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The *Drosophila dead ringer* gene is required for early embryonic patterning through regulation of *argos* and *buttonhead* expression

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SUMMARY

The *dead ringer* (*dri*) gene of *Drosophila melanogaster* is a member of the recently discovered ARID-box family of eukaryotic genes that encode proteins with a conserved DNA binding domain. *dri* itself is highly conserved, with specific orthologs in the human, mouse, zebrafish and *C. elegans* genomes. We have generated *dri* mutant alleles to show that *dri* is essential for anterior-posterior patterning and for muscle development in the embryo. Consistent with the mutant phenotype and the sequence-specific DNA-binding properties of its product, *dri* was found to be essential for the normal early embryonic expression pattern of several key regulatory genes. In *dri* mutant embryos, expression of *argos* in the terminal domains was severely reduced, accounting for the *dri* mutant head phenotype. Conversely, *buttonhead* expression was found to be deregulated in the trunk region, accounting for the appearance of ectopic cephalic furrows. Curiously, *dri* was found also to be required for maintenance of expression of the ventrolateral region of *even-skipped* stripe four. This study establishes *dri* as an essential co-factor in the regulated expression of specific patterning genes during early embryogenesis.

Key words: Pattern formation, Gene regulation, Embryogenesis, *Drosophila melanogaster*, ARID family, Head development

INTRODUCTION

The development of multicellular organisms requires a series of switches in the activity of key regulatory genes to be tightly controlled in a spatial and temporal manner. In the *Drosophila* embryo, this control is established through the combined activity of a number of genes that encode transcriptional regulators. The expression of these regulators evolves over the course of early embryogenesis to assign cells to different fates and create gene expression boundaries that organise subsequent tissue growth and differentiation. Many aspects of the nature of these gene regulatory events, are not yet understood.

dead ringer (*dri*, Gregory et al., 1996) is a founding member of a new family of proteins which share a conserved DNA binding domain, termed the ARID (A/T Rich Interaction Domain, Herrscher et al., 1995, Gregory et al., 1996). Members of this gene family include *Drosophila osa* (also referred to as *eyelid*, Treisman et al., 1997; Vazquez et al., 1999), yeast *SWI1* (O'Hara et al., 1988), the mammalian *jumonji* (Motoyama et al., 1997), *Smcx* (Agulnik et al., 1994a), *Smcy* (Agulnik et al., 1994b), *MRF1* and *MRF2* (Huang et al., 1996) genes and genes encoding Retinoblastoma binding proteins, RBP1 and RBP2 (Fattaey et al., 1993).

Sequence comparisons revealed that DRI belongs to a subgroup within this family that exhibits an extended region of similarity either side of the ARID. We refer to this motif as the extended ARID (eARID; Kortschak et al., 1998). The eARID group, which is poorly characterised, includes DRI, human DRIL1 (Kortschak et al., 1998), mouse Bright (Herrscher et al., 1995) and proteins encoded by the *C. elegans* T23D8.8 and *D. rerio dri1* and *dri2* genes (Kortschak et al., 1998). There is some evidence that members of this group are implicated in transcriptional regulatory processes. The mouse *dri* ortholog, *Bright* (for B-cell regulator of IgH transcription, Herrscher et al., 1995) encodes a B cell-specific protein which appears to bind the minor groove of a consensus MAR sequence (AT/ATC). Here Bright acts to displace a conserved human homeoprotein CUX (ortholog of *Drosophila* CUT) to activate the immunoglobulin heavy chain intronic enhancer, Eμ, specifically in B cells (Wang et al., 1999). A different relationship exists between *cut* and *dri* in *Drosophila*, where CUT and DRI bind to the same site in the *zen* minimal ventral repression region (VRR) element to repress transcription in a Dorsal and Groucho-dependent fashion (Valentine et al., 1998).

dri maternal product is distributed ubiquitously in the syncytial and cellular blastoderm embryos to be replaced by tissue-specific expression at later stages (Gregory et al., 1996). Embryonic tissues that express *dri* include the mesoderm at stage 9 and, later during embryogenesis, the dorsal part of the ring gland (*corpus allatum*), salivary gland ducts, pharyngeal muscles, rings of cells at junctions between the foregut, midgut and hindgut, as well as in two rows of cells along the hindgut.

The highly specific pattern of temporal and spatial expression and the sequence-specific DNA-binding activity of

the encoded protein suggest that *dri* plays an important role in embryogenesis. Here we demonstrate this by the generation and phenotypic characterisation of *dri* mutant alleles. We show that *dri* is essential for embryonic development, with roles in the formation of anterior-posterior structures, particularly those at the termini, and in muscle development. In addition we show that expression of *engrailed*, *wingless, even-skipped*, *argos* and *buttonhead* are disrupted to varying extents in *dri* mutant embryos, accounting for segmentation and head defects and ectopic cephalic furrow formation in these embryos. These observations establish *dri* as an essential player in the assembly of the correct pattern of tissues during embryogenesis.

MATERIALS AND METHODS

Fly stocks

$aos^{\Delta 7}$ (Freeman et al., 1992) was obtained from Steven DiNardo. P-element insertion alleles *l(2)02535* (renamed as dri^7) and *l(2)05096* (dri^8) (Cooley et al., 1988) were obtained from A. Spradling. Other stocks were obtained from the Bloomington *Drosophila* Stock Center.

