Functional Domains of the Drosophila Bicaudal-D Protein

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ABSTRACT

The localization of oocyte-specific determinants in the form of mRNAs to the pro-oocyte is essential for the establishment of oocyte identity. Localization of the Bicaudal-D (Bic-D) protein to the presumptive oocyte is required for the accumulation of Bic-D and other mRNAs to the pro-oocyte. The Bic-D protein contains four well-defined heptad repeat domains characteristic of intermediate filament proteins, and several of the mutations in Bic-D map to these conserved domains. We have undertaken a structure-function analysis of Bic-D by testing the function of mutant *Bic-D* transgenes (*Bic-D^{H}*) deleted for each of the heptad repeat domains in a *Bic-D* null background. Our transgenic studies indicate that only the C-terminal heptad repeat deletion results in a protein that has lost zygotic and ovarian functions. The three other deletions result in proteins with full zygotic function, but with affected ovarian function. The functional importance of each domain is well correlated with its conservation in evolution. The analysis of females heterozygous for *Bic-D^{H}* and the existing alleles *Bic-D^{PA66}* or *Bic-D^{R26}* reveals that *Bic-D^{R26}* as well as some of *Bic-D^{H}* transgenes have antimorphic effects. The yeast two-hybrid interaction assay shows that Bic-D forms homodimers. Furthermore, we found that Bic-D exists as a multimeric protein complex consisting of Egl and at least two Bic-D monomers.

IN early Drosophila oogenesis, a series of highly controlled divisions initiate the developmental pathway that leads to the formation of an oocyte (Spradling 1993). A cluster of 16 interconnected cystocytes is formed through four successive mitotic divisions of a cystoblast. One cystocyte develops as an oocyte and the other 15 become polyploid nurse cells. Although the mechanisms underlying oocyte determination are unclear, the initial establishment of oocyte identity is dependent on the accumulation of oocyte-specific transcripts and proteins.

The first requirement for *Bicaudal-D* (*Bic-D*) function is in the initial establishment of oocyte identity. In the strong hypomorphic alleles *Bic-D^{R26}* and *Bic-D^{PA66}*, the polarized microtubule network does not reorganize, and oocyte-specific transcripts such as *Bic-D*, *orb*, *osk*, and *fs(1)K10* fail to differentially accumulate in the presumptive oocyte; all 16 cystocytes become polyploid nurse cells. Egg chambers with 16 nurse cells grow to about stage 6 and then degenerate (Ephrussi *et al.* 1991; Suter and Steward 1991; Lantz *et al.* 1992; Theurkauf *et al.* 1993; Ran *et al.* 1994; Mach and Lehmann 1997). Another gene, *egalitarian* (*egl*), has the same loss-of-function phenotype as *Bic-D*; in *egl* ovaries, determinants also fail to localize to the prospective oocyte. The phenotypes of both mutants clearly indicate that the genes are involved in the localization of determinants in the form of mRNA and protein. Bic-D and Egl proteins are found in a complex (Mach and Lehmann 1997) and are likely to function together, but how they do it remains to be elucidated.

Sequence analysis of Bic-D protein reveals that it is similar to the myosin heavy chain tail and to intermediate filament proteins such as lamin and desmin (Suter *et al.* 1989; Wharton and Struhl 1989). These proteins contain domains of extensive heptad repeats in which the hydrophobic residues are preferentially located at positions 1 and 4 (McLachlan and Karn 1983). These heptad repeats usually mediate the packaging of one helix against another, forming coiled-coil structures made up of two or three protein molecules and resulting in homo- or heterodimers, or in multimers. The formation of coiled-coil structures can also result from the folding of one molecule in such a way that two heptad repeat domains come to lie parallel to each other (Cohen and Parry 1986).

Proteins containing coiled-coil domains have many functions and have been identified in all cell compartments. In most of these proteins, the coiled-coil domains are flanked by protein domains that control the protein's distribution or specific function. More than half of the Bic-D protein consists of heptad repeats distributed throughout, and no other protein motifs are apparent. The repeats may be classified into four well-defined regions, each greater than seven heptad repeats. Interestingly, two dominant and two recessive mutations map

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to these heptad domains (Wharton and Struhl 1989; Suter and Steward 1991).

To test the functional importance of the heptad repeat domains in Bic-D, and to further understand its function, we have undertaken a structure–function analysis of the Bic-D protein. We generated mutant Bic-D proteins in which each of the conserved heptad repeat domains was deleted. We assayed the functional importance of each of the four domains by expressing the mutant proteins in transgenic flies lacking endogenous *Bic-D* function. We found that the first and last heptad repeat domains of the Bic-D protein are essential for function, but the two centrally located domains, the second and third heptad repeat domains, are functionally less important or dispensable. Complementation tests reveal that one of the existing hypomorphic alleles, $Bic\text{-}D^{R26}$, and some of the heptad-repeat-deleted transgenes have a strong antimorphic effect. Furthermore, we found that Bic-D can form homodimers and Bic-D protein exists in a heteromeric protein complex. Our results confirm that the N-terminal half of the protein may be required exclusively for function during oogenesis, while the C-terminal domain contains the sequences required for all functional aspects. The functional importance of each domain is well correlated with its conservation during evolution.

MATERIALS AND METHODS

Fly strains: The $Bic\text{-}D^{null}$ alleles ($Bic\text{-}D^{r5}$ and $Bic\text{-}D^{r11}$) are described by Ran *et al.* (1994). The $Bic\text{-}D^{R26}$ allele and *Df(2L) TW119* (*Df119*), which uncovers the *Bic-D* locus, are described by Mohler and Wieschaus (1986). The $Bic\text{-}D^{PA66}$ allele is described by Schüpbach and Wieschaus (1991).

Construction of $Bic\text{-}D^{H}$ mutants: The classification of the four heptad repeat domains was performed by Coils programs (http://www.ch.embnet.org/software/COILS_form.html) and confirmed by visual inspection. The first heptad domain extends from amino acid (aa) 163 to aa 242, the second from aa 327 to aa 382, the third from aa 392 to aa 444, and the fourth from aa 610 to aa 743. All deletion constructs were derived from a 4-kb *Bic-D* genomic promoter fragment fused in the 5′ untranslated region to the full-length *Bic-D* cDNA in Bluescript SK^+ (K. Baksa and R. Steward, unpublished results). To delete each heptad repeat domain, *Not* I sites were created adjacent to the sequences to be deleted by *in vitro* mutagenesis using the Bio-Rad (Hercules, CA) kit (Figure 1B). The mutagenized DNA was digested with *Not* I and religated. The deletions were checked by DNA sequencing. The ~8-kb minigenes were subcloned into the pCaSpER transformation vector and were introduced into the Drosophila genome by *P*-element-mediated transformation (Robertson *et al.* 1988). The transgenic lines are named according to the position of the deletion—$Bic\text{-}D^{H1}$, $Bic\text{-}D^{H2}$, $Bic\text{-}D^{H3}$, and $Bic\text{-}D^{H4}$. $Bic\text{-}D^{H}$ refers to all four transgenes. At least two independently transformed lines for each deletion construct were tested.

To add the triple hemagglutinin (HA) epitope to the wild-type Bic-D protein, a unique *Not* I site was introduced at the C terminus of the *Bic-D* cDNA by insertional mutagenesis. The HA epitope, a 117-bp *Not* I fragment, was ligated into the *Not* I site and cloned into the pCaSpER vector. The same endogenous *Bic-D* promoter that was used for the heptad-repeat-deleted *Bic-D* transgenes was utilized.

