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Premature Translation of *oskar* in Oocytes Lacking the RNA-Binding Protein Bicaudal-C

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***Bicaudal-C* (*Bic-C*) is required during *Drosophila melanogaster* oogenesis for several processes, including anterior-posterior patterning. The gene encodes a protein with five copies of the KH domain, a motif found in a number of RNA-binding proteins. Using antibodies raised against the BIC-C protein, we show that multiple isoforms of the protein exist in ovaries and that the protein, like the RNA, accumulates in the developing oocyte early in oogenesis. BIC-C protein expressed in mammalian cells can bind RNA in vitro, and a point mutation in one of the KH domains that causes a strong *Bic-C* phenotype weakens this binding. In addition, *oskar* translation commences prior to posterior localization of *oskar* RNA in *Bic-C*$^{-}$ oocytes, indicating that *Bic-C* may regulate *oskar* translation during oogenesis.**

Anterior-posterior polarity in *Drosophila melanogaster* is established during oogenesis through the asymmetric localization of many RNAs and proteins in the egg (17, 45). Localized molecules include the *oskar* (*osk*) and *nanos* (*nos*) RNAs, which are localized at the posterior of the developing oocyte during midoogenesis and are required to specify posterior pattern information (11, 22, 48, 49). Eggs with *osk* or *nos* RNA mislocalized at the anterior produce bicaudal embryos whose posterior structures are duplicated at their anterior ends (12, 14). In addition to asymmetric RNA distribution, the localization of many maternally expressed proteins occurs through translational regulation of their RNAs (30). For example, translation of *osk* is repressed until posterior localization of its RNA at stage 9 of oogenesis. This translational repression is mediated in part by Bruno, a protein which interacts with specific sequences (termed BREs, for Bruno response elements) in the *osk* 3′ untranslated region (UTR). In oocytes produced by females with a transgene lacking BREs (*osk-BRE*$^{-}$), *osk* is prematurely translated during stages 7 and 8, resulting in a shift toward excessive posterior body patterning in progeny embryos, particularly in *osk*$^{-}$ mothers or in other mutant backgrounds which abrogate *osk* mRNA localization (23). Another protein which has recently been implicated in the translational control of maternal RNAs during oogenesis is the product of the *vasa* (*vas*) gene. *vas* encodes an RNA-binding protein with homology to the DEAD box family of RNA helicases, including the translation initiation factor eIF4A (18, 26), and *vas* activity is required for efficient translation of *osk*, *nos*, and *grk* during oogenesis (8, 15, 32, 39, 46, 47).

The *Bicaudal-C* (*Bic-C*) gene is required for a number of processes in oogenesis, including the establishment of anterior-posterior polarity in the oocyte (2, 31, 35, 41). Females heterozygous for *Bic-C* alleles produce embryos with a range of anterior-posterior patterning defects, including bicaudal embryos. These patterning defects are also seen in embryos produced by females heterozygous for a complete deletion of the gene, indicating that the dominant phenotypes result from the haplo insufficiency of the locus. Previously, we described in detail the phenotypes of 12 ethyl methanesulfonate-generated *Bic-C* alleles and ranked them by strength according to the number of bicaudal embryos that are produced by each allele (31). Consistent with their bicaudal phenotype, embryos produced by *Bic-C*$^{-}$ mothers (referred to hereafter as *Bic-C*$^{-}$ embryos) have mislocalized *osk* and *nos* RNAs at the anterior, while the localization of other RNAs, such as *bicoid*, is not affected (31). These observations suggested a role for *Bic-C* in localizing specific posterior RNAs during oogenesis.

The *Bic-C* RNA encodes a protein containing five RNA-binding domains of the KH type (31). KH domains have been found in a large number of proteins, many of which are involved in regulating RNA metabolism. These include the heterogeneous nuclear ribonucleoprotein K (33); the splicing factors MER-1 (10, 37), PSI (42), SF1 (1), and KSRP (34); the ribosomal protein S3 (16); the transcription elongation factor NusA (16); and the α-globin messenger RNP stability complex-associated proteins αCP-1 and αCP-2 (21). As in the case of *Bic-C*, mutations in genes encoding any of several KH proteins, including the human fragile X protein FMR1 (44), *Caenorhabditis elegans* GLD-1 (19), *Drosophila* How (3, 13, 29, 52), and vertebrate quaking (9, 53), have severe developmental consequences. Many KH proteins can bind either RNA or single-stranded DNA in vitro, and in a few cases this binding activity has been shown to require the KH domains (4, 6, 44). Moreover, an isolated KH domain can bind RNA (4). Using SELEX, specific RNA targets that bind with high affinity have been identified for Nova-1 and Sam68 (4, 27).

The KH domains are required for *Bic-C* function, since a strong allele of *Bic-C* contains a point mutation in a conserved residue in one of the KH domains (G296R [31]). Based on the nuclear magnetic resonance structure of the KH domain, this mutation is predicted to place a bulky charged residue in the third β sheet of the hydrophobic core of the domain and thereby to disrupt its structure (36). In addition to the KH domains, the predicted *Bic-C* protein contains a serine-glycine-

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rich region and a SAM (sterile alpha motif) domain. SAM domains have been found in more than 60 proteins and are postulated to form protein binding domains (40). Indeed Bic-C has been shown to interact with other proteins of the KH domain family when expressed in mammalian cells (6).