DNA probes, antibodies and staining methods

A rat polyclonal antibody raised against a bacterially expressed pGEX-DRI fusion protein (Gregory et al., 1996) was used to detect the distribution of DRI in embryos. *aos* cDNA (Freeman et al., 1992), *btd* cDNA (Wimmer et al., 1993), *ems* cDNA (Kalionis and O'Farrell, 1993), *otd* cDNA (Finkelstein et al., 1990), *run* cDNA (Kania et al., 1990), *sna* and *twi* cDNA (Leptin, 1991), *tll* cDNA (Pignoni et al., 1990), *wg* cDNA (Baker, 1987), polyclonal anti-BCD, anti-EVE, anti-GT, anti-H, anti-HB, anti-KNI, anti-RUN, anti-TLL (obtained from J. Reinitz), anti-KR (obtained from C. Rushlow), anti-muscle myosin (obtained from D. Kiehart) and monoclonal 4D9 anti-EN (Patel et al., 1989) antibodies were used to monitor the respective gene expression. In situ hybridisation to whole-mount embryos using digoxigenin-labeled RNA probes (Tautz and Pfeifle, 1989) were performed according to the Boehringer Mannheim protocol. Immunohistochemical stainings were carried out according to the method of Foe (1989).

Genomic structure determination and P-element localisation

The genomic structure of the *dri* locus, including the location of the two P-elements was determined using standard restriction endonuclease mapping of genomic clones, subcloning and DNA sequence analysis techniques. All exon-intron boundaries were confirmed by DNA sequence analysis.

P-element transposon replacement

We used targetted transposition of P-elements (Heslip and Hodgetts, 1994; Gonzy-Treboul et al., 1995) to replace the Pry^+ *lacZ* transposon, inserted into the *dri* locus in dri^7, with a P-element enhancer trap line expressing the yeast *GAL4* gene (Brand and Perrimon, 1993). Individuals containing the dri^7 ry^+ *lacZ* P-element insertion (59F), an X-chromosome *GAL4* mini-w^+ enhancer trap insertion and the P[Δ2-3] (99B) transposase gene were generated and crossed to w^{1118} individuals. 10978 male progeny were examined, from which 339 males were selected for possible transposition or conversion events affecting the *dri* locus, based on an altered mini-w^+ eye colour. Mutant lines underwent a series of consecutive tests: a test for 2nd chromosome linkage of the mini-w^+ transposon, a test for lethality over the original dri^7 allele and a test for excision of the Pry^+ *lacZ* transposon, i.e. reversion of ry^+ to ry^-. Finally, the GAL4 replacement lines were combined with UAS::*lacZ* and UAS::GFP reporter constructs to determine the pattern of expression of the GAL4 enhancer trap. Twenty lines exhibiting most or all of the *dri* expression pattern were generated in this way.

Drosophila genetic transformation

A full length *dri* cDNA was constructed by fusing the 5′-most extending and 3′-most extending cDNA clones isolated previously (Gregory et al., 1996). The full length cDNA, with *Eco*RI ends, was then cloned into pUAST to generate pUAS::*dri*. Transformants, generated by microinjection (Spradling and Rubin, 1982), were obtained on all three major chromosomes.

Generation of EMS-induced *dri* alleles

A screen for lethal *dri* alleles was carried out by inducing mutations with ethylmethane sulfonate (EMS) and screening for lethality against the lethal dri^7 allele. EMS treatment was as described by Roberts (1986). Among 2412 F_1 sons of EMS treated fathers, 6 failed to complement the dri^7 allele.

RESULTS

Identification of P-element insertion *dri* mutant alleles

A detailed map of the *dri* transcription unit (summarised in Fig. 1A) was generated by isolating overlapping genomic clones and characterising these clones by restriction endonuclease, Southern blot and DNA sequence analysis. The *dri* transcript was shown to consist of 12 exons that span 22 kb of genomic DNA. Two lethal and non-complementing *P-lac*Z enhancer-trap lines, *l(2)02535* and *l(2)05096* (Cooley et al., 1988), have insertions at 59F1-2, the cytological location of *dri*. These lines were found to express the reporter gene in the same developmental pattern as *dri* and to disrupt some aspects of *dri* expression, as revealed by in situ hybridisation with a *dri* cDNA probe and immunohistochemical staining with anti-β-gal antibody (Fig. 3B and results not shown). Plasmid rescue of the P-element insertions and flanking DNA, together with restriction endonuclease, Southern blot and DNA sequence analysis, showed that the P-element in lines *l(2)02535* and *l(2)05096* is inserted 471 bp and 439 bp, respectively, upstream of the *dri* transcription start site (Fig. 1A). The effect on *dri* expression and the location of the insertion elements close to the *dri* transcription start site suggested that the two P-insertion alleles were likely to be *dri* mutant alleles.

Complementation of mutant alleles by a wild-type copy of a gene is the preferred way to confirm correspondence of a complementation group with a cloned gene. We could not test for complementation using standard methods for two reasons. First, the large size of the *dri* transcription unit (Fig. 1A) prevented us from creating a genomic rescue fragment. Secondly, the complex *dri* expression pattern was not matched by the expression pattern of known enhancers, and expression of a wild-type UAS::dri^+ cDNA construct with a variety of available GAL4 drivers was found to result in lethality (results not shown). We reasoned that the lethality associated with GAL4-UAS::dri^+ expression was likely to be due to ectopic expression and therefore we generated an enhancer trap line in which GAL4 was expressed by the *dri cis*-regulatory sequences, using the observation that one P-element can be replaced by another in the same genome in the presence of the P-transposase (Heslip and Hodgetts, 1994; Gonzy-Treboul et al., 1995). We used the ry^+ marker of the *l(2)02535* insertion, the mini-$white^+$ marker of an enhancer-trap yeast GAL4 transposon located on the X chromosome and the P[Δ2-3] (99B) transposase gene to

