The role of homeotic genes in the specification of the *Drosophila* gonad

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Background: In *Drosophila*, the gonads are composed of two cell populations: the germ line, derived from the pole cells, and a somatic component, derived from the mesoderm of abdominal segments 5–8. Formation of the gonad requires the function of a specific homeotic gene, *abdominal-A* (*abd-A*). Other genes of the bithorax complex, *Ultrabithorax* (*Ubx*) or *Abdominal-B* (*Abd-B*), cannot substitute for this requirement when *abd-A* is removed.
Results: We show here that, in embryos lacking the entire bithorax complex, experimentally induced expression of either ABD-A or UBX protein in the mesoderm will rescue the expression of a gonad-specific marker, 412 RNA. Ubiquitous expression of these homeotic proteins within the mesoderm results in the formation of ectopic gonad tissue anterior to the normal location of the gonads. In the absence of any endogenous bithorax-complex gene expression, however, mesoderm expressing gonad markers still condenses preferentially in the posterior segments of the abdomen, even in the absence of pole cells.
Conclusions: The specific requirement for *abd-A* and not *Ubx* in gonad development does not reflect differences in the properties of the proteins that these genes encode, but presumably reflects differences in their regulation. In normal development, the restriction of gonad formation to the posterior abdomen does not depend on the overlap of *abd-A* and *Abd-B* expression, but must depend on the regulation of *abd-A* and *Ubx* in the sub-population of the mesoderm that forms the gonad. Factors other than homeotic gene expression provide some cues that direct gonadal mesoderm to condense in the correct location.

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Background

The *Drosophila* gonad is formed, like that of most other animals, from two distinct cellular components [1,2]. The germ line derives from the pole cells, which migrate from the posterior pole of the egg into the embryo through the wall of the posterior midgut. Here, they associate with cells from the inner layer of the mesoderm which form the somatic component of the gonad [3–5].

In the mature embryo, the gonad lies at the level of abdominal segment 5, but mesoderm from several abdominal segments contributes to its formation — at least from parasegments 10 to 13 [6,7]. The restriction of gonad formation to this part of the embryo coincides with the position where the majority of germ cells leave the hind gut, but the location of germ cells does not define where the gonad forms. The mesodermal component of the gonad forms and differentiates apparently normally in the absence of pole cells [7–9]. Other cues must therefore define which cells of the mesoderm will form gonad. This paper is concerned with the role of the homeotic genes in this process.

Embryos deficient for the bithorax complex (BX-C) do not form gonads [7]. One or more of the homeotic genes within this gene cluster must therefore be essential for some step(s) of gonadogenesis. All three of the BX-C genes are active in the region of the embryo where the gonads form. The *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*) genes are expressed in most abdominal segments from blastoderm stages onwards, although the precise pattern of expression differs somewhat from segment to segment. *Abdominal-B* (*Abd-B*) is initially expressed only in the most posterior region of the embryo (parasegment 13 back), but during stage 11 it becomes active more anteriorly — first in parasegment 12, and then at lower levels in parasegments 11 and 10 (for review see [10]).

Mutant phenotypes suggest that, during normal development, the *abd-A* gene plays the major role in gonad specification — *abd-A*$^-$ null mutants die as embryos that lack gonads [7]. Mutations that specifically affect the upstream *iab-4* regulatory region of the *abd-A* gene allow the development of adult flies, but these lack gonads [11,12]. By contrast, *Ubx*$^-$ null mutants die as embryos with apparently normal gonads (our unpublished observations), and no gonadal defects have been described in adult viable *Ubx* mutants. *Abd-B*$^-$ null mutants form gonads, although these may be somewhat reduced and disordered ([7], but see Discussion). Many adult viable *Abd-B* mutants have abnormal genitalia, so it is not clear whether some *Abd-B* mutations affect gonad function, although those with normal genitalia are fertile. (The genitalia derive from ectodermal tissue in the genital disc, not from the gonad.)

