Homologs of the mouse *Brachyury* gene are involved in the specification of posterior terminal structures in *Drosophila*, *Tribolium*, and *Locusta*

Andreas Kispert,^{1,3} Bernhard G. Herrmann,¹ Maria Leptin,^{2,4} and Rolf Reuter^{2,5}

Max-Planck-Institut für Entwicklungsbiologie, ¹Abteilung Biochemie, ²Abteilung Zellbiologie, D-72076 Tübingen, Germany

The *Brachyury* (*T*) gene is required for notochord differentiation in vertebrates. We have identified a *Drosophila* gene, the *T-related gene* (*Trg*), with high similarity to *T* within a stretch of ~200 amino acids, the DNA-binding domain of *T*. *Trg* is expressed throughout embryogenesis, first at the blastoderm stage in the hindgut primordium under the control of the terminal gap genes *tlx* and *hkb*, and then until the end of embryogenesis in the differentiating hindgut. *Drosophila* embryos deficient for *Trg* do not form the hindgut, a phenotype that can be rescued by a *Trg* transgene. Thus, a common feature of *T* and *Trg* is their requirement in specifying the development of a single embryonic structure. Homologs of *Trg* are also expressed in the developing hindgut of *Tribolium* and *Locusta* embryos suggesting a highly conserved function of *Trg* in insects. This conservation and the high similarity of *T* and *Trg* raise the question of a common evolutionary origin of the hindgut of insects and the notochord of chordates.

[Key Words: *Drosophila*; *Brachyury*; *Locusta*; *Tribolium*; gut; proctodeum]

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With the molecular characterization of genes involved in animal development it has become increasingly obvious that some gene families appear to have retained their function in a wide range of animal species. The best example of such a gene family is the *Hox* gene complex characterized by the clustered genomic arrangement of homeo box-containing genes (for review, see McGinnis and Krumlauf 1992). The *Hox* genes provide positional information along the anteroposterior body axis during development, as revealed by genetic analysis in *Drosophila*, mouse, and *Caenorhabditis elegans* (for review, see Slack et al. 1993). Homologs of other genes, first functionally characterized in the embryonic development of *Drosophila*, are now being cloned and characterized in chordates and other animal classes. In general, the molecules, or at least their functionally important domains, have been highly conserved during evolution, but the developmental processes in which they are involved are often more diverged than in the case of the *Hox* genes. For example, the numerous *engrailed* (*en*) and *wingless* (*wg* or *wnt*) homologs expressed during vertebrate development (Patel et al. 1989a; Davis et al. 1991; McMahon 1992) do not appear to function in the

establishment of segment boundaries as they do in *Drosophila*. However, in a specific step during brain development the molecules interact in a way analogous to their interaction in *Drosophila*, with *wg* being required for the maintenance of *en* expression (McMahon et al. 1992). In some developmental processes, single molecules or structural motifs have been conserved during evolution, whereas entire regulatory chains of genes are conserved in other cases.

One important gene for the development of chordates, for which no homolog has yet been described in *Drosophila* or other invertebrates, is the *Brachyury* (*T*) gene. Genetic analysis in mouse and in zebrafish revealed that *T* acts in the differentiation of the notochord and in the generation of mesendoderm during axis elongation (Beddington et al. 1992 and references therein; Halpern et al. 1993). *T* genes have been molecularly characterized for a number of chordates (Herrmann et al. 1990; Smith et al. 1991; Schulte-Merker et al. 1992; Yasuo and Satoh 1994). They are highly similar within a stretch of >200 amino acids that have been shown to be necessary and sufficient for specific DNA binding (Kispert and Herrmann 1993). Because the genes are expressed in homologous structures in the various species and have the same function in axial development in two distantly related vertebrates they are certainly also functional homologs.

We are interested in the phylogenetic origin of the *T*

Present addresses: ³Harvard University, The Biological Laboratories, Cambridge, Massachusetts 02138 USA; ⁴Universität zu Köln, Institut für Genetik, D-50931 Köln, Germany.

⁵Corresponding author.

genes and wished to determine whether molecules closely related to *T* might exist in animal classes other than the chordates, particularly in the arthropods. As an initial tool we used an antibody directed against the amino terminus of the mouse *T* protein (Kispert and Herrmann 1993, 1994). This part of the protein is sufficiently similar within the various *T* genes to allow the antibody to cross-react with both the *Xenopus* and zebrafish *T* gene products. Surprisingly, the antibody detected an antigen specifically expressed in the *Drosophila* embryo. The molecular cloning and sequencing of the gene encoding this antigen revealed a high structural similarity to the DNA-binding domain of the vertebrate *T* genes leading to the designation *Drosophila T-related gene* (*Trg*). Because *Trg* is exclusively expressed in the posterior terminal region of the *Drosophila* embryo, we wish to give a brief synopsis of the development of this body region.

The zygotic transcription factors, *tailless* (*tll*) and *huckebein* (*hkb*), are required to specify the terminal structures that originate from the region located posterior to the primordia of the segmented body (Strecker et al. 1988; Casanova 1990; Weigel et al. 1990). *hkb* is expressed during the blastoderm stage at the posterior pole in the posterior midgut primordium (Brönner and Jäckle 1991) and determines the posterior midgut (Weigel et al. 1990). *tll* is expressed during the blastoderm stage in a larger domain that includes the *hkb* domain and also comprises the primordium of the proctodeum (Pignoni et al. 1990). The proctodeum invaginates with the posterior midgut primordium and forms hindgut and Malpighian tubules. Both processes, the invagination and the determination of the proctodeal structures, depend on *tll*. *tll* and *hkb* act as gap genes, that is, they establish the posterior terminal region in the body plan and repress the expression of other gap genes acting in trunk specification. In embryos lacking *hkb*, the posterior midgut is deleted, and in embryos lacking *tll* posterior abdominal segments, the proctodeum and a minor part of the posterior midgut are not formed.

hkb and *tll* must act through a set of downstream genes to specify the organs dependent on their activities. Such a gene is *fork head* (*fkh*), which is activated by both *hkb* and *tll* in the primordium of posterior midgut and proctodeum (Weigel et al. 1989a, 1990). *fkh* seems to act as a homeotic gene and promotes development of terminal, as opposed to segmental, structures (Jürgens and Weigel 1988). A candidate downstream gene for *hkb* is *serpent* (*srp*), which also acts as a homeotic gene and specifies the development of endodermal posterior midgut as opposed to ectodermal hindgut (Reuter 1994). In addition, genes are known that are required for the correct positioning of the Malpighian tubules (Krüppel; Harbecke and Janning 1989; Skaer 1993) and for the differentiation of the anal pads [*caudal* (*cad*); Macdonald and Struhl 1986]. However, several other genes required for the partitioning of the terminal region must exist. Our results show that *Trg*, which is involved in the specification of hindgut and anal pads, is a target for regulation by *tll* and *hkb*.

Results

A *Drosophila* embryonic cDNA library was screened with the conserved region of a mouse *T* cDNA using low stringency hybridization conditions. The strongly hybridizing clones harbored a 2.6-kb cDNA as the largest insert, which we assume to represent a full-length cDNA. The insert includes the poly(A) tract at the 3' end and a long 5'-untranslated region that is blocked in all three reading frames by two or three stop codons. The nucleotide sequence of this cDNA is presented in Figure 1. It is 2630 bp in length and contains a potential open reading frame of 2163 bp. The putative start codon (position 337–339) is flanked by a sequence resembling the consensus sequence of translation start sites in *Drosophila* (Cavener 1987).

Trg contains a highly conserved T-domain

The putative *Trg* protein sequence comprises 721 amino acids. The prominent feature of this sequence is a stretch of 200 amino acids (Fig. 2), which shows extensive similarity with a domain found in the amino terminus of the chordate *T* gene products *T*, *Xbra*, *Zf-T*, and *As-T* (Herrmann et al. 1990; Smith et al. 1991; Schulte-Merker et al. 1992; Yasuo and Satoh 1994). This T-domain has been shown to confer specific DNA binding (Kispert and Herrmann 1993). Another *Drosophila* protein, the product of the gene *optomotor-blind* (*omb*), also contains a T-domain (Pflugfelder et al. 1992). However, the similarity of *Omb* to *Trg* and the chordate gene products is only moderate (Table 1). In addition, *Omb* is distinguished from the other proteins of the group by several insertions within the T-domain (Fig. 2). Although the T-domains of *Trg* and *Omb* share a particular feature (a stretch of strong divergence from the vertebrate proteins after the seventh amino acid of the T-domain), the T-domain of *Trg* is much more similar to the chordate *T*-gene products than to *Omb* (Table 1). Outside the T-domain there is only a limited similarity between the products of the chordate *T* genes and none between the chordate proteins and *Trg* or *Omb*.