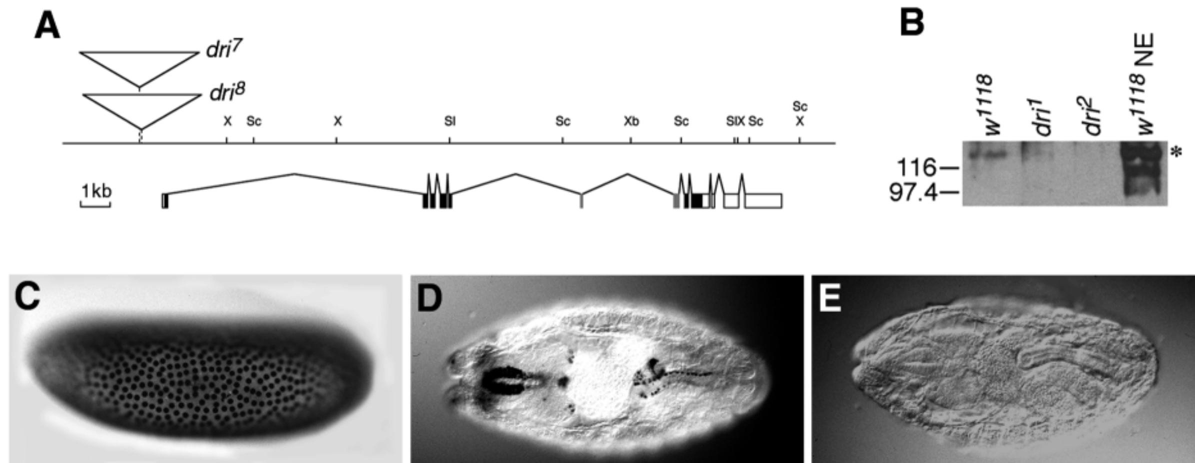

Fig. 1. Characterisation of *dri* mutant alleles. (A) Genomic map of the *dead ringer* locus showing the intron structure and sites of P-element insertions in the dri^7 and dri^8 alleles. The black boxes indicate the open reading frame of the cDNA. The grey box indicates the location of the eARID motif. Restriction site abbreviations: X, *Xho*I; Xb, *Xba*I; Sc, *Sac*I; Sl, *Sal*I. (B) Western blot analysis using anti-DRI antibody. Total embryonic extracts prepared from 0-2 hours w^{1118} embryos or germline clone-derived dri^1 and dri^2 maternal and zygotic null embryos. The w^{1118} NE lane shows an embryonic nuclear extract from w^{1118} embryos, showing the expected nuclear localisation of DRI. (C-E) Immunostains with polyclonal anti-DRI antibody. (C) A syncytial embryo derived from dri^1 heterozygous parents showing stereotypical anti-DRI staining indicating the presence of maternal *dri* product in nuclei. Note that all progeny from dri^1 heterozygous parents exhibit anti-DRI staining at this stage. (D) Dorsal view of a wild-type embryo showing expression of *dri* in a specific set of tissues (see Gregory et al., 1996 for a description of the expression pattern). (E) Dorsal view of a homozygous dri^1 embryo at the same stage as the wild-type embryo in D showing absence of zygotic *dri* product.

generate lines in which the GAL4 enhancer trap element had replaced the P-element insertion in the *l(2)02535* line (see Materials and Methods). Introduction of the UAS::*lacZ* and UAS::GFP reporter genes was used to demonstrate that the GAL4 expression patterns in the resulting lines corresponded to that of the endogenous *dri* pattern (Fig. 2). The 20 P-element replacement lines generated, termed *dri*-GAL4 lines, all failed to complement the original *l(2)02535* and *l(2)05096* lines showing that, as expected, the replacement insertion disrupted *dri* function.

Flies carrying *dri*-GAL4/*CyO* were crossed to a line carrying a UAS::dri^+ cDNA construct that was also heterozygous for *l(2)02535* and *l(2)05096*, or for *Df(2)tid*, a deficiency spanning the *dri* locus. Nineteen different *dri-GAL4* UAS::*dri* combinations showed variable rescue of the embryonic lethality associated with the complementation group defined above (12-60% of the number of *dri* mutant progeny expected for complete rescue). As predicted, viability was dependent on the presence of the UAS::dri^+construct. We conclude that the complementation group defined by these P-element alleles corresponds to the *dri* locus and renamed the *l(2)02535* and *l(2)05096* P-element lines dri^7 and dri^8.

Zygotic *dri* expression is essential for normal embryogenesis

dri^7 and dri^8 homozygotes were found to be embryonic lethal, but to have only mild phenotypes. The pattern of cuticle structures in these embryos was normal both in zygotic and germline clone mutant embryos (results not shown), but the pattern of *dri*-expressing cells in the hindgut, marked by the expression of a *lacZ* reporter gene, was highly disrupted (Fig. 3A,B). In situ hybridisation with DIG-labeled *dri* cDNA and immunohistochemical staining with specific anti-DRI antibody (Gregory et al., 1996) showed that both of these P-insertion alleles retain mRNA and protein expression in most tissues (results not shown), indicating that they were likely to be hypomorphic alleles.

In an attempt to generate amorphic alleles, we used ethylmethane sulfonate (EMS) mutagenesis to create alleles that failed to complement dri^7 and dri^8 (see Materials and Methods). Immunochemical staining with an anti-DRI antibody revealed that two of the resulting six alleles, dri^1 and $dri^{2,}$ showed no zygotic *dri* product (Fig. 1D,E). A western blot of protein derived from *dri* germline and zygotic mutant embryos also showed absence of the *dri* protein product (Fig. 1B). We conclude that dri^1 and dri^2 are amorphic alleles. This conclusion is supported by our observation that the phenotypes observed with the two alleles were indistinguishable.

Embryos homozygous or *trans*-heterozygous for these alleles, or *trans*-heterozygous for either allele and *Df(2)tid*, were embryonic lethal, but appeared to have a normal cuticle pattern (results not shown). Disruption of the pattern of *dri*-expressing hindgut cells in these lines marked, in this case, by expression of *lacZ* from the enhancer trap line 18-13 (unpublished results; Manak and Scott, personal communication), closely resembled disruptions in dri^7 and dri^8.

As reported previously, DRI is uniformly expressed throughout the mesoderm during germ band extension (Gregory et al., 1996). Anti-muscle myosin staining revealed variable levels of disruption to somatic muscle development in *dri* mutant embryos. This resulted in embryos in which groups of muscles were missing, unfused myoblasts persisted and muscle fibres were misdirected (Fig. 3D,E). Variable expressivity of the muscle phenotype made it impossible to define a specific group of muscles affected.

