 \) (Fig. 2G). In the A4.1M2-bearing, \( \text{byn} \) 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 \( \text{byn} \) 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 \( \text{byn} \) activity, structures that normally express \( \text{byn} \) (hindgut and anal

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**Fig. 1.** Range of defects in the \( \text{byn} \) allelic series. The severity of the mutant phenotype of the different \( \text{byn} \) alleles defines an allelic series consistent with that deduced from complementation analysis (Table 1). (A) Embryos homozygous for the strong \( \text{byn}^5 \) 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 \( \text{byn} \) alleles: (B) \( \text{byn}^6 \), (C) \( \text{byn}^6 \) and (D) \( \text{byn}^2 \). (E) Homozygous mutant embryos were distinguished from their phenotypically wild-type siblings by maintaining \( \text{byn} \) alleles over a TM3 balancer chromosome carrying a lacZ gene driven by the \( \text{fushi tarazu} \) promoter. Double staining with antibody to \( \beta \)-galactosidase allows phenotypically wild-type embryos to be recognized by the presence of dark stripes.

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**Fig. 2.** Differentiated characteristics of the hindgut remnant in \( \text{byn} \) mutant embryos. (A) In situ hybridization of a \( \text{byn} \) probe to a wild-type embryo stains the hindgut (hg) and anal pads (ap). (B) \( \text{byn} \) expression in a \( \text{byn}^2 \) embryo is reduced, but some \( \text{byn} \)-expressing hindgut (hg) and anal pad tissue remain. (C) \( \text{Tw} \) 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 \( \text{byn}^2 \) 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 \( \text{byn}^2 \) embryo carrying the same enhancer trap as in E.
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*10
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 byn5 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 byn5 homozygous embryos (Fig. 3G,H).

The pair-rule gene even-skipped (eve) encodes a homeo-domain 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 posterior-most. 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 byn5 homozygous embryos (Fig. 4D,F).

Zygotic expression of cad, which also encodes a homeo-domain 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 byn5 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, and 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 byn5 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 byn5, 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^+ embryos at the same stage are much shorter and largely unextended (Fig. 9B), and byn^- 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 oip and dpp in the hindgut primordium requires byn. The time elapsed between the earliest detection of byn mRNA and the onset of oip transcription (less than one hour) suggests that oip 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 ill and hkb at the posterior pole. During stage 5 ill 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 ill 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

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**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 embryos.

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**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 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.
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...
involve a modulation within the tubules themselves 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 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 bryn 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 bryn, eve and wg, have vertebrate cognates, Exv-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 bryn 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 (Exv-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), bryn 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 bryn-FGF feedback loop.

With regard to function, both bryn 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 bryn mutant embryos, but, presumably because of the abnormal program of gene expression that ensues in the absence of bryn 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 bryn 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 Bedington et al., 1992); defects in Malpighian tubules and midgut observed in bryn embryos raise the possibility that the Drosophila hindgut might also influence neighboring tissues.

In conclusion, some broad similarities between the bryn 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 bryn 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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