Trg is expressed in the posterior terminal region of the *Drosophila* embryo

The expression of *Trg* commences at the beginning of nuclear cycle 14 in the posterior terminal region from ~20 to 0% egg length (Fig. 3A,B). During cellularization, *Trg* expression recedes from the posterior pole (Fig. 3C) and becomes confined to a ring of cells of which the ventral cells form the posterior part of the ventral furrow (Fig. 3E), whereas the dorsal and lateral cells appear to constitute the primordium of most of the proctodeum and probably of the anal pads. These dorsal and lateral cells become internalized by the amnioproctodeal invagination during gastrulation (Fig. 3F,G). During germ-band extension the proctodeum continues to express *Trg*, but the expression disappears in the adjacent mesoderm that has originated from the posterior end of the

1 GAT CAT TAG CTC GGG AAC GGT GGC AAC GCA AAC ACA CGA TCG GCG GCA GCG CTA AAC TCC AAT CTC
66 GAA TCT CGA CTC GAA TCA AAT ATC TTG AAA TCT TTG AAA ACA AAT TTC ACG CGG CTG TAT CTT CCC ACT TGA
139 ATC TGA ACT CAT ACG ATA GTG ATC CTT GAT AAA GTG AAC CCT CCA GGG AAT TAG TGC TAT AAA AGT GCG TGA
211 TTT GCG AGT GGT GCG TAG TCA ACT GAA ATC TAA ACC TAA ACC ATC TTC ATA AGG AGT TTA CCA CTG
1 GAT TAT ATC GAG AGC GCC GAG TTT CGG CGT GAA GCA GGT TAC CAA ATT GCT GAC **ATG** ACC ACA TCG CAC ATC
283 L S A V D P T T G L S G N V S G G G G G G G G A G
7 CTG TCC GCC GTG GAT CCA ACA ACC GGA CTC AGT GGC AAT GTT TCG GGC OGA OGA GGA GGT GGC GGT GCT GGT
355 G G A G S G S P Q H V T H N G H G H G H G L G G
31 GGC GGT GCT GGC AGT GGT TCG CCA CAG CAT GTG ACC CAC AAT GGA CAC GCG CAT GGT CAC GCG CTG GCG GCG
427 V A A V S G G G A S V S G N G G C H R V V G G A G
55 GTG GCA GCG GTA AGC GGA GGC GGA GCC AGT GTA TCC GGA AAT GGA GGC CAT CGC GTG GTG GGC GGA GCA GGC
499 S P N E L D R N L R I S L D D R E L W L R F Q N
79 AGT CCC AAT GAA CTG GAT CGC AAT CTG CGC ATC TCG CTG GAT GAT CGG GAG CTC TGG CTG CGT TTC CAG AAT
571 L T N E M I V T K N G R R M F P V V K I S A S G
103 CTC ACC AAC GAG ATG ATC GTC ACC AAA AAC GGC AGG CGC ATG TTC CCG GTC GTG AAG ATC AGT GCC TCC GGT
643 L D P A A M M L N T V L L E F V Q I D S H R W K Y V
127 CTG GAT CCC GCC GGC ATG TAC ACC GTC CTC CTG GAG TTC GTC CAG ATC GAC TCA CAT CGC TGG AAG TAC GTC
715 N G E W V P G K A E V P P S N P I Y V H P E S
151 AAC GGT GAA TGG GTT CCT GGT GGC AAG GCA GAG GTT CCC CCG TCA AAT CCC ATC TAT GTA CAC CCC GAG TCT
787 P N F G A M M L N S G L H K Y E P R V H L V R V
175 CCC AAT TTT GGA GCC CAC TGG ATG AAG GGC ATC TCG TTT GCC AAA GTA AAG CTG ACC AAC AAG ACC AAT
859 G N G Q M M L N S G L H K Y E P R V H L V R V
199 GGC AAT GGA CAG ATA ATG CTA AAT TCG TTG CAC AAG TAC GAA CCA CGT GTG CAT CTG GTT CGC GTG GGT TCC
931 E Q R H V V T Y P F P E T Q F I A V T A Y Q N E
223 GAG CAG CGT CAT GTG GTC ACC TAT CCA TTT CCG GAA ACG CAG TTT ATC GCG GTG ACG GCG TAT CAG AAT GAG
1003 E V T S L K I K Y N P F A K A F L D A K E R P D
247 GAG GTG ACA TCG CTG AAG ATC AAG TAT AAT CCA TTT GCC AAG GCC TTT CTG GAT GCC AAA GAG CGA CCG GAT
1075 T L Y P H D T H Y G W L I P P P T H Y T A A A A
271 ACA CTG TAT CCA CAC GAT ACC CAC TAC GGC TGG CTC ATT CCA CCG CCC ACA CAT TAT ACG GCT GCG GCG GCG
1147 A V A A P P P L S I A Q S H G L V A S C P S V S
295 GCC GTG GCA GCT CCT CCG TTG TCC ATT GCC CAA AGT CAC GGC CTG GTT GCC TCA TGT CCC AGC GTT TCC
1219 S A G S V G P S S G G S C D R Y G S S L S S R S
319 TCG GCC GGA TCC GTG GGT CCA TCA TCG GGC GGC AGT TGC GAT CGC TAC GGC AGA TCC CTA TCC TCA CCG AGT
1290 V A P T R T T P Y S R P R V V S G S G S N G S A
343 GTC GCA CCC ACT CGC ACC ACA CCG TAC AGT CGA CCC AGG GTC GTT TCC GGA TCC GGA TCA AAT GGC AGT GCC
1363 G N A S C S T S P Q P P S A P Q T P T S L H S T S
367 GGC AAT GCC TCG TCC ACA TCG CCG CAG CCG CCG TCG GCT CCA CAA ACG CCC ACC AGC CTG CAC TCC ACG TCC
1435 T G S V S T S V S S S S G G G I G S A P S T G C
392 ACT GGC TCC GTG AGC ACC AGT GTG AGC AGC TCG AGC GGC GGC GGC ATC GGA TCT GCG CCG AGT ACT GGT TGC
1507 F S S S Y A S G G F M P V D A S P T A S V F S Y
415 TTC AGC AGT TCC TAC GCC CAA TCC GGC TTC ATG CCG GTC GAT GCC AGT CCC ACG GCG TCC GTG TTC TCC TAT
1579 P S S W Q N G N Y W N A T S V P G P M P M N V
439 CCC AGC AGC TGG CAG AGC AAT GGC AAT TAC TGG AAC GCC ACC AGC GTG CCG GGA CCC ATG CCC ATG AAC GTG
1651 C S G R N I S S H N S P S P T N G S P S Y T T S
464 TGC AGT GGC CCG AAC ATC TCA TCT CAT AAC TCT CCG TCG CCG AGC AAT GGA TCC CCG AGC TAT ACG ACC TCC
1723 S P S Y T I H H L T P H S H Q Y N M A Q T D I Y
488 TCG CCC AGC TAC ACT ATT CAT CAC CTG AGC CCA CAC AGT CAT CAG TAC AAC ATG GCC CAA ACG GAT ATC TAT
512 G T G V G V G G A G T T G S F Q A A Y G A A A
1795 GGA ACC GGA GTG GGC GTT GGG GGC GGG GCA GGA ACA ACG GGA TCC CCG CAA GCG GCA TAC GGT GCA GCT GCC
536 H Q V Y H F T P T S P T H Q L Y T N A V L N A P
1939 CAC CAG GTG TAC CAT CCC AGC CCC ACC TCA CCC ACT CAC CAG CTG TAC ACG AAT GCG GTT CTC AAT GCG CCC
560 S A L S Y S A S G W H N G S G A E Y G L Y Q N A
2011 TCG GCG CTA AGC TAT TCC GCC AGT GGT TGG CAC AAT GGA TCC GGG GCG GAG TAC GGA CTG TAC CAG AAC GCT
584 A A A Y Y Q P E Y I P L E I G Y A T H P L E P V
2083 GCC GCC GCG TAT TAC CAG CCG GAA TAC ATT CCC CTG GAG ATC GGC TAT GCC ACT CAT CCG TTG GAG CCC GTT
608 C V S K T L D D P Q A A M Y K P S D E Q G S V M
2155 GAT GTT TCA AAG ACA CTG CAG GAC CCG GCT GCT GAG ATC TAC AAG CCG AGC GAT GAG CAG GGC TCG GTG ATA
632 T L E C A S S S L K S S H D I K M E S S S L E H
2227 ACG CTG GAG TGC GCC AGT TCC TCG ATG AGC TCT CAC GAC ATC AAG ATA GAG TCC TCA TCC CTG GAG CAC
656 A G E R G T V G G G A A V V S V P T A V V N G A
2499 GCG GGG GAA CCG GGC ACT GTT GGC GGT GGA GCT GCC GTG GTC TCG GTG CCA ACC GCG GTG GAT GGA GCT
686 F A V A A D T W T F L T P F Q S T L Q W S D H P
2371 CCG GCC GTA GCT GCC GAC ACC TGG ACC CCG CTC ACT CCG CCA CAG AGC ACG CTG CAG TGA TCG GAT CAT CCC
704 T D K P T A F S R M T P L D V R S S
2443 ACG GAT AAA CCC ACA GCC TTT AGA CGC ATG ACC CCC CTG GAT GTT CGA TCG AGT **TAG** AAA TAA ACG CCC TAT
2515 GTT AGT AAC GCT ATT AGT AAA GCA CAC CCA ATC GCA CCT ATT GTA GAA CTA TCT GTA CCG TGT AAA
2587 CAA TCT CCT ATA AAG TCG TAA TCA AAC TAT GCA AAA AAA AAA AA 2630

Figure 1. The nucleotide and putative protein sequence of *Trg*. The sequence of the cDNA 1 of the *Trg* gene encompasses 2630 bp including 13 adenylyl residues at the 3' end. The ATG at position 337–339 represents the putative start codon of the *Trg*-encoded protein and is indicated in bold-face type. A glycine-rich stretch is located between amino acids 15 and 78. The middle of the protein (amino acids 316–492) is significantly enriched (36%) for serines and threonines. Possible polyadenylation signals at positions 2501, 2595, and 2606 are underlined.

ventral furrow (data not shown; cf. Fig. 4B). The expression is maintained in the hindgut and the anal pads until the end of embryogenesis (Fig. 3H; data not shown). The other proctodeal derivative, the Malpighian tubules, and the region from which the Malpighian tubules evaginate (the connection between the posterior midgut and hindgut) are devoid of *Trg* expression. There is also no expression in the mesoderm surrounding the hindgut.