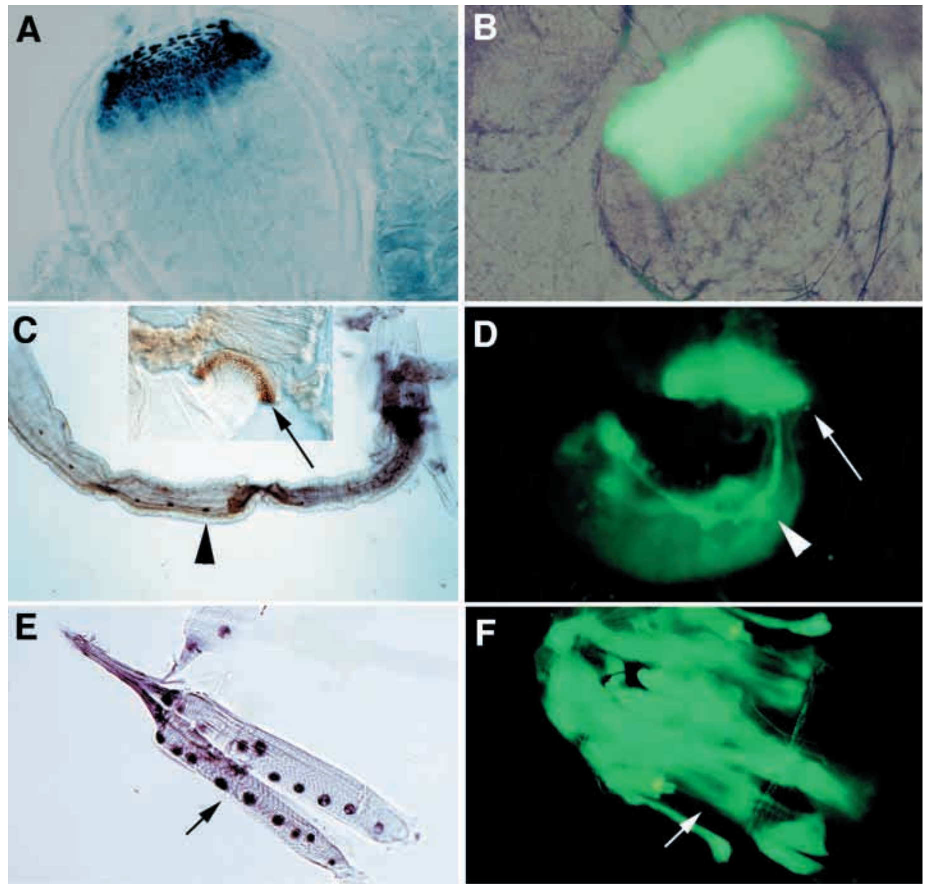

Fig. 2. Characterisation of a *dri*-GAL4 enhancer trap line. *dri*-GAL4 lines generated by P-element replacement, as described in the text, were combined with a UAS::GFP line (B,D,F) and GFP expression was compared with the *dri* expression pattern revealed by anti-DRI immunostaining (A,C,E). Expression of GFP was found to mirror that of *dri*. Examples of equivalent expression in the proventriculus (A,B), hindgut (C,D: arrows indicate midgut/hindgut junction, arrowheads indicate cells expressing *dri* along the hindgut) and pharyngeal muscles (E,F: arrows point to the single muscle fibres) are shown.

dri is required for normal anterior-posterior axis and muscle development

As noted above, maternally derived DRI is uniformly distributed throughout the syncytial cleavage divisions and during early gastrulation (Gregory et al., 1996, see Fig. 1C). It was thought likely that the presence of maternal *dri* product

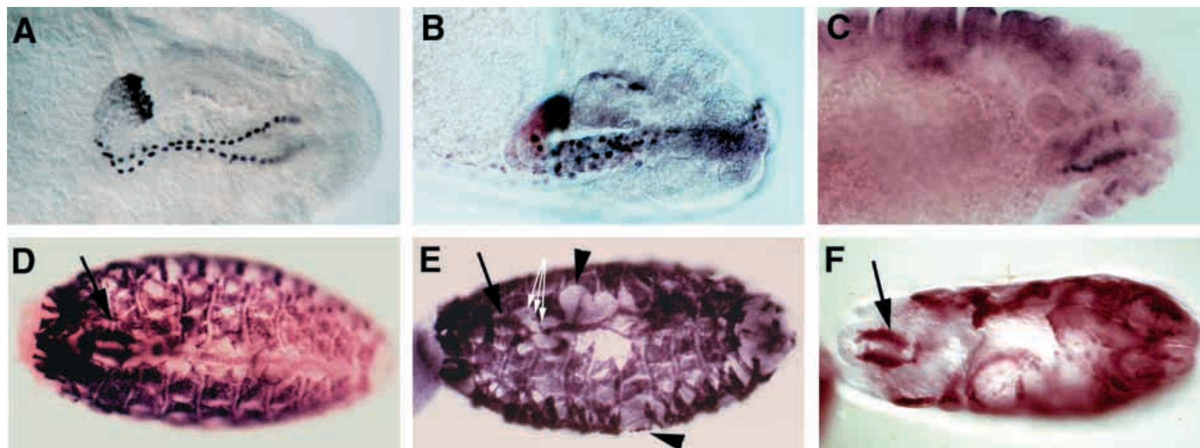

Fig. 3. The zygotic and maternal *dri* mutant phenotype. Immunochemical staining with anti-DRI (A) and anti-β-gal (B,C) antibodies. (A) Wild-type embryo showing ordered rows of *dri*-expressing cells along the hindgut. (B) Homozygous dri^7 mutant hindgut showing disordered arrangement of *dri*-expressing cells. (C) Maternal and zygotic dri^1 mutant hindgut. A transgenic 18-13 *lacZ* line was introduced paternally to label *dri*-expressing cells. The hindgut is not properly extended and *dri*-expressing cells are disordered. (D-F). Whole-mount embryos stained with anti-muscle myosin antibody. (D) Dorsal view of a w^{1118} embryo. (E) Dorsolateral view of a dri^1/dri^2 embryo derived from heterozygous parents showing loss of some groups of somatic muscles (black arrowheads) and unfused myoblasts (joined white arrows). (F) Dorsal view of a dri^2 germline clone and zygotic mutant embryo showing severe disorganisation of the somatic muscle pattern including unfused myoblasts and non-specified and misdirected muscle fibres. Black arrows in (D,E,F) point to the position of the pharyngeal muscles. In the maternal and zygotic mutant embryo (F), pharyngeal muscles have an atypical anterior position, indicative of disruption of head involution.