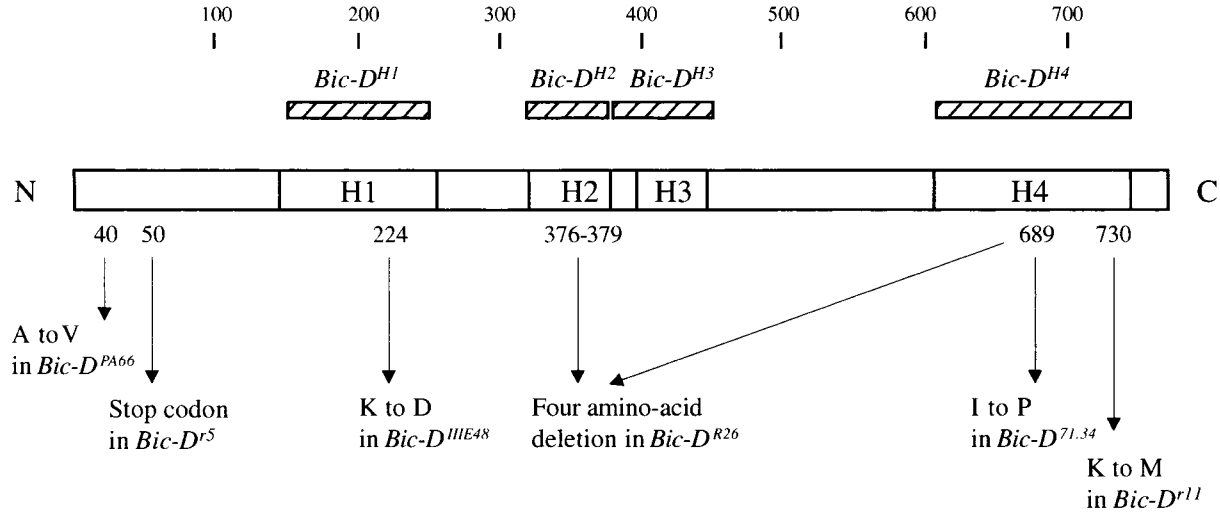
Yeast two-hybrid interaction assay: The full-length *Bic-D* cDNA was inserted in frame into the pAS1-CYH2 vector (containing amino acids 1–147 of Gal 4; GalBD) or the pACTII vector (containing amino acids 768–881; Gal4AD). As host, strain CBY 14 of *Saccharomyces cerevisiae*, carrying *LacZ* (β-galactosidase) and *HIS* (imidazole glycerol phosphate dehydratase) reporter genes, was used. Yeast was transformed by the lithium acetate method of Gietz *et al.* (1992). To ensure expression of fusion protein produced from the vectors, yeast lysates from colonies transformed with the fusion constructs were analyzed by anti-Bic-D Western blotting. For quantitative β-galactosidase assays in solution, three independently derived transformants containing appropriate plasmids were selected and grown in selective medium overnight at 30° in a shaking incubator. β-Galactosidase assays were carried out using *O*-nitrophenyl-β-d-galactopyranoside as a substrate according to the protocols provided by Clontech (Palo Alto, CA). The β-galactosidase activity (reported as Miller units) was determined by calculating the mean from three independently isolated transformants.

Germline clones: $Bic\text{-}D^{H4}$ was introduced into the $Bic\text{-}D^{null}$ FRT40A background. The $Bic\text{-}D^{null}$ FRT40A; *P[*$Bic\text{-}D^{H4}$*]* females were crossed with *hsFLP*;ovo^{D} FRT40A; *P[*$Bic\text{-}D^{H4}$*]* males. As a negative control, $Bic\text{-}D^{null}$ FRT40A females not carrying *P[*$Bic\text{-}D^{H4}$*]* were used. The progeny were collected for 24 hr and then heat shocked twice for 2 hr at 37° over a period of 2 days. $Bic\text{-}D^{null}$ FRT40A/ovo^{D} FRT40A; *P[*$Bic\text{-}D^{H4}$*]*/*P[*$Bic\text{-}D^{H4}$*]*, $Bic\text{-}D^{null}$ FRT40A/ovo^{D} FRT40A; *P[*$Bic\text{-}D^{H4}$*]* or $Bic\text{-}D^{null}$ FRT40A/ovo^{D} FRT40A virgins were selected, crossed to wild-type males, and allowed to lay eggs for 5 days. Neither genotype laid any eggs and the morphology of ovaries was indistinguishable. For the analysis of germline clones in a wild-type background, the $Bic\text{-}D^{null}$ FRT40A; *P[*$Bic\text{-}D^{H4}$*]* females were crossed with *hsFLP*; *P[arm-LacZ]* FRT40A males.

Immunoprecipitation: For each immunoprecipitation, 80 pairs of ovaries of appropriate genotypes were dissected in 1× PBS and homogenized with a Teflon pestle in 200 μl of extraction buffer [20 mm Hepes (pH 7.6), 300 mm NaCl, 1 mm EDTA, 0.5% Triton-X 100] supplemented with protease inhibitors. The homogenized ovary extracts were centrifuged at 13,000 rpm after incubation on ice for 30 min. The pellet was discarded, and the supernatant was recentrifuged two times. The resulting supernatant was incubated with 30 μl of a 1:1 suspension of protein A-Sepharose beads (Pharmacia, Piscataway, NJ) on a rotating wheel at 4° for 1 hr as a preclearing step. The beads were spun down and discarded. The precleared supernatant was incubated with 20 μl of anti-HA antibodies 12CA5, or 40 μl of a 1:1 mixture of hybridoma supernatants anti-Bic-D 1B11 and 4C2 antibodies, or 10 μl of rabbit anti-Egl antibodies (a kind gift from Ruth Lehmann), or 10 μl of monoclonal anti-α-tubulin antibodies (Sigma, St. Louis) on a rotating wheel at 4° for 1 hr, followed by the addition of 10 μl of a 1:1 suspension of protein A-Sepharose (Pharmacia) and incubation for 1 hr. The beads were washed three times with extraction buffer for 15 min, washed two times with HNET wash buffer [50 mm Hepes (pH 7.6), 250 mm NaCl, 5 mm EDTA, 0.1% Triton-X 100], washed once in HNE wash buffer [10 mm Hepes (pH 7.6), 150 mm NaCl, 1 mm EDTA), and resuspended in 40 μl of 2× SDS loading buffer.

Densitometric analyses: Densitometry of chemiluminographs of immunoblots was performed with a laser scanning chromosome 3 densitometer (Joice Loebl). For quantitative densitometric analysis, 2 μl of ovary extracts was subjected to immunoblotting using anti-Bic-D as the primary antibody and sheep anti-mouse horseradish-peroxidase-conjugated antibody as the secondary antibody. Bic-D proteins were detected using a chemiluminescence detection system (Pierce Chemical, Rockford, IL). The relative levels of $Bic\text{-}D^{HA40}$, $Bic\text{-}D^{H1}$,

A



B

Name of transgene	Deleted heptad repeat domain	Homology to a human Bic-D protein	The size of deletion
Bic-D^{H1}	H1	80%	90 amino acids (158-247)
Bic-D^{H2}	H2	62.5%	65 amino acids (317-381)
Bic-D^{H3}	H3	49%	70 amino acids (381-450)
Bic-D^{H4}	H4	89%	133 amino acids (613-745)

Figure 1.—Molecular characterization of the heptad-repeat-deleted Bic-D proteins Bic-D^{H1}, Bic-D^{H2}, Bic-D^{H3}, and Bic-D^{H4}. (A) Schematic representation of the Bic-D protein showing the location of the heptad repeat domains and the position of *Bic-D* mutations. The hatched rectangles indicate the extent of the deletions. *Bic-D*^{R26} is a revertant of the *Bic-D*$^{71.34}$ allele and contains two molecular lesions. Both *Bic-D*^{r5} and *Bic-D*^{r11} are null alleles, and both contain the *Bic-D*^{PA66} mutation because they are revertants of the *Bic-D*^{PA66} allele (Ran *et al.* 1994). (B) Deleted *Bic-D* transgenes and the size of deletions. The homology between each domain in flies and humans is also shown.

and Bic-D^{H4} were determined by comparing the densitometric values obtained for Bic-D from one copy of a transgene to the densitometric value obtained for one copy of endogenous Bic-D gene in the same lane. The ratio of the densitometric values obtained for transgenic Bic-D to the densitometric values obtained for endogenous Bic-D remained constant, with minor standard deviation when serially diluted ovary extracts were analyzed (data not shown), indicating that the densitometric values for transgenic Bic-D proteins reflect differences in the levels of these proteins. Data from four independent experiments were used to determine the relative transgenic Bic-D protein levels.

RESULTS

The molecular characteristics of heptad-repeat-deleted Bic-D proteins Bic-D^{H1}, Bic-D^{H2}, Bic-D^{H3}, and Bic-D^{H4}: A schematic representation of the Bic-D protein and the location of the heptad repeats, as well as existing mutations, are shown in Figure 1A. To investigate the functional importance of the four distinct heptad domains, each domain was deleted by *in vitro* mutagenesis (for details see materials and methods). The extent of the deletions is shown in Figure 1B with the nomenclature of the transgenic deletion lines. The deleted *Bic-D* genes were expressed under the control of an endogenous genomic *Bic-D* promoter. At least two lines for each deletion were established.