Here we characterize the Bic-C protein (BIC-C) in wild-type and *Bic-C*$^{-}$ flies. We report that multiple isoforms of BIC-C are present in ovaries and that the protein is localized to the developing oocyte. Further, we demonstrate that BIC-C can bind RNA, that a mutation in a single KH domain weakens RNA binding, and that *osk* translation is misregulated in *Bic-C* mutants. Taken together, these results suggest that *Bic-C* may act directly as a translational repressor of *osk* during oogenesis.

MATERIALS AND METHODS

Subcloning and site-directed mutagenesis. pBS*Bic-C*$^{\Delta Bgl\ II}$, which encodes the BIC-C protein in which the epitope for the anti-BIC-C antibody has been removed, was made by deleting the 813-bp *Bgl*II fragment from pBS*Bic-C* (the *Bic-C* cDNA in pBluescript II SK^{-} [31]). pBS*Bgl*II, which encodes the KH1-3 protein, was made by inserting the same *Bgl*II fragment from pBS*Bic-C* into pBluescript II SK^{-} (Stratagene). pBS*Bic-C*^{G296R} was made from pBS*Bic-C* with an oligonucleotide-directed, PCR-based mutagenesis strategy, as follows. First, pBS*Bic-C* was amplified with two pairs of primers, G296RTOP (5′ CAAGAG ATCTGAGAAGGAATCG) and BicC15 (5′ ATGGAACGATTCTGAGC) and G296RBOT (5′ CTCAGATCTCTTGACCAAAACC) and BicC1246 (5′ CGG ATACTTATGTGAGCTGGC). The products of these reactions were gel purified, mixed together, and used as template for a second round of amplification with the BicC15 and BicC1246 primers. For both rounds, 25 cycles of PCR were performed with *Taq* DNA polymerase (GIBCO/BRL) and annealing at 45°C. The final PCR product was cut with *Hpa*I and *Sma*I and used to replace the *Hpa*I-*Sma*I fragment of pBSBic-C, to make pBS*Bic-C*^{G296R}. The G296R mutation was confirmed by loss of a *Bam*HI site, and the amplified region was checked by standard dideoxy sequencing.

For expression in Cos cells, the *Bic-C* cDNA and derivative sequences were cloned into pcDNA3 (Invitrogen) by inserting the *Kpn*I-*Not*I fragment from pBS*Bic-C* into pcDNA3 cut with *Kpn*I and *Not*I. For expression in Sf9 cells with baculovirus, the 843-bp *Pst*I fragment from pBS*Bic-C* was inserted into the *Pst*I site of pVLHisB (a gift of A. Nakamura). pVLHisB was made by inserting the *Eco*RV-*Bam*HI fragment, which contains six molecules of His (6×His), from pBlueBacHisB (Invitrogen) into pVL1393 (Invitrogen) cut with *Eco*RV and *Bam*HI. For expression of the glutathione *S*-transferase–BIC-C fusion protein in *Escherichia coli*, the 813-bp *Bgl*II fragment from pBS*Bic-C* was cloned into the *Bam*HI site of pGEX-3X (Pharmacia).

Expression of fusion proteins and antibody production. Two anti-BIC-C antibodies were raised. In immunoblots of ovary extracts, both antibodies gave the same results (data not shown). The first antibody (used for immunoblotting) was raised against a glutathione *S*-transferase–BIC-C fusion protein containing amino acids 59 to 329 of BIC-C. After expression in *E. coli*, this protein was purified over glutathione Sepharose 4B according to the manufacturer's protocol (Pharmacia Biotech) and mixed 1:1 with Freund's incomplete adjuvant for injection into rabbits. The resulting serum was affinity purified against the same fusion protein coupled to Affigel-10 (Bio-Rad). Antibody was eluted in 0.1 M glycine, pH 2.3, and concentrated with a Centricon-30 microconcentrator (Amicon).

The second antibody (used for immunohistochemistry) was raised against a 281-amino-acid His-tagged portion of BIC-C containing amino acids 591 to 872. The 6×His-BIC-C protein was expressed in Sf9 cells (Invitrogen) with baculovirus as follows. Cells were infected, cultured, and harvested according to the manufacturer's protocol with the Bac-N-Blue transfection kit (Invitrogen), except that BaculoGold-linearized virus DNA (Pharmingen) and *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate liposomes (Boehringer Mannheim) were used, and cells were cultured in Grace's insect medium containing 10% fetal bovine serum (GIBCO/BRL). The protein was purified over Ni^{2+}-nitrilotriacetic acid beads (Qiagen) according to the manufacturer's instructions, except that the sonication and wash buffers contained 20 mM imidazole. In addition, after sonication, the supernatant was filtered through a 0.45-μm-pore-size filter immediately before it was mixed with the beads. Step elutions at 75, 250, and 500 mM imidazole were used. The protein was mixed 1:1 with TiterMax adjuvant (CytRx Corporation) for injection into rabbits and affinity purified against the same protein coupled to Affigel-10, as described above.