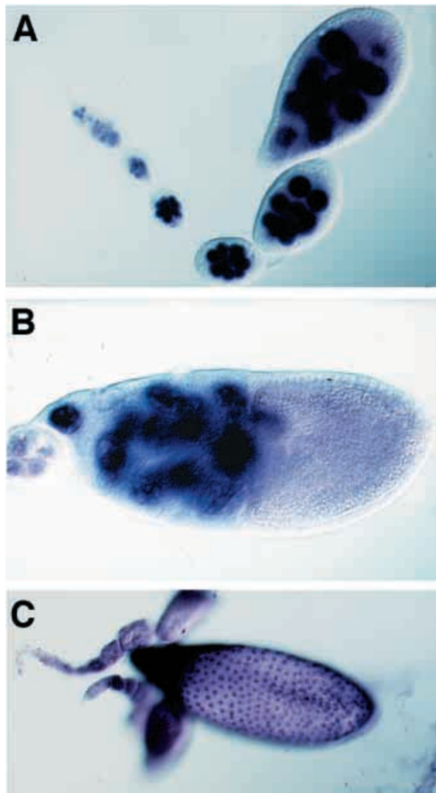

Fig. 4. *dri* is expressed in ovaries. Anti-DRI immunostaining of ovaries of a w^{1118} imago. (A,B) *dri* is expressed throughout oogenesis starting in the germarium and then specifically in the nuclei of nurse cells and oocytes. (C) Later in oogenesis, *dri* is expressed in follicle cells.

would decrease the severity of the zygotic phenotype described above. To abolish the maternal *dri* contribution we generated dri^1 and dri^2 germline clones, using the FLP-FRT-ovo^{D1} system (Chou and Perrimon, 1996). Embryos lacking both maternal and zygotic products were produced using this approach, but the efficiency of egg production was much lower than expected of a gene that plays no role in oogenesis. In addition, many eggs that were produced were unfertilised or exhibited early syncytial proliferation defects. Consistent with this, *dri* was found to be expressed during oogenesis in the germinal vesicle and in nuclei of nurse cells and follicle cells (Fig. 4).

dri mutant germline clone embryos could be rescued by a paternal dri^+ allele, as judged by the appearance of normal embryos carrying a *lacZ* marker on the dri^+ paternal chromosome and by the appearance of viable and fertile heterozygous germline clone progeny. Rescue was only partial, however, as only 16% of germline embryos with a wild-type paternal allele survived to the first instar larval stage. As predicted, embryos lacking both the maternal and the zygotic *dri* product exhibited much stronger phenotypes than those that lacked the zygotic component alone. Analysis of embryos lacking maternal and zygotic *dri* function, but with a normal nuclear distribution, revealed varying levels of disruption to segment formation, particularly in the posterior regions of the embryos (Fig. 5D,E). A majority of embryos also exhibited abnormal germ-band retraction phenotypes that were not always rescued by a wild-type paternal allele (results not shown).

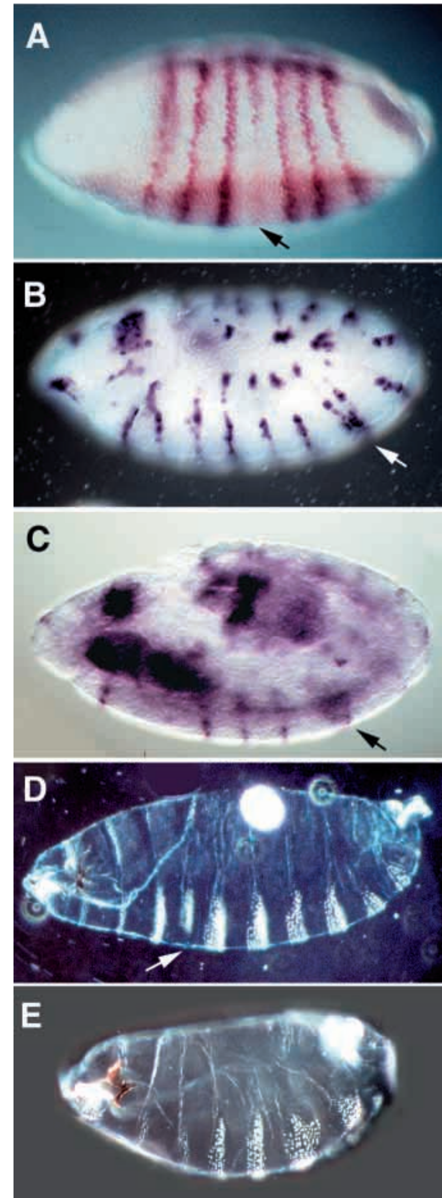

Fig. 5. *dri* is essential for maintenance of the ventrolateral region of *eve* stripe 4. (A) Anti-EVE immunostaining of a maternal and zygotic *dri* mutant embryo. The ventrolateral region of *eve* stripe 4 disappears during gastrulation (arrow). (B,C) Whole-mount in situ hybridisation of maternal and zygotic *dri* embryos with a DIG-labeled *wg* antisense RNA probe. *wg* stripes 7 and 8 are fused ventrolaterally (arrows). (C) An example of a more severe posterior phenotype in which *wg* stripes 9-14 are significantly reduced in intensity. (D,E) Cuticle patterns of maternal dri^1 mutants. (D) The ventrolateral part of the parasegment 4 setal belts is missing (arrow) as a result of the failure to maintain *eve* stripe 4. (E) An example of a more severe posterior phenotype in which most of the cuticle belts posterior to parasegment 4 are fused.