The very high similarity between mouse *T* and *Trg* within the T-domain enabled us to detect the *Trg* protein with the anti-TN_{1–123} antibody that was raised against the amino-terminal part of the mouse T-domain (Kispert and Herrmann 1993). The expression of the protein (Fig. 4) essentially shows the same pattern as the RNA. The only difference is that *Trg* protein appears slightly later than the RNA—first during cellularization when the ingrowing membranes pass the base of the nuclei. Conse-

quently, like *Trg* RNA at this time, *Trg* protein is confined to the posterior ring of cells mostly comprising the proctodeal primordium.

Regulation of *Trg* expression

The ring of cells that express *Trg* during cellularization is contained within the anterior portion of the cap of *tll*-expressing cells but is largely anterior to the cap of *hkb*-expressing cells (Fig. 5). Therefore, it appears likely that *hkb* acts as a repressor of *Trg* posteriorly and sets its posterior border of expression, whereas *tll* acts as an activator of *Trg* expression. The expression pattern of *Trg* in *hkb* and *tll* mutant embryos suggests this to be the case. In *hkb* embryos, both *Trg* RNA (Fig. 3D) and *Trg* protein (Fig. 6B) are expressed up to the posterior pole of the embryo, whereas expression at the anterior border

Figure 2. Comparison between the T-domains of the T proteins from mouse, *Xenopus*, zebrafish, and ascidian of the *Drosophila* proteins Omb and Trg. The conserved regions of Trg, the *Xenopus* Xbra (Smith et al. 1991), the mouse T (Herrmann et al. 1990), the zebrafish Zf-T (Schulte-Merker et al. 1992), the ascidian As-T (Yasuo and Satoh 1994), and the *Drosophila* Omb protein (Pflugfelder et al. 1992) are compared. In the consensus sequence only those amino acids identical in all six proteins are shown. Similar amino acids at a given position in all six proteins, or identical or similar amino acids in five proteins including Omb, are represented (●). An asterisk (*) indicates that Trg and the chordate T proteins have an identical or similar amino acid at a given position but that the corresponding amino acid in Omb is different. (.) Amino acid positions with less or no similarity. For maximum similarity, gaps (underscores) have been allowed. The following groups of amino acids were considered as similar: R and K; D and E; N and Q; S and T; A, F, L, M, I, V, W and Y.

remains unchanged. In contrast, no *Trg* expression is detectable in *tll* mutant embryos (Fig. 6C).

Some of the functions of *hkb* and *tll* appear to be mediated by *fkf* (Casanova 1990; Gaul and Weigel 1991). However, the blastoderm expression of *Trg* is independent of *fkf* function (Fig. 6D). Also, the maintenance of *Trg* expression during germ-band extension does not appear to require *fkf* activity, as *Trg* is still expressed in the developing hindgut and anal pads when these disintegrate in *fkf* mutant embryos (data not shown).

In the anterior part of the embryo there is also a do-

main in which *tll*, but not *hkb*, is expressed (Fig. 5). If *tll* and *hkb* were the only regulators of *Trg* expression, *Trg* should also be expressed in this domain. However, this is not the case, suggesting that, directly or indirectly *bicoid* (*bcd*) prevents *tll*-dependent *Trg* expression in the anterior part of the embryo. In embryos derived from *bcd* mutant mothers, the posterior terminal region is duplicated at the anterior pole (Frohnhofer and Nüsslein-Volhard 1986). This duplication includes the *Trg* expression in the blastodermal ring of cells and later in the duplicated anterior hindgut (data not shown).

The *Trg* locus

By in situ hybridization to polytene chromosomes the *Trg* locus was mapped to the region 68DE. This result was confirmed by the examination of *Trg* expression in embryos homozygous or transheterozygous for various deficiencies covering the region (Akam et al. 1978). Embryos homozygous for *Df(3L)vin²* and *Df(3L)vin⁶⁶*, but not embryos homozygous for *Df(3L)vin³* and *Df(3L)vin⁴*, express *Trg* (Fig. 7). This places *Trg* in the interval (68D6,68E3-4), which was genetically characterized previously and has probably been saturated with lethal mutations (Hoogwerf et al. 1988). Within this interval five complementation groups have been found, one of which corresponds to the *cyclin A* (*cycA*) gene (Lehner et al. 1991). Because *Trg* does not correspond to *cycA*, this probably leaves *Trg* as one of the four remaining lethal complementation groups placed in this interval.

Trg function in the development of hindgut and anal pads

To address the role of *Trg* we examined embryos lacking *Trg* function. We used embryos transheterozygous for the deficiencies *Df(3L)vin⁴* and *Df(3L)vin⁶* (Fig. 8) or homozygous for *Df(3L)vin³* (data not shown), as point mutations in *Trg* are not available at present. The mutations representing the four complementation groups mentioned above have been lost from the stock collections. However, we attribute the defects described below to the lack of *Trg* function, as they correlate strictly with the deletion of the chromosomal region encoding *Trg* and are

Table 1. Similarities between T-domains

	T	Xbra	Zf-T	As-T	Omb
Trg	88	88	86	81	66
T		96	93	83	69
Xbra			96	85	69
Zf-T				86	68
As-T					62

The percentage of amino acids identical or similar within the T-domains of Trg, of the mouse T (T), *Xenopus* T (Xbra), zebrafish T (Zf-T), ascidian T (As-T) and the *Drosophila* Omb protein are given. The glycine-rich region of Trg (amino acids 15–78) has not been considered in the calculation. Otherwise, the numbers are based on the T-domains as shown in Fig. 2.

Figure 3. The RNA expression of *Trg*. (A) *Trg* RNA is not expressed in the embryo at stage 4, cycle 13. (B) At the onset of cycle 14, transcription commences throughout the posterior terminal region of the embryo. (C) During cellularization the expression is down-regulated in the posterior tip of the embryo and becomes confined to a ring of cells that encompasses the primordium of hindgut and anal pads. (D) *Trg* RNA expression in a *hkb* mutant embryo during cellularization. *Trg* is expressed to the posterior tip of the *hkb* embryo suggesting that in the wild-type embryo *hkb* sets the posterior border of *Trg* expression (cf. C). (E) Early gastrulation. The ventral portion of the *Trg*-expressing ring of cells forms the most posterior part of ventral furrow. The most dorsal part of the ring forms the most anterodorsal aspect of the amnioproctodeal invagination. (F) Same embryo as in E but in dorsal surface view. The ring encircles the posterior midgut primordium (located around and beneath the pole cells). (G) The posterior midgut has invaginated fully. (H) After germ-band retraction *Trg* expression is confined to

the hindgut and anal pads. The Malpighian tubules (arrows) do not express *Trg*. All embryos are shown in a sagittal optical section and with their anterior to the left (except F and G, where dorsal surface views are shown).

not seen in embryos homozygous for the deficiencies *Df(3L)vin²* or *Df(3L)vin⁶⁶* nor in embryos lacking only *cycA* function. Most importantly, the defects can be rescued by a 20-kb transgene carrying the *Trg* transcription unit (Fig. 9).