RNA-binding assays. Assays were performed with proteins expressed in Cos-7 cells. Cos-7 cells were transfected with DEAE-dextran, and the cells were lysed in lysis buffer as described previously (50). Briefly, a 10-cm-diameter petri dish containing 10^6 cells was harvested, and the cells were lysed in lysis buffer (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml, 100 μg of phenylmethylsulfonyl fluoride [PMSF]). Cellular debris was removed by centrifugation. Binding assays were performed using 50 μl of 50% beads [either poly(U)-Sepharose or Sepharose 4B (Pharmacia)] and 90 μl of cell lysate with or without homopolymer competitors (Pharmacia) or additional salt. Reaction mixtures were incubated for 30 min with rocking at 4°C. Beads were then washed twice in 0.5 ml of ice-cold lysis buffer (25 mM Tris 7.4, 1% Triton X-100, 150 mM NaCl), and proteins were eluted from the beads by boiling in sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8.5% polyacrylamide), and analyzed by immunoblotting. Anti-BIC-C primary antibody was used at 1:3,000 and visualized with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Amersham) and chemiluminescence (NEN Life Science).

Mapping *Bic-C* mutations. Portions of the *Bic-C* gene were amplified from flies hemizygous for *Bic-C* mutant alleles and *Df(2L)RA5* for sequencing. Multiple primers distributed throughout the *Bic-C* gene were used to amplify the coding region. Two rounds of PCR were performed (20 cycles each with *Taq* polymerase [GIBCO/BRL] at an annealing temperature of 53 to 55°C). PCR products were either sequenced directly or subcloned into pBluescript II SK^{-} for sequencing. Sequencing was carried out with oligonucleotide primers by standard dideoxy techniques. Mutations were confirmed in the products of at least two independent amplifications. To check the sequence of the mRNA produced in *Bic-C*^{AB79}, which contains a deletion in the genomic DNA ending in an intron (31), RNA from *Bic-C*^{AB79}/*Bic-C*^{AB79} females was amplified by reverse transcription (RT)-PCR, and the product was sequenced directly by standard dideoxy techniques.

In situ hybridization and immunohistochemistry. In situ hybridizations with digoxigenin-labeled RNA probes and antibody stainings were carried out on ovaries as described previously (25), except that dimethyl sulfoxide was omitted from the fixation solution. Primary antibodies were used at the following dilutions: anti-BIC-C, 1:300; anti-OSK, 1:5,000. anti-BIC-C was preadsorbed on ovaries from OreR or *Bic-C*^{AA4}/*Bic-C*^{AA4} females, and anti-OSK was preadsorbed on ovaries from OreR females. Antibody stainings were detected with biotinylated secondary antibodies (Vector Laboratories) and diaminobenzidine and enhanced with the Vectastain ABC kit (Vector Laboratories). All immunohistochemistry was performed with the anti-BIC-C antibody raised against the His-tagged protein. Anti-OSK (24) was a gift from Paul Macdonald.

Preparation of ovary extracts and immunoblotting. Ovaries were homogenized in either phosphate-buffered saline or a buffer containing 10 mM Tris, pH 7.5, and 1 mM EGTA in the presence of protease inhibitors (0.1 mM PMSF, 10 μg of pepstatin A per ml, and 10 μg of leupeptin per ml). Anti-BIC-C primary antibody was used at 1:1,000; these antibodies were visualized with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G at 1:5,000 and chemiluminescence. All immunoblotting was performed within the linear range of chemiluminescent detection. Quantitation of immunoblots was performed with a Macintosh computer with the National Institutes of Health (NIH) Image program (developed at the NIH and available on the internet at http://rsb.info.nih.gov/nih-image/).

Fractionation of supernatants and membranes. Ovaries were dissected into phosphate-buffered saline and then homogenized in ice-cold hypotonic buffer (10 mM Tris [pH 7.5], 1 mM EGTA, 1 mM $MgCl_2$, 0.1 mM PMSF). The supernatant from a low-speed spin (1,000 × *g* for 1 min) was then spun at 100,000 × *g* for 30 min at 4°C. Supernatant and pellet protein concentrations were determined by the Bio-Rad protein assay, and 20 μg of each was analyzed by SDS–8.5% PAGE, followed by immunoblotting. For fractionation at high pH, the low-speed supernatant was incubated with 0.1 M Na_2CO_3 (pH 11) for 30 min at 4°C before the high-speed spin.

RT-PCR. Total RNA was isolated from female flies by a single-step guanidine procedure (7). RT-PCR was performed by the Titan One Tube RT-PCR system (Boehringer Mannheim) and *Bic-C*-specific primers (5′ CGGATACTTATGTG AGCTGGC and 5′ TTGATCAGCAGCTGCGT).