The protein product of each of the deleted transgenes was determined by Western blot of ovary extracts from heterozygous females carrying one copy of wild-type *Bic-D* and one copy of a transgene. Figure 4A shows a representative Western blot of extracts from wild type (lane 1) and one line of each transgene (lanes 2–5). Despite the large size of the deletions, all four proteins were expressed and the migration of each protein agrees well with the size of the deletion.

The first and last heptad repeat domains are essential for *Bic-D* function: Hypomorphic *Bic-D* alleles or germline clones of null alleles are female sterile, and homozygous *Bic-D* null mutants die as late pupae or soon after eclosion (Ran *et al.* 1994). To investigate the function of our deletion mutants, we first examined whether they can rescue the zygotic lethality of a *Bic-D*^{null} mutation.

TABLE 1

Rescue of zygotic lethality and female sterility by the *Bic-D^{H}* transgenes

Genotype	Rescue of zygotic lethality (%)	Rescue of female sterility	Fertility[a] (%)	Ventralized eggshells (%)	Level of protein expression
Bic-D^{H1}	Yes (85)	No	<1	100	Intermediate
Bic-D^{H2} [b]	Yes (95)	Yes	20	21	Similar to endogenous level
Bic-D^{H3}	Yes (91)	Yes	99	1	Similar to endogenous level
Bic-D^{H4}	No	No	NA	NA	Low

These results were obtained from at least two independent lines for each transgene. NA, not available.

[a] Fertility is defined as the total number of eggs laid by females hemizygous for each transgene divided by the total number of eggs laid by control sibling females.

[b] In *Bic-D^{H2}*, although the majority of *Bic-D^{H2}* females show ~20% fertility and 21% of eggs with ventralized eggshells, the percentage of fertility and the occurrence of ventralized eggs of an individual female in each line can vary between 5 and 80%. The percentage shown here is the average of 20 females.

Each transgene was crossed into a *Bic-D^{r5}/Df119* background. *Bic-D^{H1}*, *Bic-D^{H2}*, and *Bic-D^{H3}* rescue viability at rates ranging from 85 to 95%, but *Bic-D^{H4}* fails to rescue the zygotic lethality (Table 1), indicating that only the fourth heptad repeat domain is required for the zygotic function of *Bic-D*.

We also examined whether the deleted proteins can rescue the female-sterile phenotype of a *Bic-D^{null}* mutation. In *Bic-D^{r5}/Df119* egg chambers, oocyte differentiation fails to occur and all 16 cystocytes in the egg chamber become polyploid nurse cells (Ran *et al.* 1994). *Bic-D^{H1}* fails to complement because *Bic-D^{H1}* (*P[Bic-D^{H1}]*; *Bic-D^{r5}/Df119*) females show a similar phenotype as *Bic-D^{null}* females, although an egg is produced very occasionally (one over the entire life span of 10 females). These rare eggs show strongly ventralized chorion phenotypes and do not develop (Table 1, Figure 2D).

Bic-D^{H2} displays some complementation; the fertility of the females is ~20% that of wild type (Table 1). A total of 21% of eggs laid by *Bic-D^{H2}* (*P[Bic-D^{H2}]*; *Bic-D^{r5}/Df119*) females display a variety of ventralized chorion phenotypes ranging from partially fused appendages to no appendages. In rare cases, small eggs, collapsed eggs, and eggs with an open chorion are observed (Table 1, Figure 2). Abnormal and strongly ventralized eggs are not fertilized, but the remaining eggs develop normally.

Bic-D^{H3} complements strongly because *Bic-D^{H3}* (*P[Bic-D^{H3}]*; *Bic-D^{r5}/Df119*) females display almost wild-type fertility, but ~1% of the eggs show a weakly ventralized dorsal appendage phenotype (Tables 1 and 3). The rescue of zygotic lethality as well as sterility indicates that Bic-D^{H3}, despite missing 70 amino acids, surprisingly retains nearly full function.

The function of *Bic-D^{H4}* during oogenesis was analyzed in two ways. First, the *Bic-D^{H4}* transgene was crossed with the two hypomorphic alleles *Bic-D^{PA66}* or *Bic-D^{R26}*. These alleles are viable, but produce a strong female-sterile, 16-nurse-cell phenotype (Suter and Steward 1991). One or even two copies of *Bic-D^{H4}* did not improve the phenotype of hemizygous females of both genotypes. These results support that *Bic-D^{H4}* also loses its ovarian function.

Second, we tested if *Bic-D^{H4}* could rescue the phenotype of homozygous *Bic-D^{null}* germline clones. If Bic-D^{H4} protein provides ovarian function, the germline clone of homozygous *Bic-D^{null}* alleles should improve oocyte development and possibly result in the formation of oocytes or mature eggs in the *ovo^{D}* background. We

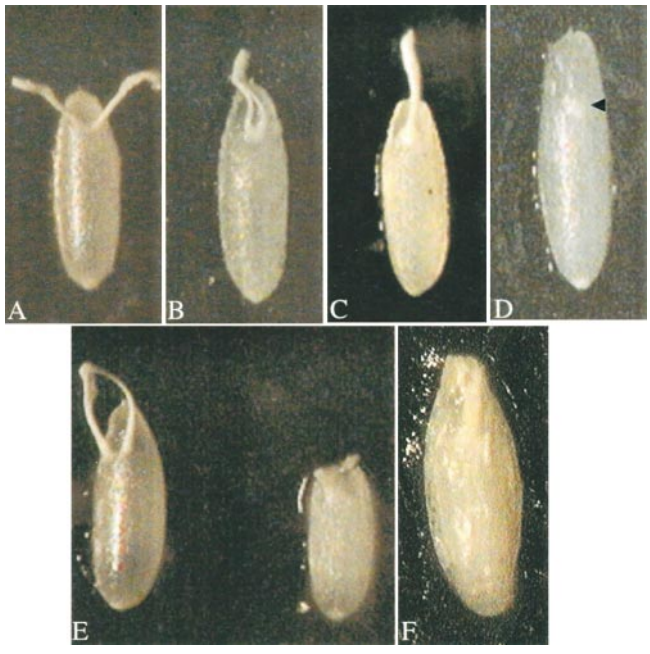


Figure 2.—Ventralized eggshells observed in *Bic-D^{H}* ovaries. (A) Wild-type egg showing normal eggshell with two dorsal appendages. (B) Weakly ventralized eggshell phenotype with partially fused appendages. (C) Intermediate eggshell phenotype with single appendage. (D) Strong eggshell phenotype with remnants of appendage material (arrowhead). (E) Normal egg and small egg with abnormal appendages (right side). (F) Collapsed egg with single appendage.