In *Trg*-deficient embryos the major part of the hindgut is missing after germ-band retraction, as is evident by the absence of hindgut tissue expressing β -galactosidase, conferred by the enhancer trap insertion A27 (Fig. 8A,B). Early gastrulation, including the invagination of the amnioproctodeum, proceeds normally in the *Trg*-deficient

embryos (data not shown). It is not until the germ band has extended almost fully that the proctodeum begins to develop aberrantly and, for example, does not express the proper stripe of En protein (Fig. 8C,D). Normally, En protein is found along the major part of the proctodeum in a stripe running in an anterior-posterior extension (Fig. 8C). In addition, the anal pads are not formed in *Trg*-deficient embryos. Zygotic *cad*, which is known to be required for the proper differentiation of the anal pads (Macdonald and Struhl 1986), is not expressed in their primordium (Fig. 8F). Only a few cells at the posterior tip

Figure 4. The expression of *Trg* protein. *Trg* protein is visualized with the anti-TN₁₋₁₂₃ antibody that is directed against the amino-terminal half of the mouse T-domain. (A) Late blastoderm, onset of ventral furrow formation. *Trg* protein is detected in a region encompassing the primordia of hindgut and anal pads and of the most posterior part of the mesoderm. (B) Germ band extension. *Trg* protein is confined to the proctodeum (pr). (C) Fully retracted germ band. *Trg* protein is found in the hindgut (hg) and the anal pads but not in the Malpighian tubules. (D) Enlarge

ment of the posterior part of the embryo in C, showing localization of *Trg* protein in the apically located nuclei (arrow) of the hindgut epithelium. The arrowheads mark the basal side of the epithelium. The level of *Trg* protein expression increases in the course of embryogenesis allowing for a better subcellular localization of the protein in later (C) than in earlier (A,B) stages. (A,C,D) Dorsal view, optical horizontal section; (B) lateral view, optical sagittal section. (ap) Anal pads; (hg) hindgut; (pmg) posterior midgut; (pr) proctodeum.

Figure 5. Spatial comparison of the expression of Trg and the terminal gap genes *hkb* and *tll*. (A) *hkb* RNA expression at the onset of cellularization. (B) *tll* RNA expression at the onset of cellularization. (C) Trg protein at almost complete cellularization. Trg is expressed in that part of the posterior terminal region where *tll*, but not *hkb*, is expressed. All embryos are shown in an optical sagittal section.

of the germ band express Cad protein and presumably constitute the precursors of more anteriorly located epidermal structures in which Cad is also expressed (Fig. 8E). Additionally, during germ-band retraction *even-skipped* is also expressed in the anal pads of wild-type embryos, but no corresponding expression is detectable when Trg function is lacking (data not shown).

The posterior end of the midgut is connected to the outside by a short tube in Trg-deficient embryos (Fig. 8B,H). This tube does not appear to be a rudimentary hindgut but seems to consist of epidermis that normally would be located at the outside of the embryo. This displacement of tissue in the Trg-deficient embryos is best seen in embryos stained for Wg protein. Normally Wg protein brackets the hindgut perfectly, as it is found at the base of the Malpighian tubules and in the anal pads (Fig. 8G). In the Trg-deficient embryos these two regions of Wg expression are separated by only a few cells (Fig. 8H). The part of the tube behind the posterior, larger Wg expression domain appears to be formed by posterior epidermis that has moved into the embryo. This interpretation is based on the observation that this part of the tube expresses *Abdominal-B* (*Abd-B*; data not shown), which in wild-type embryos is found in the posterior segmented regions of the embryo adjacent to Trg (Celniker et al. 1989; data not shown). In conclusion, we suggest that Trg plays a crucial role in the development of the hindgut and the anal pads. The lack of Trg function appears to exert a gap gene-like phenotype that specifically affects these two structures.

Genes homologous to Trg exist in short-germ insects

Because the antibody directed against the T-domain of the mouse T protein cross-reacts with the product of the *Drosophila* Trg we expected that it would also bind to T-related proteins in other insects. In both *Locusta migratoria* and *Tribolium castaneum* we detected apparently nuclear gene products with the antibody (Fig. 10). Because these are expressed exclusively in the posterior tip of the embryo (the putative primordium of the proctodeum) and in the developing hindgut, as is the case in *Drosophila*, we assume that they are homologous to *Drosophila* Trg.

A detailed comparison of the expression patterns in the different insects might allow us to begin to assess the evolution of Trg gene function. Both *Locusta* and *Tribolium* are short-germ insects that undergo a very different segmentation process compared with the long-germ insect *Drosophila* (for review, see Anderson 1972a,b). The segmental primordia of long-germ insects are deter-

Figure 6. *tll* and *hkb* regulate Trg expression. Trg protein expression at blastoderm stage is shown for wild-type (A), *hkb* (B), *tll* (C), and *fkh* (D) embryos. (A) In the wild-type embryo Trg is expressed in a circumferential ring of cells in the posterior terminal region. (B) The posterior boundary of the expression domain is set by *hkb*, as Trg expression is extended to the posterior pole in *hkb* mutant embryos. (C) *tll* acts as an activator of Trg expression, as no Trg is detectable in *tll* mutant embryos. (D) The early expression of Trg does not depend on *fkh* function.

Figure 7. The chromosomal localization of *Trg*. The *Trg* locus is uncovered by the deficiencies *Df(3L)vin*³, *Df(3L)vin*⁴, *Df(3L)vin*⁵, *Df(3L)vin*⁶, and *Df(3L)vin*⁷ but excluded from the deficiencies *Df(3L)vin*² and *Df(3L)vin*⁶⁶. This places *Trg* in the region between 68D6 and 68E3-4. The chromosomal breakpoints of the *vin* deficiencies are taken from Akam et al. (1978).

mined essentially simultaneously over the entire length of the body at the same time as the terminal primordia during the early blastoderm stage. In contrast, both *Locusta* and *Tribolium* first form a head germ and then gradually add thoracic and abdominal segment primordia. It is not known when the terminal primordia are determined.

Locusta migratoria In *Locusta* no *Trg* expression is detectable at 15% of development when the head germ has formed and only the head lobes and tail-like growth zone are visible (data not shown). *Trg* is first expressed at ~20% of development, at the very posterior tip of the embryo, when a morphological border between the thoracic and the abdominal segment primordia is visible (Fig. 10A). At this time, the first abdominal segment becomes distinguishable in the closely related species *Schistocerca americana* (Patel et al. 1989b). This expression of *Trg* suggests that the determination of the posterior terminus occurs before most of the posterior abdominal segment primordia have been laid down. Slightly later, at ~23% of development, when there is still no morphological sign of segmentation in the abdomen, but molecular methods allow the identification of three abdominal segment primordia (Patel et al. 1989b), *Trg* expression has increased in level and is found in the entire posterior tip of the embryo within the ectoderm (Fig. 10B,C). *Trg* continues to be expressed in these terminal cells (Fig. 10D), which at ~29% of development begin to invaginate perpendicular to the body axis to form the proctodeum (Fig. 10E,F). Within the cells of the proctodeal invagination the expression of *Trg* gradually declines toward the inner, more dorsally located part (Fig. 10F). Later, when the proctodeum has extended and is positioned parallel to the body axis, *Trg* expression is

still present in the developing hindgut (data not shown). However, the inner end of the proctodeal tube does not express the *Trg* gene any longer. This part might constitute the primordium of the posterior midgut of the *Locusta* embryo. We did not follow the expression of *Trg* later than 40% development.

Tribolium The expression of *Trg* in *Tribolium* embryos essentially follows the same temporal and spatial pattern as in *Locusta*. *Trg* is expressed at the posterior tip of the developing germ band before all of the abdominal segment primordia have been laid down (Fig. 10G). However, the earliest expression that we were able to detect occurs later, relative to the segmentation process, than observed in *Locusta*. The fifth abdominal segment is already morphologically distinguishable (Fig. 10G). The cells of the posterior tip later form the proctodeal invagination (60 hr of development; Fig. 10H,I) after the entire germ band has formed and all segmental primordia are morphologically distinguishable. As in *Locusta*, the protein expression declines toward the inner part of the proctodeum (Fig. 10I), which might constitute the primordium of the posterior midgut. After retraction of the germ band, *Trg* is found in the hindgut and the anus and is not expressed in any other part of the body including the tissue developing from the closed end of the proctodeum (Fig. 10K).

Discussion

A family of genes defined by the T-domain

Trg exhibits a very high similarity (88%) over a stretch of 200 amino acids (the T-domain) to the *T* genes of chordates (Fig. 2). This domain has been shown to be required and sufficient for specific DNA binding and defines a novel family of DNA-binding proteins that possibly function as transcription factors (Kispert and Herrmann 1993). There is one other *Drosophila* gene containing a T-domain, *omb* (Pflugfelder et al. 1992), which is, however, less closely related to the *T* genes of chordates than *Trg*. *omb* is distinguished by a few features, like two insertions within the T-domain, from all of the other genes of the family. In addition, the antibody directed against the 123 amino-terminal amino acids of the mouse T protein does not cross-react with *Omb* (data not shown; for embryonic *omb* expression, see Poeck et al. 1993), whereas this antibody does recognize the products of vertebrate *T* genes and of the insect *Trg* (Kispert and Herrmann, 1993; Figs. 4 and 10; data not shown). *Omb* probably constitutes a member of a particular class of T-domain proteins. We predict that in vertebrates *omb*-like genes also exist, just as *Trg* exist in insects, and we speculate that these two are not the only classes of the T-domain gene family.