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By making mosaic embryos, Cumberledge *et al.* [12] were able to show that, for gonad development, the *abd-A* gene is required in somatic cells of the gonad, but not in the germ line. The results we report here confirm this by showing that expression of ABD-A protein in the mesoderm alone is sufficient to restore at least the early stages of gonad development in an *abd-A*$^-$ mutant.

One likely role for *abd-A* in gonad formation is to select which segments (parasegments) within the mesoderm are competent to enter the gonad pathway. There is as yet no direct support for this hypothesis, however, for it is not clear whether the *abd-A* gene is expressed differentially in segments that will and will not make gonad, at the time when this choice is made. Up until stage 13, *abd-A* is expressed throughout the mesoderm of abdominal segments 8–12 [13,14]. At this early stage, however, markers for gonadal mesoderm are expressed uniformly throughout the abdomen [6,7] — it is only from stage 12 onwards that the gonadal mesoderm becomes distinct.

Thus, for example, from stage 12 onwards 412 RNA, the marker that we have used here, becomes up-regulated in gonadal mesoderm and down-regulated in the other abdominal segments. Even at stage 13, Cumberledge *et al.* [12] were unable to identify any segmental difference in *abd-A* expression that correlated with gonad formation. Moreover, they could see no change in the expression of *abd-A* in the mesoderm of embryos carrying *iab-4*$^-$ mutations that block gonad formation. If this model is correct, *iab-4*$^-$ mutations must therefore affect *abd-A* expression in a specific subset of mesodermal cells which they were not able to identify.

An alternative possibility is that ABD-A is required for gonad development, but is not itself the factor that defines which segments will contribute cells to the gonadal mesoderm. Consistent with this model, Karch *et al.* [13] report that *iab-4*$^-$ mutations do eliminate ABD-A expression in the 'gonads', though it is not clear exactly which cells they were observing. To discriminate between these hypotheses, we have expressed ABD-A ubiquitously throughout the mesoderm of early embryos, and asked whether this is sufficient to induce the formation of ectopic gonadal tissue.

For many structures in the abdomen, either the *Ubx* or the *abd-A* gene can specify normal development. Thus, the tracheae and peripheral nervous system develop normally, or very nearly so, if either of these genes is active in an abdominal segment, but are severely disrupted if both *Ubx* and *abd-A* are mutant. However, the gonad appears to be one developmental fate that is uniquely specified by *abd-A* and not *Ubx*. To examine whether this difference reflects a difference in the specificity of the UBX and ABD-A proteins, we have expressed UBX (and ABD-B) ectopically in the mesoderm and determined whether either can substitute for ABD-A.

Results

The mesodermal cells that will form the gonad are conveniently marked by the expression of 412 RNA [7]. This transcript derives from a transposable element that is not known to be required for gonad formation, but which in late embryogenesis is expressed specifically in the gonad.

The 412 RNA is expressed in the mesoderm from stage 10 onwards. Initially, it is expressed more or less uniformly from parasegments 2–14 in bilateral stripes along the length of the germ band. These stripes break into parasegmental clusters of cells as the visceral and somatic mesoderm segregate during stage 11 [3]. Levels of 412 transcript decline during stage 12, except in dorso-lateral clusters of cells in parasegments 10–12, where the labelled cells are seen to be surrounding germ cells. These dorso-lateral clusters fuse, firstly to form elongated gonadal primordia and then to form the condensed, almost spherical gonads visible in the unstained embryo [7]. Low levels of 412 RNA persist in the dorso-lateral strand of mesoderm that will not form gonad (these probably form the fat body), but under the conditions of staining used for these experiments, this low level was barely detectable after stage 13 (Fig. 1a). By selecting embryos that are between stages 14–16, 412 RNA can be used as a gonad-specific marker.

Expression of *ABD-A* in the mesoderm rescues gonad formation

Embryos deficient for the *Ubx* and *abd-A* genes of the bithorax complex (*Df(3R)Ubx*^{109} homozygotes) form no gonads. The expression of 412 RNA was initiated normally, but declined in all segments of the mesoderm before stage 14 (Fig. 1b). It behaved similarly in embryos deficient for all three bithorax complex genes ([6] and data not shown).