Disruption to the development of anterior-posterior axis structures was particularly evident in the terminal regions of the embryo. Both head and tail defects were invariably observed in *dri* maternal and zygotic mutant embryos (Figs 3C,F, 6C,D). One of the most consistent and striking phenotypes was severe disruption of the cephalo-pharyngeal skeleton (Fig. 6C,D).

Germline and zygotic *dri* mutant embryos still had a recognisable dorsal bridge, dorsal and ventral arms and mouth hooks but the H-piece and lateralgraten were missing or severely malformed. In addition, the atypical anterior position of pharyngeal muscles, visualised using anti-muscle myosin immunostaining, indicated that head involution had not proceeded properly (Fig. 3F).

Anti-muscle myosin immunostaining of embryos derived from germline clones also revealed dramatic disruption to development of the somatic musculature. Many fibres were missing, unfused myoblasts were frequently observed and some myotubes had formed aberrant attachments with epidermal cells (Fig. 3F). In addition, all dorsally closing *dri* mutant embryos lacked pericardial cells (results not shown).

The role of *dri* in segment formation gene expression patterns

The data discussed above indicate that *dri* is required for proper patterning of the embryo. We had previously shown that DRI is a sequence-specific DNA binding protein (Gregory et al., 1996), so it was likely that the phenotypes exhibited by *dri* mutant embryos resulted from disruption to the expression of developmental regulatory genes. To test this, we initially examined genes required for segment formation in the *Drosophila* embryo. Expression of the coordinate gene, *bicoid*, the gap genes, *hunchback*, *Krüppel*, *knirps* and *giant*, the primary pair-rule genes, *even-skipped*, *hairy* and *runt*, and the segment polarity genes, *wingless* and *engrailed*, was examined in embryos lacking germline and zygotic *dri* function. Most of these genes were expressed normally with respect to their role in segment formation. The variable disruption to abdominal segment formation was found to correlate with a variable reduction in expression of *engrailed* and *wingless* (*wg*) in stripes 9-14 (Fig. 5C and data not shown). The most consistent effect on expression of the segmentation genes in the *dri* maternal and zygotic mutant embryos was a disruption to the expression of *even-skipped* (*eve*) stripe 4, which was observed in nearly all embryos lacking both maternal and zygotic *dri* product. Specifically, the ventrolateral portion of *eve* stripe 4, although initiated appropriately (results not shown) was not maintained in *dri* mutant embryos (Fig. 5A), leading to the subsequent aberrant appearance of *wg* stripes 7 and 8 (Fig. 5B,C) and disruption to the parasegment 4 ventrolateral setal belts (Fig. 5D).

dri is required for normal *argos* and *buttonhead* expression

As noted earlier, terminal development was disrupted in the *dri* maternal and zygotic mutant embryos. The appearance of these defects prompted us to examine genes that play a role in the formation of terminal structures. Expression of the terminal gene *tailless*, the genes *buttonhead*, *empty spiracles*, *orthodenticle* and *argos* were examined. Of these, disruption to only *argos* (*aos*) and *buttonhead* (*btd*) expression was observed. In wild-type embryos, *aos* is initially expressed at stage 5 in two terminal domains and a domain that flanks the position of the cephalic furrow (Fig. 6A). In embryos lacking *dri* maternal and zygotic product, expression of the terminal domains was found to be almost eliminated while expression in the region of the cephalic furrow is maintained, both before and after division into two stripes at the time of cephalic furrow formation (Fig. 6B). Zygotic *aos* mutant embryos exhibit head defects which are similar to those observed in maternal and zygotic *dri* mutant embryos (Fig. 6D,E), indicating that the *dri* mutant head defects are likely to be the result of loss of anterior *aos* expression in the *dri* mutant embryos.

Analysis of *btd* expression revealed a regulatory relationship that accounted for another consistent *dri* mutant phenotype, the appearance of ectopic cephalic furrows (Fig. 7A,B). *btd* expression was found to be partially derepressed in the trunk of *dri* germline and zygotic mutant embryos (Fig. 7C,D). The cephalic furrow arises where expression of the head specific gap gene *btd* overlaps the first stripe of expression of the primary pair rule gene *eve* (Vincent et al., 1997). The repetitive appearance of ectopic cephalic furrows is therefore likely to be the result of the coincident ectopic trunk expression of *btd* with the more posterior *eve* stripes. The ectopic furrows did not progress, most probably due to the incomplete derepression of *btd* in this region.

DISCUSSION

The *dead ringer* gene of *Drosophila melanogaster* belongs to the ARID family of genes, a recently described and widely conserved gene family about which very little is known. We report here that *dri* plays an essential role in anterior-posterior embryonic patterning through regulation of gene expression.