The regulation of *Trg* expression

The *Trg* expression in mutant embryos suggests that during late blastoderm stage, *Trg* is activated by *tll* and repressed by *hkb* (Fig. 6). Because *tll* expression appears to

Figure 8. The development of hindgut and anal pads requires *Trg* function. β -galactosidase expression conferred by the enhancer trap insertion A27 (A,B) En protein expression (C,D), Cad protein expression (E,F), and Wg protein expression (G,H) is shown for wild-type embryos (A,C,E,G) and in embryos carrying a small synthetic deficiency formed by the overlap of the deficiencies *Df(3L)vin⁴* and *Df(3L)vin⁶* (B,D,F,H). (A) The enhancer trap insertion A27 normally confers β -galactosidase expression in the hindgut (hg), in a particular region of the midgut (mg), and in a part of the posterior spiracles (arrows) at stage 16 (dorsal view). The arrowhead marks the base of the Malpighian tubules. (B) In a *Trg*-deficient embryo of about the same age as that in A, the β -galactosidase-expressing loop of the hindgut is missing. The arrowheads mark the developing Malpighian tubules. (C) In the wild-type embryo, a stripe of En protein is expressed within the ventrally located part of the proctodeum (between arrows) during germ-band extension. The small arrowhead marks the epidermal En expression of the rudimentary ninth abdominal segment (a9), and the larger arrowhead indicates the transition between proctodeum and posterior midgut. (D) In a *Trg*-deficient embryo the part of the proctodeum containing the En stripe is missing. The En expression in a9 (smaller arrowhead) appears to be stronger than normal, as it crosses the midline of the mutant embryo, i.e., the focal plane of the optical sagittal section shown. In the wild-type embryo, the expression is confined to a lateral stripe in the hemisegment and excludes the middle part of the embryo. (E) Cad protein is expressed in the anal pads (ap), a few neighboring cells of the epidermis (arrow) and the developing posterior midgut (pmg) during germ-band extension. (F) In *Trg*-deficient embryos the anal pads and the corresponding Cad expression are absent, but the development of the posterior midgut is undisturbed. (G) At stage 13 (after germ-band retraction) Wg protein is expressed within the posterior terminal region in parts of the anal pads (larger arrowhead) and at the base of the Malpighian tubules at the junction between hindgut and posterior midgut (arrow). The smaller arrowhead indicates expression in the terminal epidermis. (H) The Wg expression in *Trg*-deficient embryos indicates that the major part of the hindgut between the base of the Malpighian tubules (arrow) and the cells corresponding to the anal pads (larger arrowhead) is missing.

extend beyond the anterior border of *Trg* expression (data not shown; cf. Pignoni et al. 1990) it is reasonable to assume that a particular threshold of *tll* activity is required for *Trg* expression. In the anterior part of the embryo, either *tll* activity does not surpass this threshold (because the modulating effect of *bcd* on the *tll* expres-

sion) or *bcd* itself prevents the activation of *Trg* by *tll* (data not shown). We assume that both *tll* and *hkb* are direct transcriptional regulators of *Trg* at the blastoderm stage. Other genes active in the posterior terminal region like *fkf*, which itself depends on *hkb* or *tll*, are not required for the early expression of *Trg* (Fig. 6D). Most

Figure 9. A 20-kb transgene provides *Trg* function. Wild-type embryos (A), embryos transheterozygous for the deficiencies *Df(3L)vin⁴* and *Df(3L)vin⁶* (B), or embryos transheterozygous for the deficiencies *Df(3L)vin⁴* and *Df(3L)vin⁶* and heterozygous for the transgene P-Trg20A (C) were immunostained for the Cyclin A protein. (A) In the wild-type embryo only the nervous system (arrows: brain) expresses Cyclin A at stage 14 (large arrowhead: hindgut loop). (B) In the *Trg*-deficient embryos there is no Cyclin A protein detectable and the hindgut is absent (small arrowhead). (C) *Trg*-deficient embryos (*Df(3L)vin⁴/Df(3L)vin⁶*) that are heterozygous for the *Trg* transgene P-Trg20A also show no Cyclin A expression, but the development of the hindgut (large arrowhead) and of the anal pads is rescued.

likely, *Trg* and *fkh* act in parallel, since *fkh* expression is unchanged in *Trg*-deficient embryos at the blastoderm stage or early gastrulation (data not shown).

When the proctodeum has fully invaginated, the gene *srp* takes over the role of *hkb* in repressing *Trg* in the primordium of the posterior midgut (Reuter 1994). A further gene might exist that is expressed in the mesodermal germ layer and turns off *Trg* in those cells that invaginate at the posterior tip of the ventral furrow. However, *Trg* could also be positively regulated, and there might be a particular gene that is required for the maintenance of *Trg* expression in hindgut and anal pads. There is no indication that *fkh* regulates late *Trg* expression, since in *fkh* mutant embryos *Trg* expression decays concomitantly with the cellular integrity of the proctodeum. It is conceivable that *Trg* itself is required for the maintenance of its own expression and possesses autoregulatory properties. A similar function has been sug-

gested for the maintenance of *T* gene expression in the notochord (Herrmann 1991).

The function of *Trg* in *Drosophila*

The loss of *Trg* function causes a gap gene-like phenotype in the *Drosophila* embryo. Based on morphological criteria and the expression pattern of tissue-specific markers, we conclude that the anal pads and the major part of the hindgut are missing in *Trg*-deficient embryos (Fig. 8). This phenotype is specifically associated with the deletion of the region (68D6;68E3-4) and can be rescued by a *Trg* transgene.

Among the genes whose expression is affected in *Trg*-deficient embryos is *cad*, which may normally mediate some of the functions of *Trg*, as *cad* is itself required for the proper differentiation of the anal pads (Macdonald and Struhl 1986). The Malpighian tubules and their base, which like the hindgut are derived from the proctodeum, develop essentially normal in *Trg*-deficient embryos. Thus, only those structures that normally express *Trg* are affected.

Trg is not only expressed early in the primordia of hindgut and anal pads, but also later in the definitive organs, a pattern that is unusual and reminiscent of homeotic gene expression. It is conceivable that *Trg* acts as a selector gene and positionally specifies these body parts like a homeotic gene, although its lack-of-function phenotype is that of a gap gene. Such behavior is observed for homeotic genes acting in the *Drosophila* head, like *Deformed* (*Dfd*), the homeotic character of which is revealed only upon ectopic expression. In contrast, the lack of *Dfd* function causes a gap in the body plan and not a homeotic transformation (Kuziora and McGinnis 1988).

The phenotypic analysis of *srp* mutant embryos led to the conclusion that a selector gene must exist that is responsible for hindgut-specific morphogenesis and differentiation (Reuter 1994). *srp* embryos develop an additional hindgut in place of the posterior midgut. This phenotype can be regarded as a homeotic transformation when posterior midgut and hindgut are considered as serial homologs. The transformation in *srp* mutant embryos is accompanied by an expansion of *Trg* expression into the posterior midgut primordium at stage 8. This is consistent with the possibility that *Trg* might be the hindgut selector gene. However, it remains to be determined whether or not *Trg* alone has the capacity to direct hindgut development when expressed ectopically in the midgut primordia.

Trg function in short-germ insects

The proteins detected in *Locusta* and *Tribolium* by the anti-TN₁₋₁₂₃ antibody are most likely encoded by genes homologous to *Trg* in *Drosophila*. First, the cross-reactivity of the antibody suggests a structural similarity to the vertebrate *T* genes as we have shown for *Trg* in *Drosophila*. Second, both in *Locusta* and *Tribolium*, *Trg* is expressed exclusively, as in *Drosophila*, in the poste-

Figure 10. Expression of *Trg* homologs in *Locusta* and *Tribolium* embryos. The protein products of *Trg* in *L. migratoria* (A–F) and in *T. castaneum* (G–K) have been detected with the anti-TN_{1–123} antibody. (A) Approximately 21% development. The posterior tip of the embryo expresses *Trg*. The approximate border between thoracic and abdominal primordia (t3 a1) has been determined by morphological criteria. (B) Approximately 23% development. *Trg* expression is confined to the posterior tip. The arrowhead marks the sharp border of expression (t3: primordium of the third thoracic segment). (C) Enlargement of the tail tip of the embryo shown in B. The expression is predominantly ectodermal. (D) Approximately 26% development. Segmentation can be distinguished morphologically up to about abdominal segment 7. Cells not expressing *Trg* (arrow) form a layer apparently posterior to the *Trg* expression domain and are probably serosa cells that connect to the posterior tip of the germ band (a4: primordium of the fourth abdominal segment). (E) Approximately 29% development. The proctodeum that expresses *Trg* has started to invaginate (arrowhead). (F) Enlargement of the proctodeum of the embryo shown in E. The expression of *Trg* declines in strength toward the dorsal, inner side of the proctodeum. The arrowhead indicates the proctodeal opening. (G) Posterior region of a *Tribolium* embryo during anatrepsis (~40 hr of development; [a5] primordium of the fifth abdominal segment). *Trg* is expressed at the posterior tip of the germ band (arrow) and the serosal membrane (arrowhead). (H) Embryo after completion of the segmentation process (~60 hr of development). The arrowhead indicates the opening of the proctodeum which expresses *Trg*. (hd) Head; (md) mandibular segment; (t1) first thoracic segment; (a1 and a9) first and ninth abdominal segment. (I) Enlargement of the proctodeum of the embryo shown in H. The expression of *Trg* declines in strength towards the dorsal, inner side of the proctodeum. The arrow indicates the border between proctodeum and adjacent mesoderm. (K) Enlargement of the posterior region of an embryo after full retraction of the germ band (~100 hr of development). *Trg* is expressed around the anus (between open arrowheads). It is confined to the ectodermal epithelium of the hindgut (small solid arrowhead), whereas the mesoderm surrounding the hindgut (arrows) is devoid of the protein. The primordium of the midgut that is contiguous with the hindgut (large solid arrowhead) also does not express *Trg*. Scale bars correspond to 25 μ m.

rior terminal region, where the primordium of the proctodeum is presumably located, in the proctodeum itself, and finally in the proctodeal derivatives hindgut and anus. These findings suggest that *Trg* has a homologous and phylogenetically well-conserved function in the specification of the proctodeum and its derivatives in insects. The proctodeum in “lower” (grasshopper) and in

“higher” (beetles and flies) insects appears to be determined by similar molecular mechanisms.