We have expressed ABD-A throughout the mesoderm of these deficiency embryos, using GAL4-mediated induction that was itself driven by a mesoderm-specific promoter from the *twist* gene [15,16]. This system causes ubiquitous expression in the mesoderm during stages 8–11. Thereafter, levels of induced protein fall, except in a few persistent *twist*-expressing cells which will form the adult muscles. The ectoderm does not express GAL4 (except for a few mesectodermal cells along the midline) and thus no ABD-A is induced in the epidermis that overlies the gonadal mesoderm, or in segmental neurons.

The pattern of 412 RNA expression was altered in embryos ectopically expressing ABD-A protein. Transcript levels did not decline uniformly in the anterior mesoderm, but remained high — comparable to levels in the gonadal mesoderm of normal embryos (Fig. 1c). The 412-expressing cells condensed into lumps connected by strands. The number and position of these condensed lumps varied from embryo to embryo, but frequently included a fragment in the thorax and another

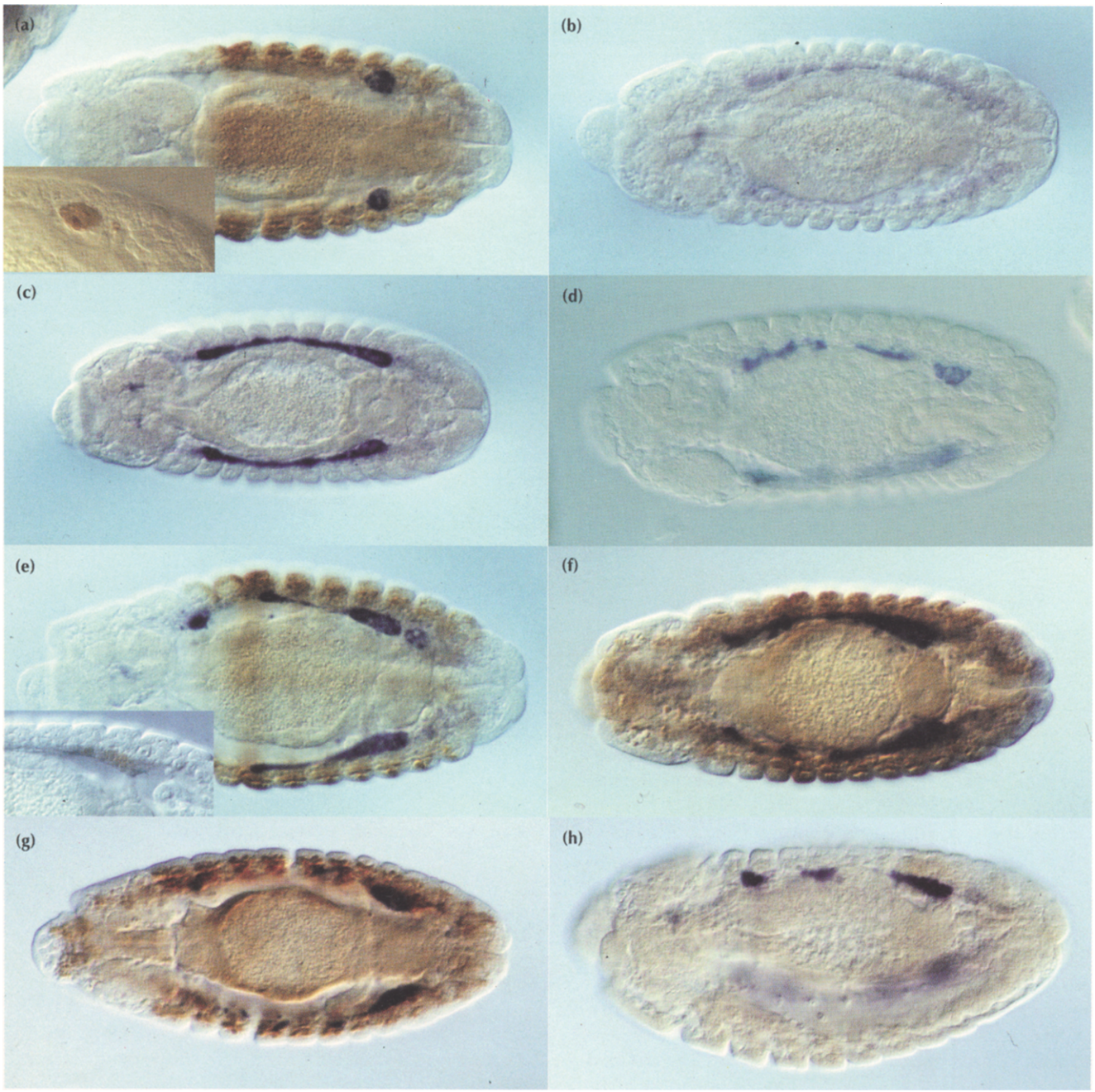

Fig. 1. Formation of gonadal mesoderm by induced homeoprotein expression. All embryos are at stage 14–15 and have been antibody-stained to reveal UBX protein and then subjected to *in situ* hybridization to reveal 412 RNA. **(a)** Wild-type embryo. By this stage, 412 RNA is present at high levels only in the condensed gonadal mesoderm that engulfs the pole cells. Inset: wild-type gonad stained with anti-Vasa antibody, to show the pole cells. **(b)** *Ubx*⁻ *abd-A*⁻ deficiency homozygote. No gonadal mesoderm forms. Traces of 412 RNA persist in all segments of the mesoderm, as they do in the non-gonadal mesoderm of wild-type embryos. **(c)** *Ubx*⁻ *abd-A*⁻ embryo expressing ABD-A ectopically under *twist*-GAL4 induction. High levels of 412 RNA persist in the normal gonadal primordium and more anteriorly. **(d)** *Df(3R)P9* embryo (*Ubx*⁻ *abd-A*⁻ *Abd-B*⁻) expressing ABD-A ectopically under *twist*-GAL4 induction. The effect is indistinguishable from (c), although this embryo is from a different experiment, which was stained less strongly. **(e)** BX-C⁺ embryo expressing ABD-A ectopically under the same conditions as above. Gonadal mesoderm forms ectopically in anterior segments. Inset: similar embryo stained with anti-Vasa antibody, to show the pole cells within the elongated gonadal sheath. **(f)** BX-C⁺ embryo expressing UBX protein ectopically under *twist*-GAL4 regulation. Ectopic gonadal mesoderm was seen in 16/16 embryos. **(g)** *Ubx*⁻ *abd-A*⁻ embryo (*Df(3R)Ubx*^{109}/*Ubx*⁻ *abd-A*⁻ *Abd-B*⁻) expressing UBX protein ectopically as above. This embryo was selected to show the unusually well-formed gonads. More typically, embryos of this genotype resemble those illustrated in (c,d,h). **(h)** Embryo lacking pole cells and deficient for the entire bithorax complex (*Df(3R)P9* homozygote), expressing ABD-A protein ectopically in the mesoderm. Even in this genotype, the 412-expressing mesoderm still condenses preferentially in the correct location.

large fragment in the posterior abdomen, which ensheaths the pole cells. Occasionally, embryos developed a single condensed lump that appeared very like a normal gonad.

Similar ectopic lumps and strands of gonadal tissue were formed when ABD-A protein was expressed ectopically in wild-type embryos (Fig. 1e). Staining such embryos with anti-Vasa antibodies (Vasa protein is a marker for pole-cell development) showed that the pole cells failed to condense normally, remaining distributed within an elongated gonadal sheath, even in stage 16 embryos (Fig. 1e, inset). However, pole cells were not observed in the condensed lumps of gonadal mesoderm that remained in the thoracic region.

We conclude that ABD-A protein expressed in the mesoderm is able to rescue the early stages of gonad formation. Moreover, ectopic expression leads to the formation of ectopic gonadal tissue in anterior segments. These cells behave like normal gonadal mesoderm, in that they condense with one another, but only in the abdominal segments are they colonized by pole cells.