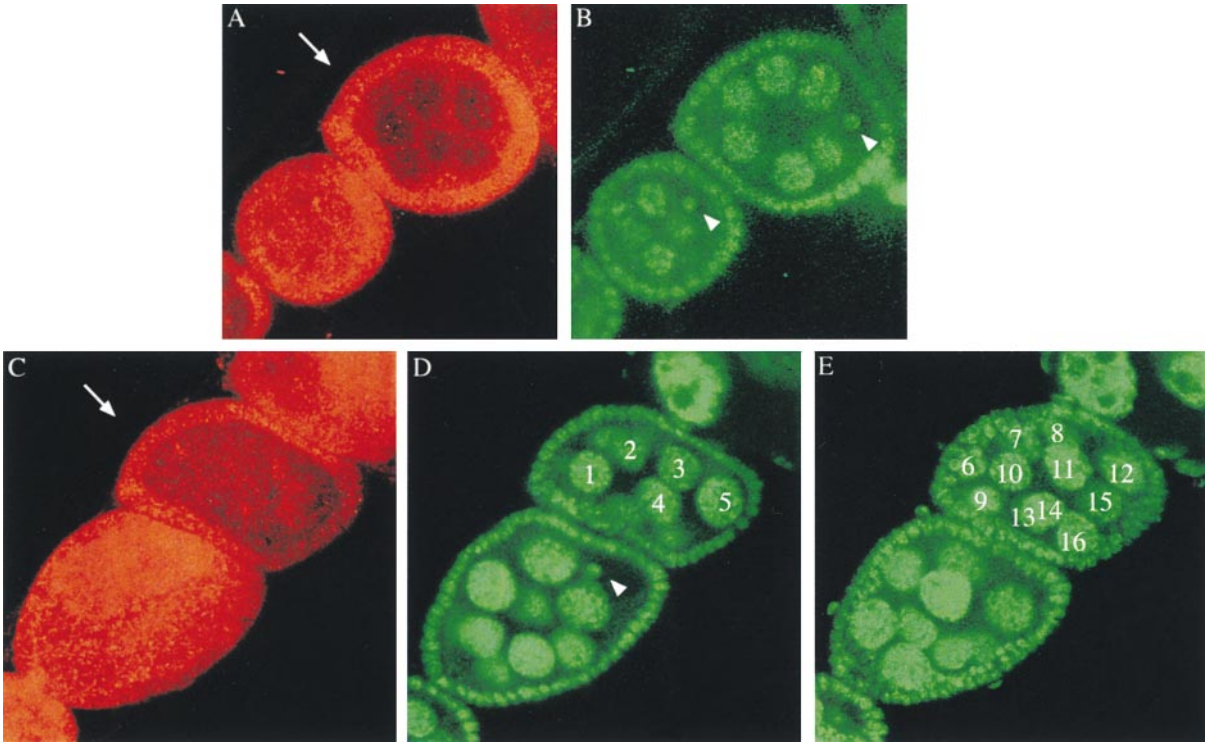


Figure 3.—Homozygous *Bic-D*^{null} clone in *Bic-D*^{H4} background fails to differentiate an oocyte. The ovaries were double stained for LacZ protein (A and C) and for DNA (B, D, and E). (A and B) Mosaic wild-type control ovariole. Control wild-type egg chamber lacking LacZ staining (arrow) contains one diploid oocyte and 15 polyploid nurse cells. The arrowhead points to the diploid oocyte nucleus, which is seen as a compact, fluorescent dot by DNA staining. (C–E) Mosaic mutant ovariole. *Bic-D*^{null} germline clone in *Bic-D*^{H4} background identified by the lack of LacZ staining (arrow) contains 16 polyploid nurse cells, indicating that *Bic-D*^{H4} fails to provide ovarian function. (D and E) Different optical sections of germline clone shown in C reveal that all 16 germline nuclei are polyploid.

generated mosaic clones homozygous for the *Bic-D*^{null} allele in the presence or absence of a *Bic-D*^{H4} transgene, using the *ovo*^{D}/FRT technique (Chou *et al.* 1993; Chou and Perrimon 1996). Both mosaic egg chambers failed to produce mature eggs, and the dissected ovaries of both genotypes were indistinguishable.

To clearly identify mutant egg chambers, we induced germline clones in a wild-type background, using *arm-lacZ* as a marker. Mosaic ovaries were double stained with anti-β-galactosidase antibodies, identifying germline clones by the absence of LacZ staining, and the DNA stain Yo-Pro, allowing the examination of the ploidy of nuclei in mutant egg chambers. Control egg chambers (total >80) of the genotype (*hsFLP*; + FRT40A/*arm-LacZ* FRT40A) always contained 15 polyploid nurse cells and 1 diploid oocyte (Figure 3, A and B). However, *Bic-D*^{null} germline clones (total >80) in the presence of one or two copies of *Bic-D*^{H4} showed no difference in phenotypes when compared to *Bic-D*^{null} germline clones (>70) in the absence of *Bic-D*^{H4} (Figure 3, C–E). The failure to complement *Bic-D*^{PA66} and *Bic-D*^{R26}, as well as the analysis of *Bic-D*^{null} egg chambers in the presence or absence of *Bic-D*^{H4}, show that Bic-D^{H4} protein does not retain any ovarian function.

Bic-D protein levels and function: Western blot analysis shows that protein levels of *Bic-D*^{H2} and *Bic-D*^{H3} are similar to those expressed by one copy of the endogenous gene, but the level is decreased in *Bic-D*^{H1} and more severely decreased in *Bic-D*^{H4} (Figure 4A). Because the expression levels of *Bic-D*^{H1} and *Bic-D*^{H4} are significantly lower than that of the endogenous *Bic-D*, the loss of function of *Bic-D*^{H1} and *Bic-D*^{H4} could be caused by either the functional importance of the deleted heptad domains or the lower levels of the deleted proteins. To distinguish between these possibilities, we needed to determine the lowest level of Bic-D that supports normal function in the zygote and the ovary. We took advantage of a hemagglutinin (HA)-tagged wild-type transgene (*Bic-D*^{HA40}; see materials and methods) that produced low levels of protein but rescued both the zygotic and ovarian phentoype.

We performed Western blots of ovaries from females heterozygous for *Df119*, which uncovers the *Bic-D* locus, carrying one copy of a transgene (Figure 4B). The relative levels of Bic-D^{HA40}, Bic-D^{H1}, and Bic-D^{H4} were determined by comparing the densitometric values obtained for Bic-D expressed by the transgene to the values obtained for the endogenous Bic-D. Densitometric analysis showed that the amount of Bic-D^{HA40} expressed by one copy of a transgene was ~12% of wild-type Bic-D protein

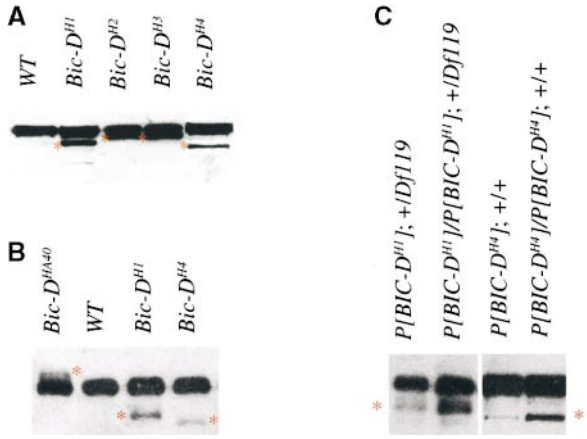


Figure 4.—Relative levels of Bic-D^{HA40}, Bic-D^{H1}, and Bic-D^{H4} proteins. (A) Western blot of the heptad-repeat-deleted Bic-D proteins. *Bic-D* mutant ovaries were derived from females carrying one copy of endogenous wild-type *Bic-D* and one copy of a transgene. The immunoblot was probed with a monoclonal anti-Bic-D antibody. Lower bands are observed only in the deletion mutants, and the shift in migration correlates with the size of the deletion. (B) Transgenic ovaries were derived from females carrying one copy of endogenous wild-type *Bic-D* and one copy of a transgene. Relative protein levels were determined by scanning the immunoblot with a laser scanning densitometer (see materials and methods). (C) Doubling of a transgene resulted in an approximately twofold increase in abundance of the deleted proteins, determined by densitometric analyses. Lane 1, ovary extracts from females carrying one copy of *Bic-D^{H1}* and one copy of endogenous *Bic-D*; lane 2, ovary extracts from females carrying two copies of *Bic-D^{H1}* and one copy of endogenous *Bic-D*; lane3, ovary extracts from females carrying one copy of *Bic-D^{H4}* and two copies of endogenous *Bic-D*; lane 4, ovary extracts from females carrying two copies of *Bic-D^{H4}* transgene and two copies of endogenous *Bic-D*.

expressed by one copy of endogenous *Bic-D*. The amount of Bic-D^{H1} was 14% and the amount of Bic-D^{H4} was 9% of wild-type Bic-D protein. Two copies of *Bic-D^{H1}* or *Bic-D^{H4}* resulted in an approximately twofold increase in abundance of the deleted proteins, as determined by densitometric analyses (Figure 4C).

Two copies of *Bic-D^{H4}* did not rescue the zygotic and ovarian *Bic-D^{null}* phenotype, and two copies of *Bic-D^{H1}* failed to rescue the ovarian-null phenotype. Because two copies of these transgenes produce significantly more protein than one copy of *Bic-D^{HA40}* that has wild-type function, the loss of function of *Bic-D^{H1}* or *Bic-D^{H4}* is most likely caused by the deletion of an essential domain, not by insufficient levels of proteins.

***Bic-D^{R26}* acts as an antimorph:** The rescue of female sterility of a *Bic-D^{null}* mutation by *Bic-D^{H2}* and *Bic-D^{H3}* indicates that these transgenes retain some function. To investigate if they show intragenic complementation, we examined the interactions between *Bic-D^{H2}*, *Bic-D^{H3}*, *Bic-D^{PA66}*, and *Bic-D^{R26}*.