Several aspects of *Trg* expression in *Locusta* and *Tribolium* are of further interest. *Trg* is not detectable during blastoderm stage. This might be attributable to a lower affinity of the antibody for these proteins than for *Trg* in *Drosophila*. It is, however, more likely that the

determination of the posterior terminus in the two short-germ insects occurs comparatively later than in *Drosophila* and that this is reflected in the later onset of *Trg* expression. In short-germ insects the thoracic and abdominal segments are determined and formed sequentially in an anterior to posterior order. Interestingly, in both *Locusta* and *Tribolium*, *Trg* expression appears at the posterior tip of the germ band well before the posterior abdominal segments have formed. This suggests that the proctodeal primordium is determined significantly earlier than the primordia of these abdominal segments.

Is Trg a homolog of the T genes?

Within the T-domain, there is a very high degree of similarity between *Drosophila* *Trg* and the *T* genes of vertebrates. This implies a close phylogenetic relationship between the genes. Is there also a phylogenetic relationship between the developmental processes that these genes regulate? In higher chordates (vertebrates) *T* is expressed both in the notochord and in the area from which mesendoderm is generated, that is, in the primitive streak (mouse, chick), germ ring (fish), or marginal zone (frog) and later, during axis elongation, in the tail bud. These two domains of expression reflect the twofold function of the *T* gene during vertebrate development. *T* is required both for the differentiation of the notochord and for the formation of mesendoderm during posterior axis elongation (for review, see Beddington et al. 1992). The phylogenetically older aspect of *T* function most likely reflects its role in notochord development, as the *T* homolog of a lower chordate (ascidian) is expressed exclusively in the cells of the presumptive and definitive notochord (Yasuo and Satoh 1994). The expression of *Trg* in the proctodeum of insects is certainly not related to the expression of the *T* genes in the mesendoderm generation zone of vertebrates. The part of the insect embryo that might be considered to be a structure similar to the primitive streak, or the germ ring, is the ventral furrow or gastral groove, and *Trg* is not expressed there (with the exception of the posterior tip of the furrow). Thus, the question remains whether there is a phylogenetic relationship between the function of *T* genes in the vertebrate notochord and the insect proctodeum. Did these structures originate from a common precursor? It has been hypothesized that the notochord evolved from the stomochord of hemichordates, a structure that evaginates from the anterior part of the gut (for further discussion, see Siewing 1969). Phylogenetically, the notochord would then be derived from the gut. Connecting this notion to the *T*-gene function leads to the speculation that *T* specified a particular part of the gut early in evolution. In the branch leading to the arthropods *T* then became concerned with the specification of the proctodeum, whereas in parallel in the branch leading to the chordates, *T* became concerned with the specification of a gut derivative, first the stomochord and then the notochord.

The current assignment of notochord and insect hindgut to different germ layers, mesoderm and ectoderm,

respectively, is somewhat arbitrary in both cases and is not an argument against their possible common origin. Often the notochord and definitive endoderm are formed from the same progenitor, the head process in mouse, for example (Poelmann 1981), or certain blastomeres in ascidian (Ortolani 1957) that might imply a closer relationship of notochord to endoderm than to mesoderm. Also the assignment of the insect proctodeum to ectoderm and not to endoderm is not based on its (interior) position within the embryo after the gastrulation movements, but on the epithelial properties that the late organ shares with the epidermis (for further discussion, see Skaer 1993). At this point, of course, we can only raise the question of whether there is a phylogenetic relationship between notochord of chordates and proctodeum of arthropods. In this context it would be useful to search for *Brachyury* class T-domain genes in species that phylogenetically link chordates and arthropods and to investigate the expression pattern and function of such genes during development.

Materials and methods

Isolation of Trg cDNAs and sequence analysis

A 687-bp fragment (*Nco*I–*Eco*RI) encompassing the first 229 codons of the open reading frame of the mouse *T* gene was isolated from the plasmid pBP4.T(1–229) (Kispert and Herrmann 1993), labeled with [³²P]dCTP by random priming and used as a hybridization probe to screen a plasmid cDNA library prepared from 8- to 12-hr *Drosophila* embryos (Brown and Kafatos 1988). A strongly hybridizing clone (*Trg*1, 2.6-kb *Hind*III–*Eco*RI insert) was isolated under reduced stringency conditions, that is, at 50°C in Church buffer (Church and Gilbert 1984). By screening the same filters with a 1.6-kb *Sac*I fragment of *Trg*1 under high stringency conditions (68°C in Church buffer), 10 additional *Trg* clones were isolated of which 3 had inserts identical to *Trg*1, whereas the others (*Trg*2) contained a 1.8-kb *Hind*III–*Eco*RI insert comprising the 3' part of the *Trg*1 insert.

Both strands of *Trg*1 and the ends of *Trg*2 were sequenced using the dideoxy method of Sanger (Sanger et al. 1977) with a T7 sequencing kit (Pharmacia, Sweden). Sequence analysis was performed on a Macintosh personal computer using the "MacMolly Tetra" program (Softgene, Germany).

In situ hybridization to polytene chromosomes

The 3-kb *Eco*RI insert from the *Trg*1 cDNA clone was labeled with biotinylated dUTP (Bio-16-dUTP, Boehringer Mannheim, Germany) by random priming. Salivary gland chromosome spreads were prepared from Oregon-R third-instar larvae using standard techniques (Langer-Safer et al. 1982). The probe was hybridized to the acetylated and alkali-denatured chromosomes in 0.6 M NaCl, 50 mM NaPO₄ (pH 7.0), 5 mM MgCl₂, and 5% dextran sulfate in Denhardt's solution. The chromosome preparations were washed, and the probe detected as described by Engels et al. (1986) using the streptavidin/biotinylated alkaline phosphatase DNA detection system (BRL, USA). The chromosomes were viewed without counterstaining with a Zeiss Photomicroscope III equipped with phase-contrast optics.

Germ-line transformation and genetic analysis

The bacteriophage P1 clone 2-73 containing genomic DNA from the chromosomal region 68E2,3 was kindly provided by

Daniel Hartl (Smoller et al. 1991). Using the *Trg1* cDNA as a probe, we identified a 20-kb *KpnI* fragment of the genomic insert that contains exclusively the 8-kb transcription unit of *Trg* together with 9 kb of 5'-flanking and 3 kb of 3'-flanking DNA. This fragment was subcloned into *KpnI*-digested pCaSpeR4 (Pirrotta 1988). The recombinant clone and the helper plasmid p π 25.7wc (Karess and Rubin 1984) were injected into *w¹¹¹⁸* embryos as described by Spradling (1986). Two independently transformed fly lines were obtained from the F1 generation, one of which (P-*Trg20A*) carried the transgene on the second chromosome, the other (P-*Tr20B*) on the third chromosome.

To test the capacity of the transgene to rescue the hindgut phenotype of the *Trg*-deficient embryos we crossed the transgene on the second chromosome into the background of *Df(3L)vin⁶* and backcrossed with *Df(3L)vin⁴* females. We examined the embryonic progeny that did not express Cyclin A and, therefore, were transheterozygous for the deficiencies *Df(3L)vin⁶* and *Df(3L)vin⁴*. About 50% of these embryos develop normal hindgut and anal pads. Therefore, we conclude that a single copy of the *Trg* transgene rescues the hindgut and anal pad phenotype of *Trg*-deficient embryos. Similar results were obtained with the *Trg* transgene on the third chromosome, which was recombined onto a *Df(3L)vin⁶* chromosome and tested in *trans* to *Df(3L)vin⁴*.