RESULTS

Characterization of the BIC-C protein. In order to characterize the BIC-C protein, we raised an antiserum against a glutathione *S*-transferase fusion protein containing amino acids 59 to 329 of BIC-C (KH 1–3, see Fig. 4A). In immunoblots, three polypeptides, one of approximately 120 kDa and two minor isoforms of approximately 160 and 116 kDa (Fig. 1A), were specifically detected by the antiserum in ovaries from wild type but not *Bic-C*$^{-}$ females. In addition, the antiserum recognizes a 100-kDa protein which is still present in *Bic-C*$^{-}$ ovaries. Expression of either the wild-type or G296R mutant (31) *Bic-C* cDNA in Cos cells produced a single polypeptide that migrates slightly faster than the major isoform in *Drosophila* extracts (Fig. 1B). In order to examine the subcellular localization of BIC-C, particulate and cytosolic components of ovaries were separated by centrifugation and BIC-C protein

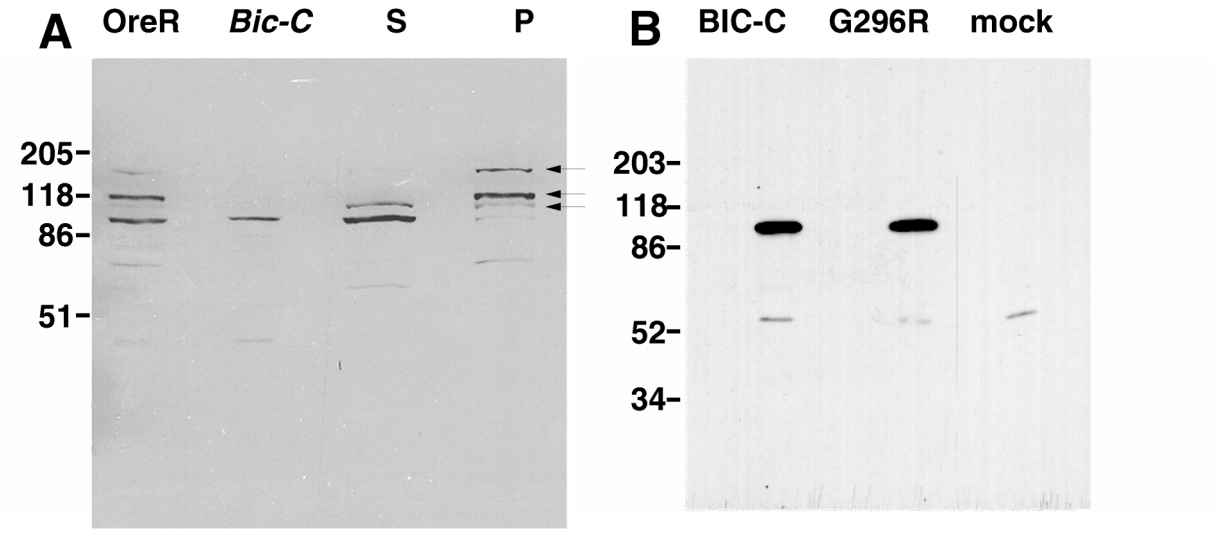

FIG. 1. Multiple isoforms of the BIC-C protein. (A) Ovary extract from the indicated flies (20 μg) was analyzed by SDS–8.5% PAGE followed by immunoblotting with an affinity-purified antibody raised against a glutathione *S*-transferase fusion protein containing the KH 1–3 region of the protein (Fig. 4A). OreR, wild-type Oregon-R; *Bic-C*$^{-}$, transheterozygotes of *Df(2L)RA5* and *Df(2L)GW19*, deletion mutations which both remove *Bic-C* (2). In addition to three BIC-C-specific bands (indicated by arrows), the antibody also recognizes a polypeptide of approximately 100 kDa, which is still seen in *Bic-C*$^{-}$ ovaries. Ovary extracts from Oregon-R flies were also fractionated into supernatant (S) and pellet (P). Note that the cross-reacting band was found in the supernatant, while the majority of the BIC-C protein was found in the pellet. (B) Extracts from Cos cells expressing the wild-type (BIC-C) or G296R mutant (G296R) proteins were analyzed by SDS–8.5% PAGE followed by immunoblotting. "Mock" represents a control expressing pBS*Bic-C*$^{\Delta Bgl\ II}$, a BIC-C derivative with the epitope for the BIC-C antibody deleted. Molecular size markers are indicated in kilodaltons.

was detected by immunoblotting. The 120-kDa BIC-C protein was associated primarily with the particulate fraction (Fig. 1A). In contrast to the majority of the BIC-C protein, the 100-kDa cross-reacting band was found primarily in the supernatant. After incubation at high pH (0.1 M Na_2CO_3 [pH 11]), all the BIC-C protein was found in the supernatant (data not shown), suggesting that BIC-C is a peripheral membrane protein (28) or that it is associated with high-molecular-weight alkali-labile RNP complexes.