Both the fertility and the frequency of ventralized eggs were the same in heteroallelic *Bic-D^{H2}/Bic-D^{PA66}* (*P[Bic-D^{H2}]*; *Bic-D^{PA66}/Df119*) females as in hemizygous *Bic-D^{H2}* (*P[Bic-D^{H2}]*; *Bic-D^{r5}/Df119*) females. However, when *Bic-D^{R26}* was introduced into the *Bic-D^{H2}* background, strong enhancement of the *Bic-D^{H2}* phenotype was observed (Figure 5A). First, the heteroallelic *Bic-D^{H2}/Bic-D^{R26}* (*P[Bic-D^{H2}]*; *Bic-D^{R26}/Df119*) females rarely lay eggs, while the hemizygous *Bic-D^{H2}* females lay 20% of expected eggs. Second, the few eggs laid are virtually all strongly ventralized in that the eggs have either a single appendage or remnants of appendage materials, while the eggs laid by hemizygous *Bic-D^{H2}* females range from wild type to strongly ventralized, with ~20% showing a ventralized phenotype (Figure 2). Third, the occurrence of abnormal eggs, such as collapsed eggs, small eggs, and eggs with open chorion, is increased. In *Bic-D^{H2}*, abnormal eggs are observed at a low frequency (2%), but in *Bic-D^{H2}/Bic-D^{R26}*, abnormal eggs can reach up to 20% of eggs laid (data not shown).

We also examined interaction of *Bic-D^{H3}* with *Bic-D^{PA66}* and *Bic-D^{R26}* (Figure 5B, Table 2). The heteroallelic *Bic-D^{H3}/Bic-D^{PA66}*(*P[Bic-D^{H3}]*; *Bic-D^{PA66}/Df119*) females exhibit similar fertility and eggshell phenotype as hemizygous *Bic-D^{H3}* (*P[Bic-D^{H3}]*; *Bic-D^{r5}/Df119*). The *Bic-D^{H3}/Bic-D^{R26}* (*P[Bic-D^{H3}]*; *Bic-D^{R26}/Df119*) females display similar fertility as *Bic-D^{H3}* hemizygotes. However, the frequency of ventralized eggs is significantly increased in the *Bic-D^{H3}/Bic-D^{R26}* females, although the degree of ventralization remains weak: almost 100% of the eggs from transheterozygous females are ventralized, while only ~1% of the eggs laid by the *Bic-D^{H3}* females display ventralized eggshells.

The observed strong phenotypic enhancement seen in the *Bic-D^{H2}/Bic-D^{R26}* and *Bic-D^{H3}/Bic-D^{R26}* females suggests that Bic-D^{R26} is antimorphic because it interferes with the function of Bic-D^{H2} and Bic-D^{H3}. If this is true, females carrying two copies of *Bic-D^{R26}* in the presence of one copy of *Bic-D^{H3}* should show a more severe phenotype. As shown in Table 2, more severe phenotypes are indeed observed. First, the fertility of such females was decreased to ~19%, compared to 98% observed in females carrying one copy of each allele. Also, the occurrence of abnormal eggs, such as collapsed eggs and small eggs, is increased. For example, the frequency of collapsed eggs is up from 2% in the *Bic-D^{H3}/Bic-D^{R26}* females to 43% in females carrying two copies of *Bic-D^{R26}* and one copy of *Bic-D^{H3}*.

The antimorphic effect of *Bic-D^{R26}* in combination with *Bic-D^{H2}* and *Bic-D^{H3}* could be due to a specific interaction between the deleted proteins and Bic-D^{R26} because *Bic-D^{R26}/+* females do not show a female-sterile phenotype. Alternatively, Bic-D^{R26} could also interact with wild-type Bic-D, with one copy of wild-type *Bic-D* producing protein levels high enough to suppress the antimorphic effect of Bic-D^{R26}. To address this issue, we investigated whether Bic-D^{R26} shows an antimorphic effect in the presence of low levels of wild-type protein. We introduced one copy of *Bic-D^{R26}* into *Bic-D^{null}* females carrying one copy of *Bic-D^{HA40}* (*P[Bic-D^{HA40}]*; *Bic-D^{r5}/Df119*) that behave like wild type despite a low level of *Bic-D^{HA40}*

expression. *Bic-D*^{R26}/*Bic-D*^{HA40} females lay normal numbers of eggs, but virtually all eggs are ventralized, which is similar to eggs laid by *Bic-D*^{H3}/*Bic-D*^{R26} females (Figure 5, B and C). These results suggest that Bic-D^{R26} has a general antimorphic effect.

Interactions among the *Bic-D*^{H} transgenes: The antimorphic effect of *Bic-D*^{R26} suggests that some of the heptad-repeat-deleted transgenes may also act in a similar fashion. Therefore, we tested for complementation between the heptad-repeat-deleted *Bic-D* transgenes and found that *Bic-D*^{H1} and *Bic-D*^{H4} have antimorphic effects.

Bic-D^{H1} enhances the phenotype of *Bic-D*^{H2} and *Bic-D*^{H3} females (Table 3). The *Bic-D*^{H1}/*Bic-D*^{H2} females lay fewer eggs than hemizygous *Bic-D*^{H2} females, and all eggs are ventralized. The degree of ventralization becomes more severe: 55% of ventralized eggs laid by *Bic-D*^{H1}/*Bic-D*^{H2} females display a strong eggshell phenotype, while only 19% of ventralized eggs laid by *Bic-D*^{H2} females exhibit a strong eggshell phenotype. The *Bic-D*^{H1}/*Bic-D*^{H3} females lay fewer eggs (69% of expected) than *Bic-D*^{H3} females (97% of expected), and the frequency of ventralized eggs is increased as the phenotype becomes stronger.

Like *Bic-D*^{H1}, *Bic-D*^{H4} also produces antimorphic effects in hemizygous *Bic-D*^{H2} or *Bic-D*^{H3} backgrounds (Table 4). For example, the *Bic-D*^{H4}/*Bic-D*^{H2} females do not lay eggs, while hemizygous *Bic-D*^{H2} females lay 27% of expected eggs. The *Bic-D*^{H4}/*Bic-D*^{H3} females lay fewer eggs (18% of expected) than hemizygous *Bic-D*^{H3} females.

Bic-D exists in a multimeric protein complex: Given the presence of extensive heptad repeat domains in Bic-D, which usually serve as protein-protein interaction domains, as well as the antimorphic effect of Bic-D^{R26}, Bic-D^{H1}, and Bic-D^{H4}, we postulated that Bic-D may exist in a multimeric protein complex. To test this possibility, we performed coimmunoprecipitation experiments of ovary extracts containing both wild-type and HA-tagged Bic-D proteins. Ovary extracts of flies containing one copy of endogenous *Bic-D* and one copy of HA-tagged *Bic-D* were immunoprecipitated with anti-HA antibodies. Immunoprecipitated proteins were analyzed by Western blot and probed with anti-Bic-D antibodies. The extracts from the transgenic flies revealed two bands, one of the size expected for Bic-D, and the other, a novel band just above the endogenous Bic-D, corresponding to HA-tagged Bic-D (Figure 6, lane 1). Control wild-type extracts without a transgene contained only