UBX as well as ABD-A protein can induce gonad formation

To determine whether the ability to rescue gonad formation and specify ectopic gonad is unique to the ABD-A protein, we also expressed UBX and ABD-B proteins under the same conditions. UBX protein had very similar effects to those of ABD-A — ubiquitous expression throughout the mesoderm led to the formation of ectopic gonad tissue (Fig. 1f). In *Ubx*$^-$ *abd-A*$^-$ deficiency embryos, UBX protein in the absence of ABD-A could restore the formation of an encapsulated gonad, which contained germ cells (Fig. 1g).

The ectopic expression of ABD-B throughout the mesoderm blocked the formation of the gonad primordium, even in BX-C$^+$ embryos (compare Fig. 2a,b). We conclude that ABD-B cannot substitute for ABD-A in specifying gonadal mesoderm. However, the ability to repress gonad formation in a wild-type background is probably indirect. ABD-B protein represses the *abd-A* gene [13,14], and will therefore block gonad formation. To eliminate this effect, we have co-expressed ABD-A and ABD-B proteins under *twist*-GAL4 induction. Under these conditions, 412 RNA expression was restored (Fig. 2c), suggesting that the presence of ABD-B protein does not act directly to block specification of the gonad mesoderm.

Other factors contributing to the localization of the gonad

In *Ubx*$^-$ *abd-A*$^-$ deficiency homozygotes expressing ectopic UBX or ABD-A proteins, the gonadal mesoderm most frequently condensed in approximately the correct location for the gonad, despite the absence of any normally regulated *Ubx* or *abd-A* function (Fig. 1c,g). This suggests that there is some other signal helping to locate the gonad correctly. The *Abd-B* gene is not providing this function, for the same preference was seen when ABD-A was expressed ectopically in *Df(3R)P9* homozygotes, which lack all BX-C genes (Fig. 1d). Although it is difficult to compare the very variable distributions of gonadal tissue found in these deficiency embryos, we observed no obvious difference between the embryos with and without the *Abd-B* gene.

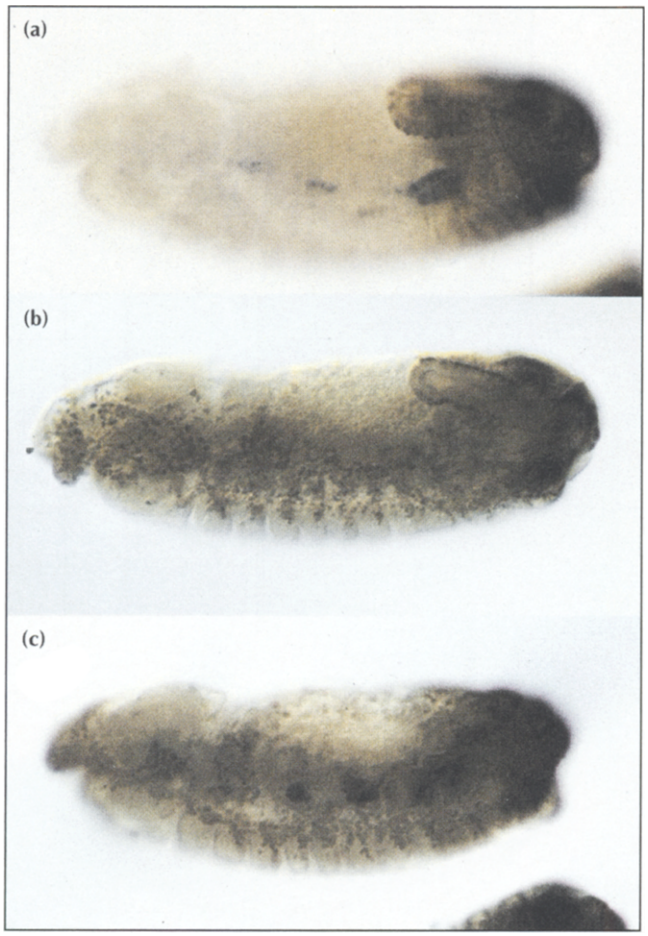

Fig. 2. The repression of gonadal mesoderm by ABD-B protein. Stage 14–15 embryos were stained for ABD-B protein and then subjected to *in situ* hybridization to reveal 412 RNA. **(a)** Embryo expressing ABD-A ectopically in the mesoderm, showing ectopic gonadal mesoderm in anterior segments. **(b)** Embryo expressing ABD-B ectopically in the mesoderm. No gonadal mesoderm is detectable. **(c)** Embryo expressing ABD-B ectopically, and inferred to be also expressing ABD-A (see Materials and methods). Gonadal mesoderm is restored.