BIC-C protein localizes to the oocyte early in oogenesis. Next we investigated the distribution of BIC-C protein in developing ovaries (Fig. 2). The pattern of BIC-C expression is similar to that of the *Bic-C* RNA, except that the RNA is detectable in a single cell (the presumptive oocyte) as early as

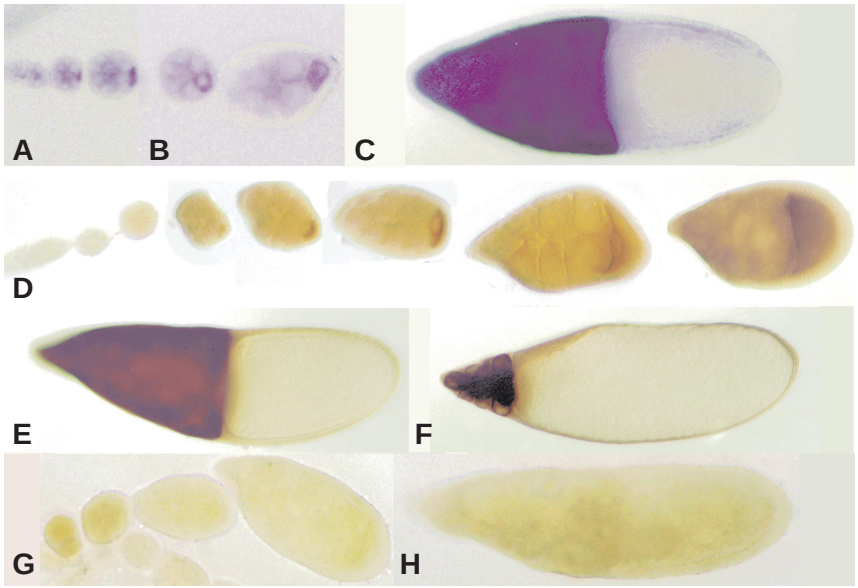

FIG. 2. (A to C) Wild-type ovaries hybridized with a digoxigenin-labeled probe to visualize *Bic-C* RNA. Note the accumulation of *Bic-C* RNA in a single cell from the earliest vitellarial stages of oogenesis. (D to F) Wild-type ovaries stained with anti-BIC-C antibody. For immunohistochemistry, an antibody raised against a His-tagged 281-amino-acid portion of BIC-C was used (see Materials and Methods for details). (D) Stages of oogenesis from germarium to stage 8; stages are those described by King (24). Oocyte accumulation of BIC-C first becomes detectable at stage 4. (E) Stage 10 egg chamber showing abundant BIC-C protein in the nurse cells. (F) Stage 12 egg chamber showing abundant BIC-C protein in the nurse cells. (G and H) *Bic-C*$^{\Delta A4}$ ovaries stained with the same antiserum as in panels D to F. Note the absence of localized signal.