Fly stocks

bcd^{E1} is a small deletion of the *bcd* locus (Berleth et al. 1988). *hkb^A* is commonly used as *hkb* deficiency (Weigel et al. 1990). *tll^S* is a small deficiency of the *tll* locus, used here as *tll* null allele (Strecker et al. 1988). *fkh^{XT6}* is a small deficiency of the *fkh* locus (Weigel et al. 1989b). A27 is an enhancer trap insertion on the second chromosome (O'Kane and Gehring 1987). *Df(3L)vin²*, *Df(3L)vin³*, *Df(3L)vin⁴*, *Df(3L)vin⁵*, *Df(3L)vin⁶*, *Df(3L)vin⁷*, and *Df(3L)vin⁶⁶* are deficiencies in the *vin* regions (Akam et al. 1978; Crosby and Meyerowitz 1986). The smallest deficiency, *Df(3L)vin³*, in addition to affecting the development of anal pads and hindgut, also often gave a variable range of other severe developmental aberrations even in *trans* to the other *vin* deficiencies. Therefore, we did not use it for further investigation of the phenotype of *Trg*-deficient embryos. *l(3)183* and *neo114* are alleles of *l(3)rsg11* or *l(3)68Ea* (Hoogwerf et al. 1988), both synonyms for the *cycA* gene (Lehner and O'Farrell 1989). *l(3)183* and *neo114* do not affect the expression of *Trg*. Unfortunately, other mutant stocks described in the genetic analysis of the region 68C8;69B5 (Hoogwerf et al. 1988) have been lost from the stock collections.

Antibodies and probes

The following antibodies were used in the study: anti-Caudal (Mlodzik and Gehring 1987); anti-Cyclin A (Lehner and O'Farrell 1989); anti-digoxigenin (Boehringer Mannheim, Germany); anti-Engrailed (4D9; Patel et al. 1989a); anti-Even-skipped (3C10; Patel et al. 1992); anti- β -galactosidase (Sigma, St. Louis); and anti-TN₁₋₁₂₃ (Kispert and Herrmann 1993), the antibody bound specifically to *Trg* protein in *Drosophila* embryos [anti-TN₁₋₁₂₃ did not show any cross-reaction to the Omb protein in the *Drosophila* embryo that contains a distinct T-domain and is expressed in the optic lobe primordium and in cells of the developing ventral and peripheral nervous system (Poeck et al. 1993)]; anti-Wingless (Van Den Heuvel et al. 1989); biotinylated anti-rabbit-, anti-rat-, and anti-mouse-IgG (Jackson, Bar Harbor).

The *hkb* cDNA used for the in situ detection of *hkb* mRNA was a gift from G. Brönnner (Brönnner and Jäckle 1991) and was isolated as a *HindIII*-*EcoRI* fragment from a pNB40 cDNA

clone. The *tll* cDNA was kindly provided by Frank Sprenger (MPI, Tübingen, Germany) and isolated as an *EcoRI* fragment from a Bluescript cDNA clone. For the detection of *fkh* RNA we used the 4-kb *XhoI* fragment containing most of the *fkh* transcription unit (Weigel et al. 1989a), and for *Trg* RNA the 3-kb *EcoRI* fragment of the *Trg1* cDNA.

Immunohistochemical detection of protein and RNA in *Drosophila* embryos

Drosophila embryos were dechorionated with commercial bleach (5% NaOCl), fixed with 4% formaldehyde in PBS/heptane and devitellinized by vortexing in heptane/methanol and subsequently in methanol. The immunostaining followed standard protocols of washes and blocking agents, and the bound antibodies were detected histochemically with the Vectastain ABC kit (Vector Labs, USA) using diaminobenzidine as chromogen. Before the embryos were incubated with the anti-TN₁₋₁₂₃ antibody, they were treated after the devitellinization with 0.3% H₂O₂ in methanol for 20 min at room temperature and stored (for a few days to several months) in methanol at -20°C. These two steps helped to decrease the background of the immunostaining with anti-TN₁₋₁₂₃.

Probes were labeled with digoxigenin-dUTP (Boehringer Mannheim, Germany) and used for in situ hybridization as described by Tautz and Pfeifle (1989).

For photomicrography the embryos were mounted individually either in water-free glycerol (in situ hybridizations) or in Epon Durcupan (Fluka, Switzerland) and were photographed using either Kodak Ektachrome 160T or Agfapan APX 100 film on a Zeiss Axiophot equipped with Zeiss 20 $\times$ (n.a. 0.50) or 40 $\times$ (n.a. 0.75) Plan-Neofluar objectives and differential interference contrast optics. The staging of the embryos followed the descriptions of Foe and Alberts (1983) for nuclear cycles and of Campos-Ortega and Hartenstein (1985) for stages after cellularization.

Handling of *Locusta* and *Tribolium* embryos

Locusta embryos were obtained from a population of *L. migratoria* maintained by the Department of Zoology at the University Tübingen and were kindly provided by Klaus Hartfelder. They were fixed and processed for immunostaining essentially as described by Tear et al. (1990) for *Schistocerca*, with the following differences. The eggs were dissected in PBS, treated with 0.3% H₂O₂ in methanol for 20 min at room temperature, postfixed, and stored in methanol at -20°C. The staining reaction was performed using 0.2 mg/ml of diaminobenzidine and 0.003% H₂O₂ in PBS plus 0.1% Triton X-100. The staging of the locust embryos followed the description by Bentley et al. (1979) for the closely related grasshopper *Schistocerca nitens*.

Tribolium embryos were obtained from a population of *T. castaneum* maintained by the Department of Zoology at the Ludwig-Maximilian University München and were kindly provided by Reinhard Schröder and Diethard Tautz. They were fixed and processed for immunostaining like *Drosophila* embryos. The staging of the *Tribolium* embryos follows the description of Stanley (1965), as summarized by Sokoloff (1972) for the closely related species *T. confusum*, and refers to development at 25°C.

After immunostaining, both *Locusta* and *Tribolium* embryos were dissected from surrounding yolk and membranes in water-free glycerol and mounted individually in glycerol for microphotography.

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References

- Akam, M.E., D.B. Roberts, G.P. Richards, and M. Ashburner. 1978. *Drosophila*: The genetics of two major larval proteins. *Cell* 13: 215–225.
- Anderson, D.T. 1972a. The development of hemimetabolous insects. In *Developmental systems: Insects* (ed. S.J. Counce and C.H. Waddington), pp. 95–163. Academic Press, London, UK.
- . 1972b. The development of holometabolous insects. In *Developmental systems: Insects* (ed. S.J. Counce and C.H. Waddington), pp. 165–241. Academic Press, London, UK.
- Beddington, R.S.P., P. Rashbass, and V. Wilson. 1992. *Brachyury*—A gene affecting mouse gastrulation and early organogenesis. *Development* (Suppl.) 7: 157–165.
- Bentley, D., H. Keshishian, M. Shankland, and A. Toroian-Raymond. 1979. Quantitative staging of embryonic development of the grasshopper, *Schistocerca nitens*. *J. Embryol. Exp. Morphol.* 54: 47–74.
- Berleth, T., M. Burri, G. Thoma, D. Bopp, S. Richstein, G. Frigerio, M. Noll, and C. Nüsslein-Volhard. 1988. The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J.* 7: 1749–1756.
- Brönner, G. and H. Jäckle. 1991. Control and function of terminal gap gene activity in the posterior pole region of the *Drosophila* embryo. *Mech. Dev.* 35: 205–211.
- Brown, N.H. and F.C. Kafatos. 1988. Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* 203: 425–437.
- Campos-Ortega, J.A. and V. Hartenstein. 1985. *The embryonic development of Drosophila melanogaster*. Springer, Berlin, Germany.
- Casanova, J. 1990. Pattern formation under the control of the terminal system in the *Drosophila* embryo. *Development* 110: 621–628.
- Cavener, D.R. 1987. Comparison of the consensus flanking translational start sites in *Drosophila* and vertebrates. *Nucleic Acids Res.* 15: 1353–1361.
- Celniker, S.E., D.J. Keelan, and E.B. Lewis. 1989. The molecular genetics of the bithorax complex of *Drosophila*: Characterization of the products of the *Abdominal-B* domain. *Genes & Dev.* 3: 1424–1436.
- Church, G.M. and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci.* 81: 1991–1995.
- Crosby, M.A. and E.M. Meyerowitz. 1986. Lethal mutations flanking the 68C *glue* gene cluster on chromosome 3 of *Drosophila melanogaster*. *Genetics* 112: 785–802.
- Davis, C.A., D.P. Holmyard, K.J. Millen, and A.L. Joyner. 1991. Examining pattern formation in mouse, chicken and frog embryos with an *En*-specific antiserum. *Development* 111: 287–298.
- Engels, W.R., C.R. Preston, P. Thompson, and W.B. Eggleston. 1986. *In situ* hybridization to *Drosophila* salivary chromosomes with biotinylated probes and alkaline phosphatase. *Focus* 8: 6–8.
- Foe, V.E. and B.M. Alberts. 1983. Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *J. Cell Sci.* 61: 31–70.
- Frohnhofer, H.G. and C. Nüsslein-Volhard. 1986. Organization of anterior pattern in the *Drosophila* embryo by the maternal gene *bicoid*. *Nature* 324: 120–125.
- Gaul, U. and D. Weigel. 1991. Regulation of *Krüppel* expression in the anlage of the Malpighian tubules in the *Drosophila* embryo. *Mech. Dev.* 33: 57–68.
- Halpern, M.E., R.K. Ho, C. Walker, and C.B. Kimmel. 1993. Induction of muscle pioneers and floor plate is distinguished by the zebrafish *no tail* mutation. *Cell* 75: 99–111.
- Harbecke, R. and W. Janning. 1989. The segmentation gene *Krüppel* of *Drosophila melanogaster* has homeotic properties. *Genes & Dev.* 3: 114–122.
- Herrmann, B.G. 1991. Expression pattern of the *Brachyury* gene in whole-mount *T^{Wis}/T^{Wis}* mutant embryos. *Development* 113: 913–917.
- Herrmann, B.G., S. Labeit, A. Poustka, T.R. King, and H. Lehrach. 1990. Cloning of the *T* gene required in mesoderm formation in the mouse. *Nature* 343: 617–622.
- Hoogwerf, A.M., M. Akam, and D. Roberts. 1988. A genetic analysis of the *rose-gespleten* region (68C8-69B5) of *Drosophila melanogaster*. *Genetics* 118: 665–670.
- Jürgens, G. and D. Weigel. 1988. Terminal versus segmental development in the *Drosophila* embryo: The role of the homeotic gene *fork head*. *Wilhelm Roux's Arch. Dev. Biol.* 197: 345–354.
- Karess, R.E. and G.M. Rubin. 1984. Analysis of P transposable element functions in *Drosophila*. *Cell* 38: 135–146.
- Kispert, A. and B.G. Herrmann. 1993. The *Brachyury* gene encodes a novel DNA binding protein. *EMBO J.* 12: 3211–3220.
- . 1994. Immunohistochemical analysis of the *Brachyury* protein in wild-type and mutant mouse embryos. *Dev. Biol.* 161: 179–193.
- Kuziora, M.A. and W. McGinnis. 1988. Autoregulation of a *Drosophila* homeotic selector gene. *Cell* 55: 477–485.
- Langer-Safer, P.R., M. Levine, and D.C. Ward. 1982. Immunological method for mapping genes on *Drosophila* polytene chromosomes. *Proc. Natl. Acad. Sci.* 79: 4381–4385.
- Lehner, C.F. and P.H. O'Farrell. 1989. Expression and function of *Drosophila* Cyclin A during embryonic cell cycle progression. *Cell* 56: 957–968.
- Lehner, C.F., N. Yakubovich, and P.H. O'Farrell. 1991. Exploring the role of *Drosophila* Cyclin A in the regulation of S phase. *Cold Spring Harbor Symp. Quant. Biol.* 51: 465–475.
- Macdonald, P.M. and G. Struhl. 1986. A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. *Nature* 324: 537–545.
- McGinnis, W. and R. Krumlauf. 1992. Homeobox genes and axial patterning. *Cell* 68: 283–302.
- McMahon, A.P. 1992. The *Wnt* family of developmental regulators. *Trends Genet.* 8: 236–242.
- McMahon, A.P., A.L. Joyner, A. Bradley, and J.A. McMahon.