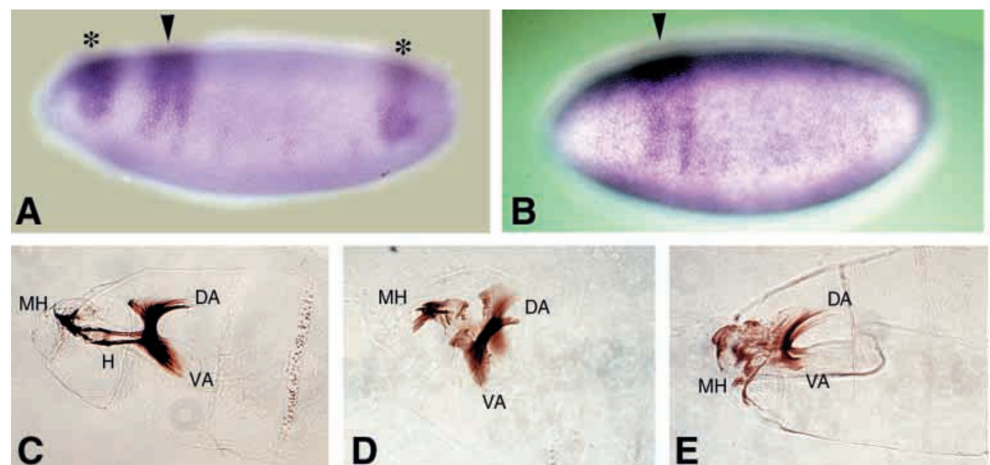

Fig. 6. *dri* is essential for normal expression of the terminal domains of *aos*. (A,B) Whole-mount in situ hybridisation with a DIG-labeled *aos* antisense RNA probe. (A) Pattern of expression of *aos* at the onset of cellularisation in a wild-type embryo. The arrowhead indicates a central domain marking the position of the cephalic furrow. Asterisks indicate the terminal domains. (B) The terminal domains of expression are almost eliminated in the absence of maternal and zygotic DRI. Morphology of the first larval instar head in (C) a wild-type embryo, (D) a dri^2 germline clone embryo, and (E) a homozygous mutant $aos^{\Delta 7}$ embryo. (D) and (E) show similar defects, namely missing or malformed H-piece structures. MH, mouth hook; DA, dorsal arm; VA, ventral arm; H, H-piece.

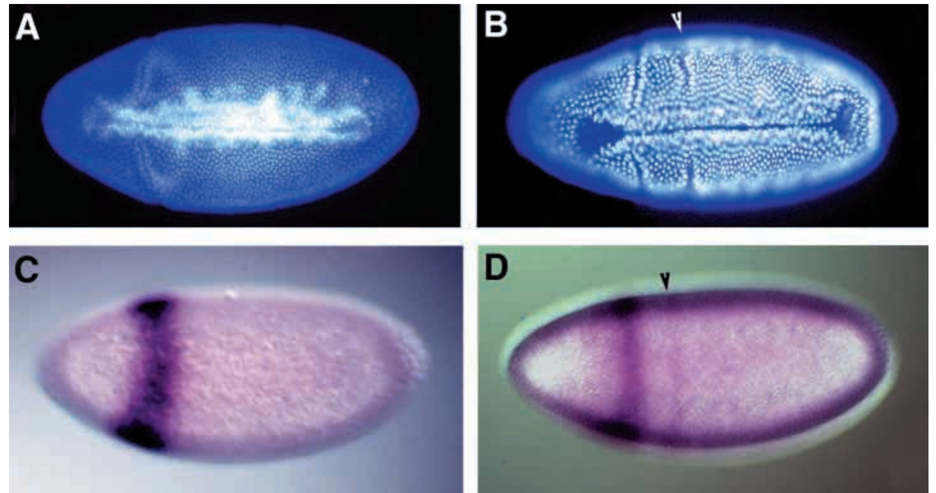

Fig. 7. *dri* is necessary for *btd* repression in the trunk region of the embryo. (A,B) Hoechst 33258 stained embryos showing the cephalic furrows. (A) A wild-type embryo showing the normal position of the cephalic furrow. (B) A maternal dri^1 mutant embryo showing the appearance of an ectopic cephalic furrow (arrowhead). (C,D) Whole-mount in situ hybridisation with DIG-labeled *btd* antisense RNA. (C) Wild-type expression of *btd*. The posterior boundary of *btd* expression demarcates the anterior border of the cephalic furrow. (D) A maternal dri^1 mutant embryo showing derepression of *btd* in the trunk of the embryo (arrowhead).

Three observations pointed to the likelihood of a significant role for *dri* in *Drosophila* development. The first was recognition that the gene encoded a sequence-specific DNA-binding protein (Gregory et al., 1996). The second was the highly tissue-specific pattern of expression during development (Gregory et al., 1996 and results not shown) and the third was the identification of highly conserved orthologs in the nematode *Caenorhabditis elegans*, zebrafish *Danio rerio*, mouse and human genomes (Herrscher et al., 1995; Kortschak et al., 1998). In order to test for such a role, we have generated and characterised amorphic *dri* alleles.

Phenotypic characterisation of *dri* mutant embryos derived from heterozygous parents revealed that loss of zygotic *dri* expression results in embryonic lethality, with mild developmental defects apparent in the hindgut and muscles. Removal of the *dri* maternal component, however, yielded much more dramatic phenotypes and showed that *dri* plays roles in a variety of embryonic patterning processes. Embryos lacking both maternal and zygotic *dri* function exhibited varying levels of disruption to segment formation and severe disruption to the formation of terminal structures and muscles.

Disruption to muscle development is not surprising, given the pattern of *dri* expression. Following a general early nuclear distribution, DRI is observed specifically in mesodermal cells in late stage 9 embryos (Gregory et al., 1996). Formation of both somatic muscle and pericardial cells was disrupted to varying extents in *dri* mutant embryos. The *dri* mutant muscle phenotypes resembled those of the segmentation mutants, *sloppy-paired* (*slp*) (Riechmann et al., 1997) and *wingless* (*wg*) (Volk and VijayRaghavan, 1994). Precursors of somatic muscle and pericardial cells are specified in a segmentally repeated pattern in the mesoderm (Riechmann et al., 1997; Rugendorff et al., 1994). The muscle defects could therefore have been a secondary consequence of the variable segmental defects observed in *dri* mutant embryos, for example in leading to the incorrect specification of founder cells. Muscle disruption was, however, consistently observed irrespective of the degree of disruption to normal epidermal segmentation. *dri* zygotic mutant embryos, for example, exhibit no observable segmental defects but do exhibit muscle defects. We conclude, therefore, that *dri* is essential for normal muscle formation.