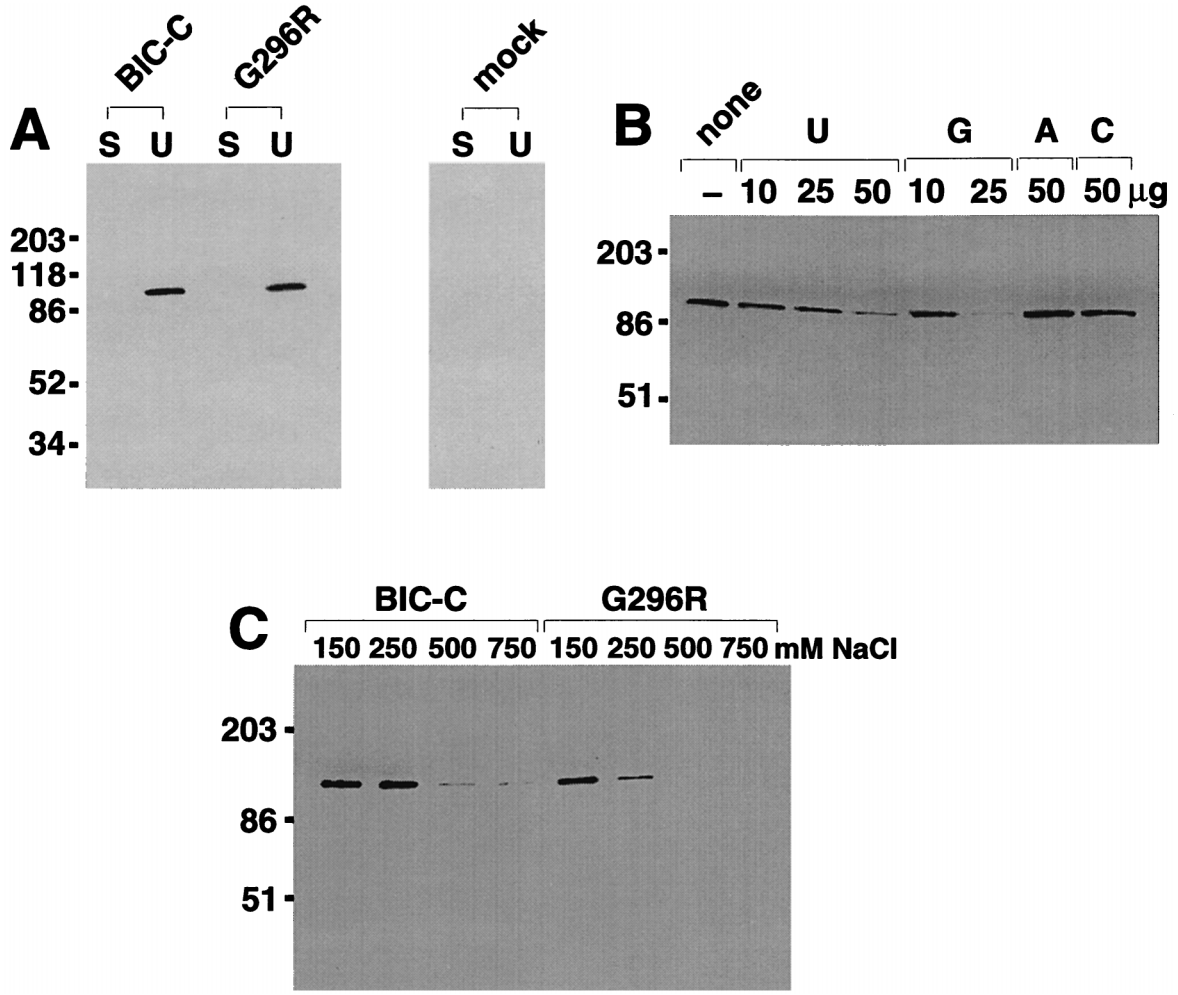

FIG. 3. Poly(U)-Sepharose binding assays. (A) Extracts from Cos cells expressing the indicated BIC-C proteins, as in Fig. 1B, were incubated with either Sepharose 4B (S; control) or poly(U)-Sepharose (U) at 150 mM NaCl for 30 min at 4°C. After being washed, proteins were eluted from the beads by boiling in sample buffer and analyzed by SDS–8.5% PAGE followed by immunoblotting. Wild-type and G296R proteins were expressed at similar levels (Fig. 1B). (B) Poly(U)-Sepharose binding assays of BIC-C expressed in Cos cells were carried out in the presence of the indicated amount of homopolymer [poly(U), poly(G), poly(A), or poly(C)] as the competitor. Similar results were seen in at least three independent experiments. (C) Dependence of poly(U)-Sepharose binding on salt concentration. Cos cell extracts expressing either BIC-C or G296R protein were used for poly(U)-Sepharose binding assays at the indicated concentration of NaCl. Proteins bound to the beads were analyzed by SDS–8.5% PAGE followed by immunoblotting. Molecular size markers are indicated in kilodaltons.

germarium region 2A (31) (Fig. 2A to C). In contrast, specific accumulation of BIC-C protein in the oocyte is first detectable at stages 3 and 4 of oogenesis but remains very faint until stage 5, when the protein level increases substantially (Fig. 2D). In stages 4 to 6, BIC-C protein is visible throughout the oocyte cytoplasm but is enriched at the posterior pole of the oocyte (Fig. 2D). During stages 7 to 9, BIC-C protein is abundant in the oocyte cytoplasm, with some enrichment at the anterior of the oocyte and around the oocyte cortex (Fig. 2D). In stage 10 and in later stages, the protein is expressed at high levels in the nurse cells (Fig. 2E and F). No localized staining was detected in ovaries from females homozygous for *Bic-C*^{AA4} (Fig. 2G and H) or in ovaries from females heterozygous for *Bic-C*^{AA4} and with a deficiency which removes *Bic-C* (*Df*(*2L*)*RA5*; data not shown).

BIC-C protein binds RNA. KH-domain-containing proteins are involved in many aspects of RNA metabolism, including mRNA splicing, translation, and RNA stability, and many KH proteins can bind either RNA or single-stranded DNA in vitro (5, 16). Since the physiological RNA targets for BIC-C are unknown, we carried out binding assays to homopolymeric RNA, as such RNA is an in vitro substrate for many RNA-binding proteins (20, 43). Poly(U)-Sepharose binding assays were performed with protein expressed in mammalian Cos cells (proteins are described in the legend for Fig. 4A). Wild-type BIC-C and G296R proteins were expressed at similar levels in the Cos cells (Fig. 1B). Extracts from cells transfected with an expression construct expressing either BIC-C or a BIC-C derivative with the epitope deleted (mock control) were incubated with either poly(U)-Sepharose or Sepharose 4B, and proteins bound to the beads were analyzed by immunoblotting. No BIC-C protein bound detectably to Sepharose 4B or in the mock-transfected control. However, BIC-C and G296R were retained on poly(U)-Sepharose at 150 mM NaCl (Fig. 3A). In addition, a fragment of BIC-C containing amino acids 59 to 329 (KH 1–3, see Fig. 4A), comprising the first three KH domains and 37 flanking amino acids, bound weakly to poly(U) (data not shown).

Many KH proteins bind preferentially to certain RNA homopolymers. For example, the fragile X protein FMR1 and the