1992. The midbrain-hindbrain phenotype of *Wnt-1^{-/-}/Wnt-1⁻* mice results from stepwise deletion of *engrailed-expressing* cells by 9.5 days postcoitum. *Cell* **69**: 581–595.
- Mlodzik, M. and W.J. Gehring. 1987. Expression of the *caudal* gene in the germ line of *Drosophila*: Formation of an RNA and protein gradient during early embryogenesis. *Cell* **48**: 465–478.
- O'Kane, C.J. and W.J. Gehring. 1987. Detection in situ of genomic regulatory elements in *Drosophila*. *Proc. Natl. Acad. Sci.* **84**: 9123–9127.
- Ortolani, G. 1957. Il territorio precoce della corda nelle ascidie. *Acta Embryol. Morphol. Exp.* **1**: 33–36.
- Patel, N.H., E. Martin-Blanco, K.G. Coleman, S.J. Poole, M.C. Ellis, T.B. Kornberg, and C.S. Goodman. 1989a. Expression of *engrailed* proteins in arthropods, annelids, and chordates. *Cell* **58**: 955–968.
- Patel, N.H., T.B. Kornberg, and C.S. Goodman. 1989b. Expression of *engrailed* during segmentation in grasshopper and crayfish. *Development* **107**: 201–212.
- Patel, N.H., E.E. Ball, and C.S. Goodman. 1992. Changing role of *even-skipped* during the evolution of insect pattern formation. *Nature* **357**: 339–342.
- Pflugfelder, G.O., H. Roth, B. Poeck, S. Kerscher, H. Schwarz, B. Jonschker, and M. Heisenberg. 1992. The *lethal(1)optomotor-blind* gene of *Drosophila melanogaster* is a major organizer of optic lobe development: Isolation and characterization of the gene. *Proc. Natl. Acad. Sci.* **89**: 1199–1203.
- Pignoni, F., R.M. Baldarelli, E. Steingrímsson, R.J. Diaz, A. Patapoutian, J.R. Merriam, and J.A. Lengyel. 1990. The *Drosophila* gene *tailless* is expressed at the embryonic termini and is a member of the steroid receptor superfamily. *Cell* **62**: 151–163.
- Pirrotta, V. 1988. Vectors for P-mediated transformation in *Drosophila*. In *Vectors, a survey of molecular cloning vectors and their uses* (ed. R.L. Rodriguez and D.T. Denhardt), pp. 437–456. Butterworth, Boston, MA.
- Poeck, B., A. Hofbauer, and G.O. Pflugfelder. 1993. Expression of the *Drosophila optomotor-blind* gene transcript in neuronal and glial cells of the developing nervous system. *Development* **117**: 1017–1029.
- Poelmann, R.E. 1981. The head-process and the formation of the definitive endoderm in the mouse embryo. *Anat. Embryol.* **162**: 41–49.
- Reuter, R. 1994. The gene *serpent* has homeotic properties and specifies endoderm versus ectoderm within the *Drosophila* gut. *Development* **120**: 1123–1135.
- Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**: 5463–5467.
- Schulte-Merker, S., R.K. Ho, B.G. Herrmann, and C. Nüsslein-Volhard. 1992. The protein product of the zebrafish homologue of the mouse *T* gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116**: 1021–1032.
- Siewing, R. 1969. *Lehrbuch der vergleichenden Entwicklungsgeschichte der Tiere*. Verlag Paul Parey, Hamburg/Berlin, Germany.
- Skaer, H. 1993. The alimentary canal. In *The developmental biology of Drosophila melanogaster* (ed. M. Bate and A. Martinez-Arias), pp. 941–1012. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Slack, J.M.W., P.W.H. Holland, and C.F. Graham. 1993. The zootype and the phylotypic stage. *Nature* **361**: 490–492.
- Smith, J.C., B.M.J. Price, J.B.A. Green, D. Weigel, and B.G. Herrmann. 1991. Expression of a *Xenopus* homolog of *Brachyury (T)* is an immediate-early response to mesoderm induction. *Cell* **67**: 79–87.
- Smoller, D.A., D. Petrov, and D.L. Hartl. 1991. Characterization of bacteriophage P1 library containing inserts of *Drosophila* DNA of 75–100 kilobase pairs. *Chromosoma* **100**: 487–494.
- Sokoloff, A. 1972. Developmental and post-embryonic studies. In *The biology of Tribolium*, pp. 163–206. Clarendon Press, Oxford, UK.
- Spradling, A.C. 1986. P element-mediated transformation. In *Drosophila: A practical approach* (ed. D.B. Roberts), pp. 175–197. IRL Press, Oxford, UK.
- Stanley, M.S.M. 1965. "The development of the confused flour beetle *Tribolium confusum* Duval." Ph.D. thesis, University of Utah, Salt Lake City, UT.
- Strecker, T.R., J.R. Merriam, and J.A. Lengyel. 1988. Graded requirement for the zygotic terminal gene, *tailless*, in the brain and tail region of the *Drosophila* embryo. *Development* **102**: 721–734.
- Tautz, D. and C. Pfeifle. 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**: 81–85.
- Tear, G., M. Akam, and A. Martinez-Arias. 1990. Isolation of an *abdominal-A* gene from the locust *Schistocerca gregaria* and its expression during early embryogenesis. *Development* **110**: 915–925.
- Van Den Heuvel, M., R. Nusse, P. Johnston, and P.A. Lawrence. 1989. Distribution of the *wingless* gene product in *Drosophila* embryos: A protein involved in cell-cell communication. *Cell* **59**: 739–749.
- Weigel, D., G. Jürgens, F. Kuettner, E. Seifert, and H. Jäckle. 1989a. The homeotic gene *fork head* encodes a nuclear protein and is expressed in the terminal regions of the *Drosophila* embryo. *Cell* **57**: 645–658.
- Weigel, D., H.J. Bellen, G. Jürgens, and H. Jäckle. 1989b. Primordium specific requirement for the homeotic gene *fork head* in the developing gut of the *Drosophila* embryo. *Wilhelm Roux's Arch. Dev. Biol.* **198**: 201–210.
- Weigel, D., G. Jürgens, M. Klingler, and H. Jäckle. 1990. Two gap genes mediate maternal terminal pattern information in *Drosophila*. *Science* **248**: 495–498.
- Yasuo, H. and N. Satoh. 1994. An ascidian homolog of the mouse *Brachyury (T)* gene is expressed exclusively in notochord cells at the fate restricted stage. *Dev. Growth Differ.* **36**: 9–18.

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