We have not been able to elucidate the specific role played by *dri* in muscle development. As discussed below, variable expressivity is a characteristic of *dri* mutant phenotypes, making it difficult to assign precise roles. We did not observe significant changes in *twist* and *snail* expression, as indicators of normal mesoderm formation, nor observe dramatic changes in ventral furrow formation, although we did observe embryos which had undergone aberrant gastrulation, including embryos that exhibited a ‘twist’ phenotype. As the muscle defects were much greater in embryos lacking maternal as well as zygotic *dri* products, *dri* must participate in mesoderm patterning during early stages of embryogenesis. Based on other aspects of gene regulation in which *dri* is involved, it is reasonable to postulate that *dri* will be necessary for expression of one or more genes involved in muscle patterning or differentiation.

dri mutant embryos also failed to form normal terminal structures. This disruption appears not to act through *tailless*, which is expressed normally in *dri* mutant embryos. Expression of *aos* was, however, severely disrupted in the terminal regions. In germline and zygotic *dri* mutant embryos at stage 5, the terminal domains of *aos* expression were barely detectable while expression at the site of cephalic furrow formation was unaffected. *aos* embryos were found to exhibit head defects that were similar to those observed in *dri* maternal and zygotic mutant embryos, so the role of *dri* in head formation appears to be mediated, at least in part, by regulation of *aos*. There are no reports of studies of *aos* transcriptional regulation in blastoderm embryos, but the role of DRI in *aos* transcriptional regulation must be as a co-factor for position-specific transcriptional regulators, given that DRI is present ubiquitously in nuclei at the stage at which terminal *aos* expression occurs. Similarly, DRI must be acting as a co-factor in the regulation of *btd* expression, except that in this case the absence of DRI leads to derepression of *btd*. This derepression correlates with the appearance of ectopic cephalic furrows in *dri* mutant embryos, which would be expected to occur where *eve* and *btd* expression coincides (Vincent et al., 1997).

The regulation of *aos* and *btd* by *dri* is consistent with the function of DRI as a sequence-specific DNA binding protein and with the involvement of DRI and its mouse ortholog, Bright, in transcriptional regulation (Herrscher et al., 1995; Valentine et al., 1998; Wang et al., 1999). The specific

molecular roles of DRI are poorly understood. One important conclusion that can be made from the studies reported here is that DRI is unlikely to be acting as a general transcription co-factor or chromatin modifier, as transcription of only a small number of the genes examined was disrupted in *dri* mutant embryos. In terms of specific molecular function, DRI was shown to act in conjunction with Dorsal to recruit Groucho and repress the *zen* minimal ventral repression region (VRR) element (Valentine et al., 1998). It should be noted, however, that while ventral derepression of the VRR element in *dri* mutant embryos was virtually complete, derepression of *zen* itself was only partial and spatially restricted (Valentine et al., 1998), indicating a level of redundancy in the regulation of *zen* that was lost in the isolation of the VRR. This is consistent with the lack of any major dorsal-ventral patterning defects in *dri* mutant embryos, although variable gastrulation defects were observed, consistent with some level of disruption to dorsal-ventral patterning. *btd* expression is regulated by three maternal organiser systems which control body pattern formation: the anterior morphogens *bicoid* and *hunchback*, the dorsoventral morphogen, *dorsal*, and the terminal system gene *tailless*. These genes most likely act through the 1 kb *cis*-acting control region located about 3 kb upstream of the promoter (Wimmer et al., 1995). A 790 bp fragment in the 3′ *cis*-control region apparently contains a repressor binding site, since truncation of this fragment results in a posterior expansion of the *btd*-specific head stripe (Wimmer et al., 1995). Analysis of the DNA sequence of the 790 bp *btd cis*-acting fragment revealed potential binding sites for DRI, but the role of such sites in *btd* repression has yet to be tested.

The simplest interpretation of the results reported here is that DRI can act as an activator or repressor, depending on the context within which it finds itself. These different actions must depend on the combination of regulators acting on the respective position-specific *cis*-regulatory sequences. As noted above, the differential regulation of *aos* and *btd* domains by *dri* occurs at a time when DRI is found in all somatic nuclei of the embryo, so that DRI must be acting to permit the proper function of other developmental regulatory factors. However following gastrulation, *dri* expression becomes exquisitely tissue and stage specific (Gregory et al., 1996) raising the possibility that it may specify spatial-specific expression at later stages of development.

We cannot explain the variability in segmentation phenotype in *dri* mutant embryos. These defects ranged from near fully penetrant loss of maintenance of the ventrolateral portion of *eve* stripe 4 to variable loss of *wg* expression, most noticeably in the posterior half of the trunk region. *dri* is not alone in exhibiting such variability. *Drosophila Dichaete* mutants also exhibit phenotypic variability (Russell et al., 1996). Dichaete is a member of the SOX family of HMG proteins and is likely, therefore, to modify the local architecture of chromatin. Although the structure of DRI and of a DRI-DNA complex have not been determined, the mouse Bright protein appears to bind to the minor groove of DNA and, like the HMG proteins of which Dichaete is a member, to kink the DNA (Herrscher et al., 1995). Furthermore, the other known *Drosophila* ARID family member, *osa*, is a member of the Trithorax Group of genes implicated in the modification of chromatin structures required for epigenetic regulation (Vazquez et al., 1999). We propose, therefore, that DRI, like Dichaete, Bright and presumably OSA, is acting to establish stable chromatin structures. These structures may favour, but not be essential for, the formation of complexes in which other transcriptional regulators act, so that the absence of factors such as Dichaete or DRI introduces an element of chance into the stable formation of such complexes and, consequently, variability in gene expression and mutant phenotype.

In conclusion, we have demonstrated that the *dead ringer* gene of *Drosophila melanogaster* is essential for the formation of normal anterior-posterior structures and for muscle development during embryonic development. We have demonstrated that this effect is mediated through the regulation of other key developmental regulatory genes. These studies establish *dead ringer* as an essential component of regulatory mechanisms that control embryonic patterning in *Drosophila melanogaster*.

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