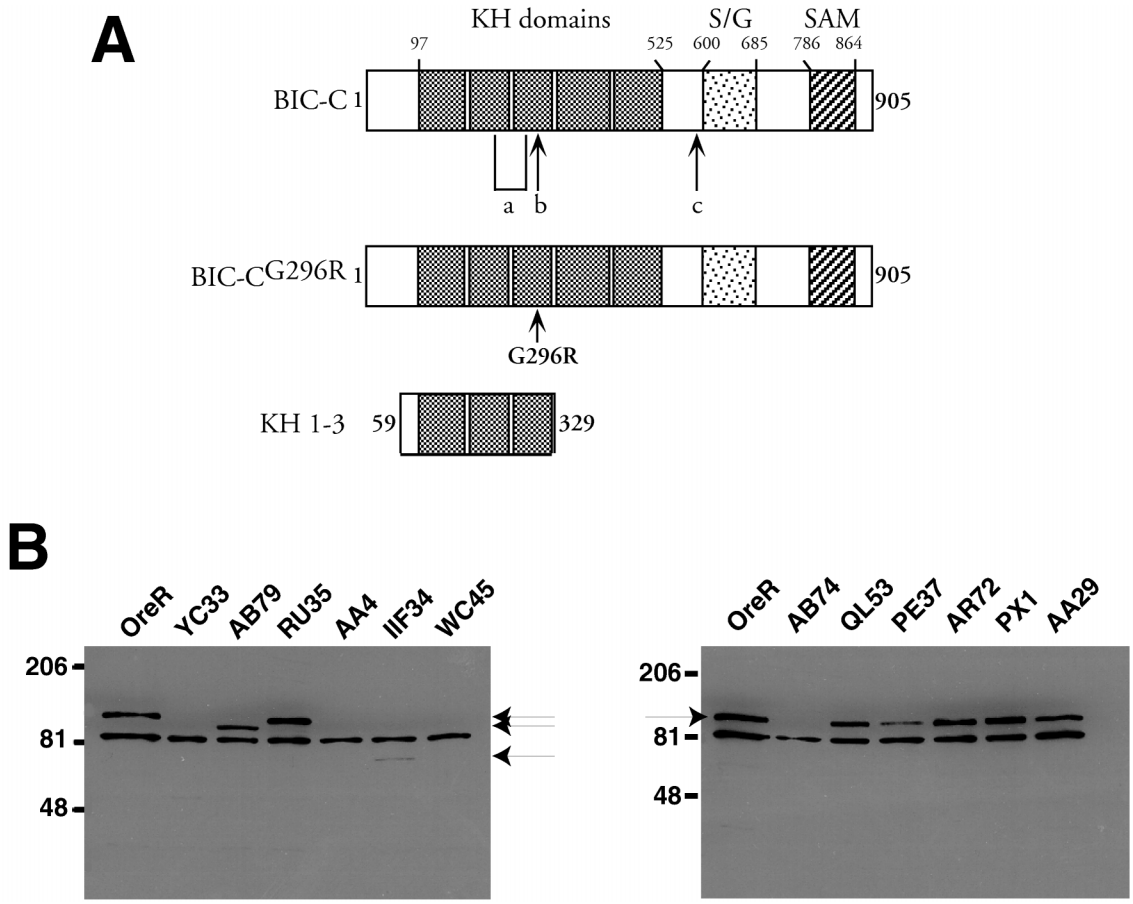

FIG. 4. Characterization of the BIC-C protein in *Bic-C* alleles. (A) A schematic representation of the BIC-C protein is shown. S/G, serine-glycine-rich region. Letters indicate the sites of mutations found in the following *Bic-C* alleles: a, *AB79*, deletion of amino acids 226 to 278; b, *RU35*, G296R; c, *IIF34*, protein truncated at 574 plus 27 new amino acids. Schematics of two other proteins used in RNA-binding experiments, BIC-C^{G296R} and KH 1–3, are also shown. Numbers represent amino acid positions in the protein. (B) Western analysis of BIC-C protein in *Bic-C* alleles. Ovaries were taken from either Oregon-R (OreR) or transheterozygotes of the indicated *Bic-C* allele and *Df(2L)RA5*. Ten micrograms of ovary extract was analyzed by SDS–8.5% PAGE followed by immunoblotting. *Bic-C* alleles are presented in decreasing order of strength for the dominant embryonic patterning phenotype (31) from left to right. The arrowheads indicate BIC-C proteins; the 100-kDa band is the cross-reacting protein also recognized by the anti-BIC-C antibody. Under these conditions, only the major BIC-C isoform (120 kDa) is seen. Molecular size markers are indicated in kilodaltons.

NOVA protein bind strongly to poly(G) and poly(U) but not to poly(C), whereas heterogeneous nuclear ribonucleoprotein K binds only to poly(C). To test whether BIC-C binds to polyribonucleotides differentially, poly(U)-Sepharose binding assays were carried out with poly(U), poly(G), poly(A), or poly(C) as the competitor. Poly(G) and poly(U) competed the most effectively for BIC-C binding to poly(U)-Sepharose, while poly(A) and poly(C) competed poorly, if at all, at similar concentrations (Fig. 3B). Thus, like other KH proteins, BIC-C shows specificity for RNA homopolymers in vitro.

The severe G296R mutation renders the RNA-binding activity of BIC-C more salt labile. A strong allele of *Bic-C*, *Bic-C^{RU35}*, changes a consensus glycine (G296) in the third KH domain to an arginine (31). To test whether the phenotype of this allele could be correlated with defects in RNA binding, we asked whether BIC-C protein carrying the G296R mutation (G296R) still bound poly(U)-Sepharose under high-salt conditions. Although G296R bound at 150 mM salt, binding was greatly reduced at 250 mM salt and completely abolished at 500 mM salt, whereas the wild-type BIC-C protein still bound detectably at NaCl concentrations up to 750 mM (Fig. 3C). Using the NIH Image software package, we quantitated and averaged the signal on the blot in Fig. 3C with that on two further independent replicates. We found binding at 250 mM NaCl to be 77% ± 11% (mean ± standard deviation) of that at 150 mM for the wild-type protein, but only 14% ± 6% for BIC-C^{G296R}. Thus, the G296R mutation found in the *Bic-C^{RU35}* allele substantially increases the salt lability of BIC-C binding to poly(U)-Sepharose, suggesting that RNA-binding activity is required for the in vivo function of *Bic-C*. For FMR1, an asparagine-to-isoleucine substitution in one of the KH domains has a similar effect on in vitro binding to homopolymeric RNA, as binding to poly(U) is reduced at 250 mM NaCl but not at 100 mM NaCl (44).