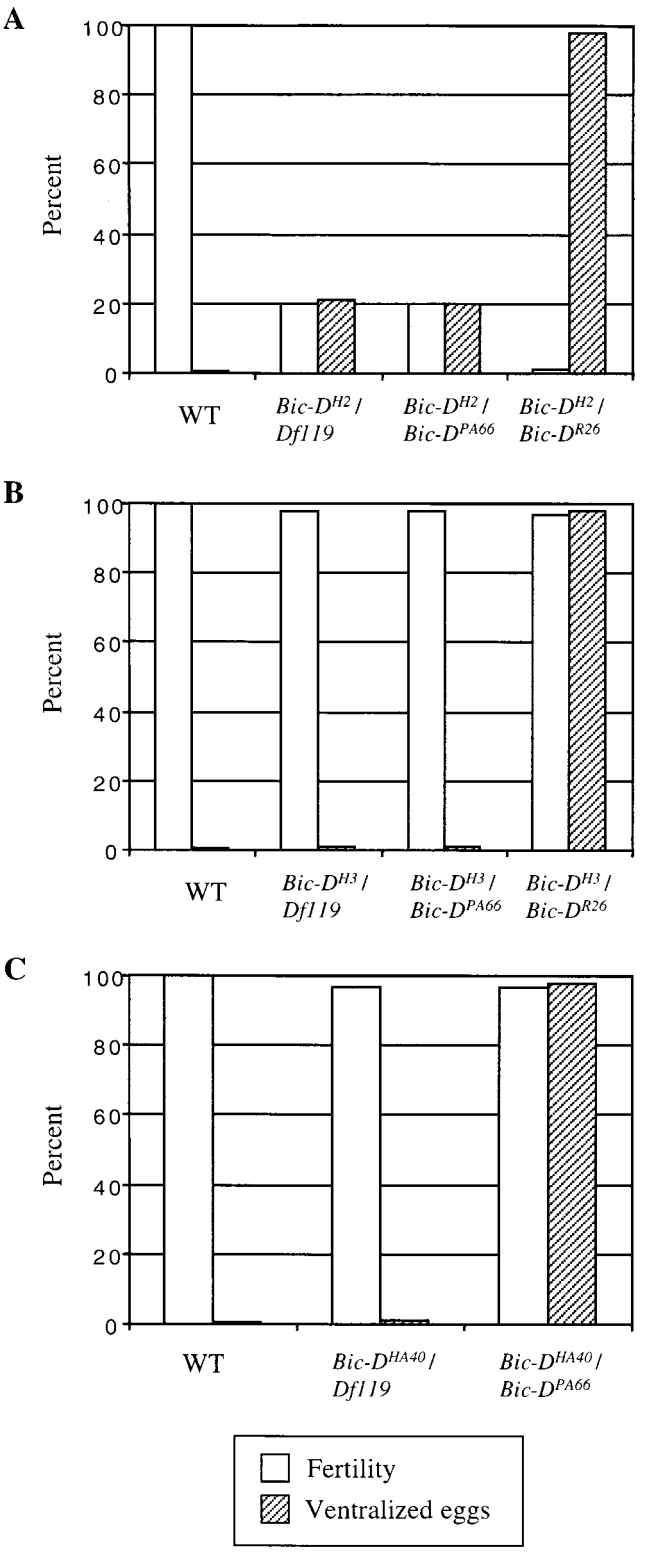


Figure 5.—*Bic-D*^{R26} acts as an antimorph. (A) The interaction of *Bic-D*^{H2} with *Bic-D*^{PA66} and *Bic-D*^{R26}. The fertility and the frequency of ventralized eggs of heteroallelic *Bic-D*^{H2}/*Bic-D*^{PA66} (*P[Bic-D*^{H2}*]*; *Bic-D*^{PA66}/*Df119*) females are similar to those of the hemizygous *Bic-D*^{H2} (*P[Bic-D*^{H2}*]*; *BicD*^{r5}/*Df119*) females. However, the heteroallelic *Bic-D*^{H2}/*Bic-D*^{R26} (*P[Bic-D*^{H2}*]*; *Bic-D*^{R26}/*Df119*) females lay few eggs (~1% of expected), and all eggs display ventralized eggshells. (B) The allelic interaction of *Bic-D*^{H3} with *Bic-D*^{PA66} and *Bic-D*^{R26}. In contrast to the hemizygous *Bic-D*^{H3} (*P[Bic-D*^{H3}*]*; *Bic-D*^{r5}/*Df119*) females and heteroallelic *Bic-D*^{H3}/*Bic-D*^{PA66} (*P[Bic-D*^{H3}*]*; *Bic-D*^{PA66}/*Df119*) females, almost 100% of eggs laid by the heteroallelic *BicD*^{H3}/*Bic-D*^{R26} (*P[Bic-D*^{H3}*]*; *Bic-D*^{R26}/*Df119*) females are ventralized. (C) Almost 100% of eggs laid by hemizygous *Bic-D*^{HA40} (*P[Bic-D*^{HA40}*]*; *Bic-D*^{r5}/*Df119*) display wild-type appendages. However, most eggs laid by heteroallelic*Bic-D*^{HA40}/*Bic-D*^{R26} (*P[Bic-D*^{HA40}*]*; *Bic-D*^{R26}/*Df119)* females are ventralized. The results are based on the analysis of 20 females.

TABLE 2

Frequency of ventralized or abnormal eggs laid by heteroallelic combinations of *Bic-D*^{H3} and *Bic-D*^{R26}

Genotype[a]	Total number of eggs (% of expected)[b]	Ventralized egg shells (%)	Collapsed eggs (%)	Eggs with open chorion (%)	Small eggs (%)
Bic-D^{H3}	1658 (99)	<1	<1	<1	<1
Bic-D^{H3} / *Bic-D*^{R26}	1138 (98)	98	2	2	
Bic-D^{H3} / *Bic-D*^{R26} / *Bic-D*^{R26}	356 (19)	95[c]	43	2	5

[a] The genotype of *Bic-D*^{H3} females is *P[Bic-D*^{H3}*]*; *Bic-D*^{r5} / *Df119*. The genotype of *Bic-D*^{H3} / *Bic-D*^{R26} females is *P[Bic-D*^{H3}*]*; *Bic-D*^{R26} / *Df119*. The genotype of *Bic-D*^{H3} / *Bic-D*^{R26} / *Bic-D*^{R26} females is *P[Bic-D*^{H3}*]*; *Bic-D*^{R26} / *Bic-D*^{R26}.

[b] The total number of eggs laid by females of the genotype divided by the total number of eggs laid by control sibling female flies.

[c] Higher frequency of collapsed eggs hampers the precise determination of the frequency of ventralized eggs. The majority of abnormal eggs, such as collapsed and small eggs, display ventralized eggshell phenotypes.

the Bic-D band (Figure 6, lane 2). Reprobing of the same immunoblot with anti-HA antibodies detected only the novel band in the transgenic extracts and did not show any band in the wild-type extracts (Figure 6, lanes 1′ and 2′). These results show that the anti-HA antibody is capable of immunoprecipitating the tagged Bic-D as well as the endogenous Bic-D, indicating that Bic-D forms a multimeric complex.

The ability of Bic-D to form a multimeric complex is not affected in Bic-D^{R26}. Anti-HA antibodies precipitate both HA-tagged Bic-D and Bic-D^{R26} proteins in ovary extracts from *HA-Bic-D* / *Bic-D*^{R26} females (Figure 7A, lane 5). Bic-D and Bic-D^{R26} have been shown to be coimmunoprecipitated with anti-Egl antibodies (Mach and Lehmann 1997). Consistent with this, we found that Egl is also present in immunoprecipitates of anti-HA antibodies (Figure 7B, lanes 4 and 5). These results suggest that the Bic-D / Egl complex contains at least two Bic-D monomers.

Our genetic and immunoprecipitation results suggest that Bic-D exists *in vivo* as a heteromeric protein complex that contains Egl and at least two Bic-D monomers, but it is unclear whether Bic-D self-associates. We tested whether Bic-D can form homodimers by yeast two-hybrid interaction. Full-length *Bic-D* cDNA was fused to the DNA-binding domain (BD) and the transcriptional activation domain (AD) of Gal4 to produce Gal4BD-Bic-D and Gal4AD-Bic-D fusion proteins. Cotransformation of Gal4BD-Bic-D and Gal4AD-Bic-D resulted in growth of the yeast host on histidine-minus medium and high activation of β-galactosidase activity, 100- to 120-fold above baseline (Table 5). Neither Gal4BD-Bic-D nor GalAD-Bic-D alone showed growth on the selective medium, nor did they show detectable β-galactosidase activity. The Bic-D-Bic-D interaction is apparently specific because Bic-D failed to interact with either Gal4BD or Gal4AD alone. These results indicate that Bic-D can interact with itself, forming homodimers.