Although pole cells are not required for the correct formation of the gonad, we reasoned that, in the absence of localized homeotic gene expression, the pole cells may be providing some signal that helps to position the gonad. To test this possibility, we expressed ABD-A ectopically in the mesoderm of embryos that were simultaneously deficient for the entire bithorax complex and lacking pole cells — *Df(3R)P9* homozygotes, derived from *oskar*^{301} mothers and reared at 25 °C, a temperature at which (in our stocks) abdominal segments are normal but no pole cells are formed ([17] and see Materials and methods). In these embryos, the largest single clumps of 412-expressing mesoderm still formed in the normal location for the gonad (within the 4th–6th abdominal segments) in 20/25

cases scored. Thus, the pole cells do not play a detectable role, even in the absence of homeotic gene expression.

Discussion

In normal development, function of the *abd-A* gene is essential to initiate formation of the gonad primordium. However, ectopic expression of either the UBX or the ABD-A protein is able to rescue the initial stages of gonad development in deficiency embryos. We conclude that the specific requirement for the *abd-A* gene reflects the different regulation of these two genes *in vivo*. The *abd-A* gene must contain a specific enhancer element, in the *iab-4* regulatory region [11], which ensures expression in the correct cells at the time when its function is required. The *Ubx* gene cannot contain an equivalent tissue-specific element, for although *Ubx* is generally derepressed in the abdominal segments of *abd-A*$^-$ mutants, it is unable to substitute for this particular function of the *abd-A* gene.

The primary factor restricting the gonadal mesoderm to parasegments 10–12 appears to be the distribution of ABD-A protein. In the early embryo, ABD-A is ubiquitously expressed in the mesoderm of parasegments 8-12. All segments probably retain the potential to form gonadal mesoderm at this stage. It is not until later in development that different fates are specified within the mesoderm [13]. The expression of *abd-A* clearly persists in segments anterior to parasegment 10 — in the somatic mesoderm, the gut musculature and the heart [13,14]. The restriction of *abd-A* expression must therefore be specific to particular cells within the mesoderm.

It is interesting to note that the restriction of the gonad to the posterior abdominal mesoderm is not characteristic of all insects. In the Hemipteran *Pyrrhocoris apterus*, separate gonadal primordia form in each segment from A2 to A8 ([18], cited in [19]). In the apterygote Thysanura, segmental gonad primordia extend into the thorax [20]. Our experiments in *Drosophila* suggest that these differences may reflect differences in the activity of tissue-specific regulatory elements within the homeotic genes.

The role of *Abd-B* in gonad formation is still not clear. Some gonadal tissue certainly forms in *Abd-B* mutants, though Brookman *et al.* [7] reported that the primordium was reduced and disorganized. No reduction was obvious in our *Abd-B*^{M1}/*Ubx*$^-$ *abd-A*$^-$*Abd-B*$^-$ embryos, which had condensed gonad primordia (data not shown). Brookman *et al.* [7] suggest that the limits of the gonad primordium might be determined by the overlap of *abd-A* and *Abd-B* expression. ABD-B protein is normally detectable in the mesoderm of parasegments 10–13, but not more anteriorly. Thus, it potentially provides a spatial cue in the right location to define the anterior boundary of the gonad. In support of their argument, they observed that ectopic gonad tissue forms anteriorly in *extra sex combs* mutant embryos, a genotype in which *Ubx*, *abd-A* and *Abd-B* are all derepressed. However, our experiments rule out this explanation — ABD-A is able to induce gonadal mesoderm, in the absence of ABD-B. The *Abd-B* gene may play some role in localizing gonad formation by repressing *abd-A* in the most posterior mesoderm.