DISCUSSION

The functional importance of the heptad repeat domains and their conservation: The transgenic studies of the deleted Bic-D proteins show that the two terminal

TABLE 3

Phenotypes produced by females hemizygous or heteroallelic for *Bic-D*^{H1}, *Bic-D*^{H2}, and *Bic-D*^{H3}

Genotype[a]	Total number of eggs (% of expected)[b]	Number of eggs with ventralized eggshells (%)	Eggshell phenotype (%)		
			Weak	Intermediate	Strong
+ / *Df119*	1587 (100)	0	0	0	0
Bic-D^{H1}	1 (<<1)	1 (100)	0	0	100
Bic-D^{H2}	381 (24)	125 (33)	74	6	19
Bic-D^{H1} / *Bic-D*^{H2}	36 (2.2)	36 (100)	28	17	55
Bic-D^{H3}	1451 (97)	14 (<1)	98	2	0
Bic-D^{H1} / *Bic-D*^{H3}	822 (69)	56 (7)	59	21	20
Bic-D^{H2} / *Bic-D*^{H3}	1478 (98)	15 (1)	97	3	0

[a] The genotype of *Bic-D*^{H1} females is *P[Bic-D*^{H1}*]*; *Bic-D*^{r5} / *Df119*. The genotype of *Bic-D*^{H2} females is *P[Bic-D*^{H2}*]*; *Bic-D*^{r5} / *Df119*. The genotype of *Bic-D*^{H1} / *Bic-D*^{H2} females is *P[Bic-D*^{H1}*]*; *P[Bic-D*^{H2}*]*; *Bic-D*^{r5} / *Df119*. The genotype of *Bic-D*^{H3} females is *P[Bic-D*^{H3}*]*; *Bic-D*^{r5} / *Df119*. The genotype of *Bic-D*^{H1} / *Bic-D*^{H3} females is *P[Bic-D*^{H1}*]*; *P[Bic-D*^{H3}*]*; *Bic-D*^{r5} / *Df119*. The genotype of *Bic-D*^{H2} / *Bic-D*^{H3} females is *P[Bic-D*^{H2}*]*; *P[Bic-D*^{H3}*]*; *Bic-D*^{r5} / *Df119*.

[b] The total number of eggs laid by females of the genotype divided by the total number of eggs laid by control sibling female flies.

TABLE 4

Phenotypes produced by females heteroallelic for *Bic-D^{H4}*, *Bic-D^{H2}*, and *Bic-D^{H3}*

Genotype[a]	Total number of eggs (% of expected)[b]	Number of eggs with ventralized eggshells (%)	Eggshell phenotype (%)		
			Weak	Intermediate	Strong
+/*Df119*	1112 (100)	0	0	0	0
Bic-D^{H2}	298 (27)	71 (24)	61	21	18
Bic-D^{H4}/*Bic-D^{H2}*	0	0	0	0	0
Bic-D^{H3}	1087 (96)	5 (<1)	100	0	0
Bic-D^{H4}/*Bic-D^{H3}*	205 (18)	5 (2)	60	40	0

[a] The genotype of *Bic-D^{H2}* females is *P[Bic-D^{H2}]*; *Bic-D^{r5}*/*Df119*. The genotype of *Bic-D^{H4}*/*Bic-D^{H2}* females is *P[Bic-D^{H4}]*; *P[Bic-D^{H2}]*; *Bic-D^{r5}*/*Df119*. The genotype of *Bic-D^{H3}* females is *P[Bic-D^{H3}]*; *Bic-D^{r5}*/*Df119*. The genotype of *Bic-D^{H4}*/*Bic-D^{H3}* females is *P[Bic-D^{H4}]*; *P[Bic-D^{H3}]*; *Bic-D^{r5}*/*Df119*.

[b] The total number of eggs laid by females of the genotype divided by the total number of eggs laid by control sibling female flies.

heptad repeat domains of Bic-D protein are essential for function and that the two centrally located domains are functionally less important or dispensable (Table 1). The first heptad domain is required exclusively for ovarian function: *Bic-D^{H1}* rescues the zygotic lethality of a *Bic-D^{null}* mutation, but fails to complement female sterility. The last heptad domain is essential for zygotic as well as ovarian function: *Bic-D^{H4}* fails to rescue the zygotic lethality and the female sterility of a *Bic-D^{null}* mutation. The second heptad domain retains only partial function: *Bic-D^{H2}* rescues the zygotic lethality, but females lay only 20% as many eggs as wild type and show eggshell phenotypes. The third heptad repeat deletion retains almost full wild-type function in both the ovary and the zygote. Interestingly, we did not identify any domain that functions specifically in the zygote, suggesting that the ovarian function of Bic-D may represent a special adaptation.

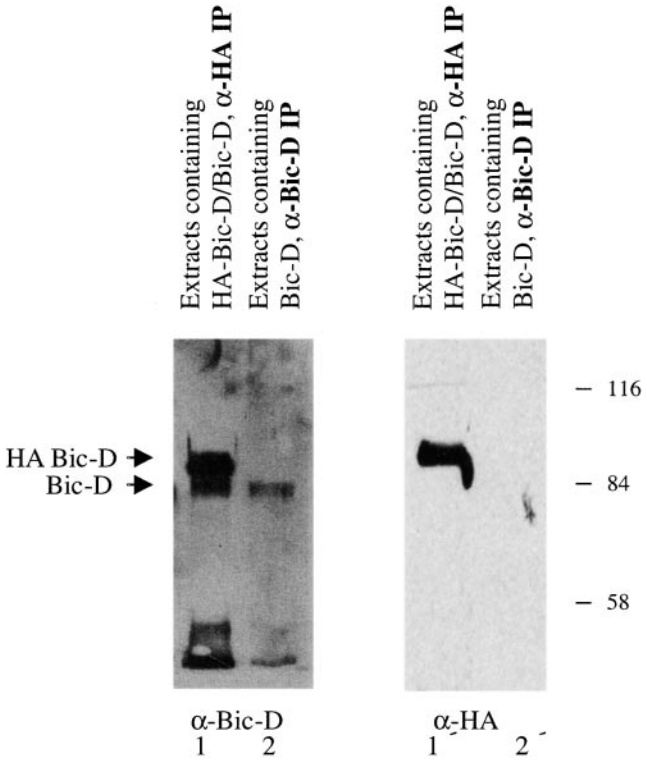


Figure 6.—Bic-D proteins exist in a multimeric complex. HA-tagged Bic-D (HA-Bic-D) protein was immunoprecipitated from ovary extracts containing wild-type Bic-D and HA-Bic-D protein with anti-HA antibodies (lane 1). Wild-type Bic-D protein was immunoprecipitated from wild-type extracts with anti-Bic-D antibodies (lane 2). The blot was probed with an anti-Bic-D antibody (left) and reprobed after stripping with anti-HA antibodies (right).

We determined that levels as low as 12% of wild-type Bic-D protein are enough to provide full zygotic and ovarian function. All the deleted transgenes produce in two copies ≥18% of wild-type protein levels, which are high enough to rescue the mutant phenotypes if the proteins were fully functional. We conclude, therefore, that the phenotypes observed by the four deleted transgenes are not due to low levels of protein, but to the essential function of the deleted domains.

The functional importance of each heptad repeat domain is well correlated with its conservation during evolution (Figure 1B). A *Bic-D* homologue has been sequenced in humans (Baens and Marynen 1997). The first and last heptad domains show the highest homology in flies and humans, 80 and 89%, respectively. The second heptad domain shows an intermediate homology (62.5%), while the third heptad domain is the least conserved (49%). The high conservation of heptad repeats 1 and 4 suggests that human *Bic-D* may also have functional homology and that it may have, like the Drosophila gene, two distinct functions.

Structural organization of Bic-D protein: Existing alleles of *Bic-D* agree with our observation that the two functionally essential domains of Bic-D reside at each end of the protein, with the center possibly having a spacer function. The mutation in *Bic-D^{PA66}* maps to the N-terminal domain. It is an alanine-to-valine change at aa 40 and eliminates the phosphorylation of a nearby serine. This phosphorylation is essential only for the ovarian function of the protein (Suter and Steward 1991).

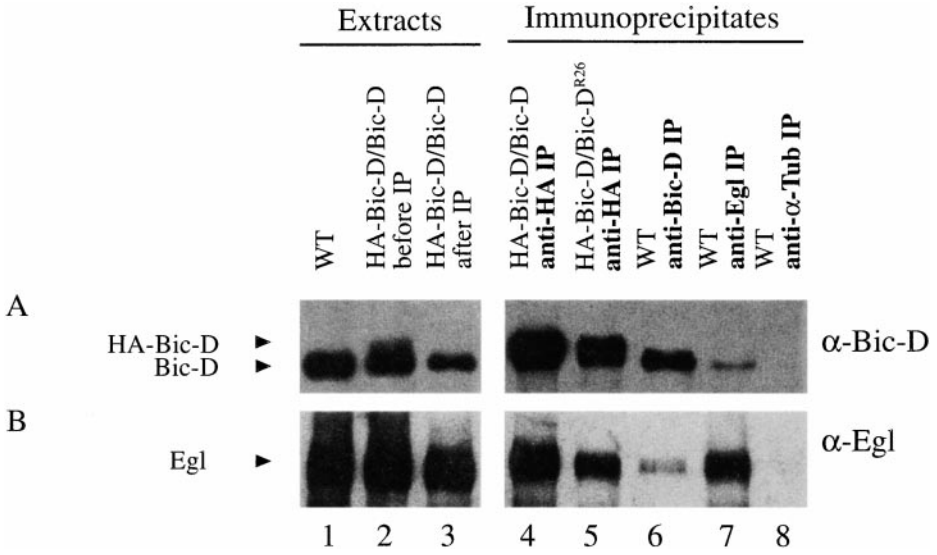


Figure 7.—Bic-D^{R26} maintains the ability to form a multimeric protein complex. Immunoblots of ovary extracts (lanes 1–3) and immunoprecipitates (lanes 4–8) probed with anti-Bic-D antibodies (A) and anti-Egl antibodies (B). Lane 1, wild-type ovary extracts. Lane 2, ovary extracts from females with one copy of the *Bic-D^{HA40}* transgene and one copy of endogenous *Bic-D*. Lane 3, supernatant, and lane 4, immunoprecipitate (10 μl loaded) of the same ovary extracts as in lane 2 precipitated with anti-HA antibodies. Anti-HA antibodies are able to immunoprecipitate most of the soluble Bic-D. Lane 5, immunoprecipitate of ovary extracts from females with one copy of the *Bic-D^{HA40}* transgene and one copy of endogenous *Bic-D^{R26}* (*P[Bic-D^{HA40}]; Bic-D^{R26}/Df119)*, immunoprecipitated with anti-HA antibodies (5 μl loaded). Lanes 6–8, control immunoprecipitates of wild-type ovary extracts without transgenes. Lane 6, anti-Bic-D antibodies (5 μl loaded). Lane 7, anti-Egl antibodies (5 μl loaded). Lane 8, anti-α-tubulin antibodies (5 μl loaded).

Bic-D^{r11}, a complete loss-of-function allele, has a single aa change of lysine730 to methionine located in the fourth heptad domain. The importance of this domain is further supported by the phenotype of *Bic-D$^{71.34}$*. In this dominant mutation, a single aa change of isoleucine689 to proline (Wharton and Struhl 1989) may result in a novel interaction of Bic-D with other proteins, specifically in late oogenesis and early embryogenesis.

Another maternal effect allele, *Bic-D^{R26}*, was isolated as a revertant of the dominant *Bic-D$^{71.34}$* allele and contains a deletion of four amino acids in the second heptad domain in addition to the single aa change in the fourth heptad repeat domain. In *Bic-D^{R26}*, the dominant embryonic phenotype is completely suppressed, but it has a much stronger ovarian phenotype than *Bic-D^{H2}*. This difference may be due to our deletion eliminating the entire conserved heptad repeat region, leaving no partial repeats, unlike the deletion in *Bic-D^{R26}*, which leaves behind three amino acids of a heptad repeat. It has been suggested that a deletion of four residues in heptad repeats could cause the local overwinding of the coiled-coil structure, resulting in a global effect (Brown *et al.* 1996).

No alleles that map to the third heptad repeat domain have ever been identified, supporting the notion that this domain has a largely redundant function.

On the basis of all these observations, it appears that the two termini of Bic-D serve as effector domains of the protein, with the C-terminal domain being essential for all functional aspects of Bic-D. Given that two ovary-specific mutations and the first and second heptad deletions affect only the ovarian function, a large part of the N-terminal half of the protein may specifically function in the ovary by interacting with ovary-specific factors. The second and third heptad repeat domains seem to mainly act as a linker that connects effector domains at both ends. Deleting part of this linker apparently can be tolerated.

A functionally similar protein organization is found in the yeast protein Zip1p, a component of synaptonemal complex (SC; Sym and Roeder 1995). Sequence analysis predicts that it encodes a protein consisting of an extensive helical coiled-coil domain (from aa 180 to aa 748) flanked by a globular domain at both termini (aa 1–179 and aa 749–875). Deletion analyses of the protein suggest that the coiled-coil domain may act as a spacer between the two effector domains at both termini because the protein is able to tolerate a large internal deletion and retain its function (Sym and Roeder 1995; Tung and Roeder 1998). A proposed model for the organization of Zip1p protein within the SC is that Zip1p protein forms a dimer and, in turn, two dimers form a tetramer that traverses the width of SC from one lateral element to the other lateral element (Tung and Roeder 1998).

TABLE 5

Self-association of Bic-D in yeast two-hybrid assay

Gal4BD fusion	Gal4AD fusion	His$^-$ test	β-Gal activity (Miller units)
Gal4BD		−	− (<0.1)
	Gal4AD	−	− (<0.1)
Gal4BD	Gal4AD	−	− (<0.1)
Gal4BD-Bic-D		−	− (<0.8)
Gal4BD-Bic-D	Gal4AD	−	− (<0.7)
	Gal4AD-Bic-D	−	− (<0.3)
Gal4BD	Gal4AD-Bic-D	−	− (<0.4)
Gal4BD-Bic-D	Gal4AD-Bic-D	+	+ (90)

Like Zip1, Bic-D has its functional domains at the ends of the protein, even though they are not structurally distinct. Given that Bic-D is required in oogenesis for the localization of mRNAs, it may function as a linker between the cytoskeletal network and the RNA localization machinery. Further experiments are necessary to investigate if indeed the central domain of Bic-D functions as a linker, or if it is largely redundant for the function of the protein.

Bic-D exists in a heteromeric protein complex: We found that *Bic-D*^{R26}, *Bic-D*^{H1}, and *Bic-D*^{H4} show antimorphic effects in hemizygous *Bic-D*^{H2} or *Bic-D*^{H3} backgrounds. The anti-HA antibody is capable of immunoprecipitating the HA-tagged Bic-D as well as endogenous Bic-D and Egl from ovary extracts, suggesting the existence of a multimeric Bic-D protein complex containing Egl and at least two Bic-D monomers. Because HA-Bic-D, Bic-D^{R26}, and Egl can be coimmunoprecipitated, the antimorphic effect of Bic-D^{R26} protein is most likely caused by the formation of nonfunctional Bic-D/Egl complex. By yeast two-hybrid interaction analyses, we found that Bic-D can form homodimers *in vivo*. Consistent with this, we also found that Bic-D was eluted from ovary extracts in fractions with an estimated molecular mass of 200 kD, the size of a dimer, on sucrose density gradients (data not shown); a similar elution profile was also reported for bacterially purified Bic-D protein (Stuurman *et al.* 1999).

It is likely that Bic-D functions as a dimer in the zygote as well as during oogenesis; therefore, Bic-D^{H1}, Bic-D^{H2}, and Bic-D^{H3} may retain the capability of forming homodimers. Because *Bic-D*^{H4} enhances the phenotype of hemizygous *Bic-D*^{H2} or *Bic-D*^{H3}, it probably also retains its capacity to dimerize. These observations suggest that Bic-D does not contain a defined sequence responsible for dimerization; rather, it appears that the coiling of the protein occurs along the entire length of the protein, and the protein may form a rod.

Although our results support that Bic-D forms a heteromeric protein complex, many questions remain concerning the Bic-D/Egl complex and its exact function. The structure of the Bic-D protein suggests that it may represent a linker between an RNA-binding protein and the cytoskeleton or between an RNA-binding protein and a motor. Alternatively, Bic-D may be an integral part of the cytoskeletal network essential for the integrity of many different cell types. Further biochemical purification and characterization of the Bic-D/Egl complex from ovary extracts will help us to address these questions.

A role of Bic-D in the formation of dorsal-ventral eggshell axis: The distribution and level of Gurken (Grk) activity in the oocyte is central to establishing the dorsal-ventral pattern of the egg and eggshell (Schüpbach and Roth 1994; Ray and Schüpbach 1996). Many genes function to localize Grk activity to the dorsal-anterior side of the growing oocyte, and there are many that control the translation of *grk* mRNA. It appears that there is a close correlation between the levels of *Bic-D* and *grk* function: the fertility of females directly correlates with the severity of the ventralized egg phenotype. In *Bic-D*^{H1} that fails to complement the female sterility of a *Bic-D*^{null} allele, the occasional eggs laid are strongly ventralized. In *Bic-D*^{H2} that shows intermediate complementation, a range of ventralized eggshell phenotypes are observed in ~20% of eggs laid. In *Bic-D*^{H3} that strongly complements, only a small fraction of eggs (<1%) display ventralized eggshell phenotypes and the extent of ventralization is weak. The correlation suggests that Bic-D influences the localization and possibly the translation of *grk* mRNA within the oocyte.

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