The later development of the gonad is a complex process involving interactions between germ line and somatic cells, and probably involving many distinct patterning steps. Our experiments do not address what requirement there may be for the function of the homeotic genes during these later stages of gonadogenesis. It is likely that both *abd-A* and *Abd-B* are required for specific steps in gonadal morphogenesis and differentiation.

In a recently published paper [21], Boyle and Dinardo also address some of the issues raised here. They show that heat-shock-induced expression of ABD-A protein will induce ectopic gonadal tissue, that *iab-4* mutants eliminate *abd-A* expression in only a subset of mesodermal cells, and that *Abd-B* is required for the appearance of a distinct subpopulation among the cells of the gonadal mesoderm.

Conclusions

In normal development, the homeotic gene *abd-A* is essential for the development of gonads. The *Ubx* gene, which encodes a similar homeodomain protein, will not substitute, even though it is expressed in the same segments. Our results suggest that the failure of *Ubx* to substitute for *abd-A* in normal development does not depend on differences in the structure of these two proteins, but on differences in their normal regulation within segments.

Ectopic expression of either *Ubx* or *abd-A* causes the formation of gonadal tissue anterior to the normal domain. Taken together with the normal requirement for *abd-A* but not for *Ubx*, this suggests that the spatial regulation of *abd-A* in the mesoderm determines where gonadal tissue is specified in the embryo.

Materials and methods

Fly stocks

The GAL4 and UAS stocks used are described in [16,22]. The lines used were: *P[twist-Gal4]*108.4 (X chromosome); *P[UAS-abd-A]*21.6 (X chromosome); *P[UAS-Ubx]*36.2 (chromosome 3). The *Ubx*$^-$ *abd-A*$^-$ *Abd-B*$^-$ chromosome is described in [23]. Other mutations are described in [24]. All crosses were maintained at 25 °C and reared on cornmeal-agar medium.

Histochemistry

Anti-UBX monoclonal antibody was FP3.38 [25]. Anti-Vasa antibody was a gift from A. Williamson and R. Lehmann (unpublished, see [26]). The 412 probe was prepared from a 412 cDNA clone [7]. For antibody and *in situ* double stainings, embryos were first antibody-stained using a rapid, 6 h protocol developed by L. Martin-Bermudo and based on the ABC system (Vector Labs). Primary and secondary antibody incubations were for only 1 h at room temperature, and the

number and length of the washing steps were reduced compared with standard protocols. After antibody staining, the embryos were hybridized with a digoxigenin-labelled 412 DNA probe, essentially as described [27].

Identification of genotypes

Df(3R)P9 and *Df(3R)Ubx^{109}* homozygotes were identified among the progeny of heterozygous parents by the absence of ectodermal UBX protein. Only one half of these deficiency homozygotes are expected to carry both the *Gal4* and *UAS* transgenes. These were recognized by the ectopic expression of 412 RNA, which is never observed at stage 14 in deficiency homozygotes. Counting populations of embryos confirmed that ectopic expression occurred in approximately 50 % of embryos. For the embryos shown in Figure 1f,g, the genotype was confirmed directly by the presence of ectopic UBX protein in anterior mesoderm. For example, the embryo shown in Figure 1g came from a population in which all (16/16) stage 14–15 embryos with ectopic UBX showed extensive ectopic 412 expression. In the same population, similar embryos without ectopic UBX resembled the embryo shown in Figure 1a (14/15), or showed only a small patch of ectopic 412 expression, immediately adjacent to the normal gonad (1/15).

The penetrance of the *oskar^{301}* phenotype (Fig. 1h) was checked by examining embryos from the same population at blastoderm stage for the absence of pole cells, and by confirming that all adults from the cross were sterile. The embryos shown in Figure 2 were all from the same cross, stained in parallel. Those with ectopic ABD-B protein were identified directly by staining with anti-ABD-B antibody. Half of these will also express ABD-A. We inferred the identity of these from the re-appearance of ectopic 412 expressing mesoderm. In crosses producing 100 % of embryos that express only ABD-B ectopically, no gonadal or ectopic 412 RNA persists.

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