Characterization of the BIC-C protein in *Bic-C* alleles. Previously we characterized 12 *Bic-C* alleles phenotypically (31). In order to characterize these alleles at the molecular level, we determined which alleles express BIC-C protein. Ovary extracts from flies carrying each of the mutant *Bic-C* alleles and a complete deletion of the gene [*Df(2L)RA5*] were analyzed by immunoblotting (Fig. 4B). In general, the severity of the alleles could be correlated with the amount of protein they produce, as strong alleles produced reduced levels of protein, while weaker alleles expressed protein at levels comparable to those of the wild type. Four strong alleles (*Bic-C^{YC33}*, *Bic-C^{AA4}*, *Bic-C^{WC45}*, and *Bic-C^{AB74}*) do not produce any detectable

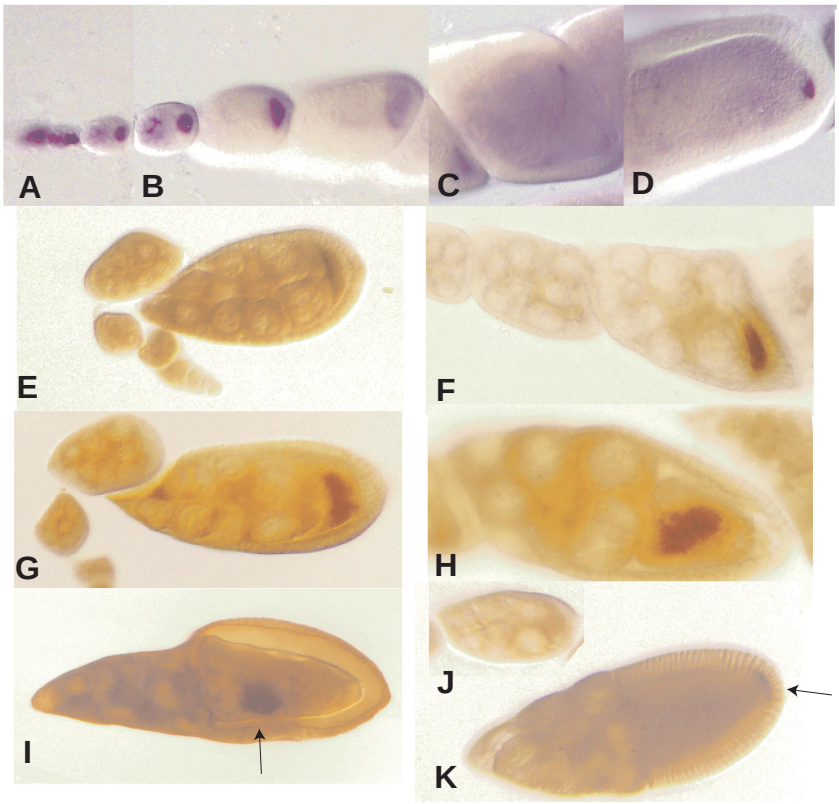

FIG. 5. (A to D) Egg chambers from *Bic-C*^{YC33}/*Bic-C*^{YC33} females hybridized with a digoxigenin-labeled probe for *osk* RNA. Posterior accumulation of *osk* RNA is apparent in the stage 10 oocyte shown in panel D. Similar results were obtained with *Bic-C*^{AA4}/*Bic-C*^{AA4} egg chambers. (E to I) Egg chambers from *Bic-C*/*Bic-C* females stained with an antibody recognizing OSK protein. (E) Stage 8 *Bic-C*^{AA4}/*Bic-C*^{AA4} oocyte staining positively for OSK in anterior and central regions. (F) Stage 8 *Bic-C*^{AB79}/*Df*(2L)*RA5* oocyte staining positively for OSK in anterior and central regions. (G) Stage 9 *Bic-C*^{AA4}/*Bic-C*^{AA4} oocyte with abundant OSK in central regions. (H) Stage 10 *Bic-C*^{AB79}/*Df*(2L)*RA5* oocyte with abundant OSK in central regions. (I) Degenerating stage 10 *Bic-C*^{AA4}/*Bic-C*^{AA4} egg chamber, with a region of high OSK concentration near the center of the oocyte (arrow). Other similarly staged oocytes show a more diffuse distribution of OSK, but OSK is always excluded from the posterior pole of *Bic-C* oocytes. *Bic-C*^{AA4}/*Df*(2L)*RA5* and *Bic-C*^{RU35}/*Df*(2L)*RA5* egg chambers gave similar results (data not shown). (J and K) α-OSK staining as in panels E to I of wild-type egg chambers. Note the absence of signal in the stage 8 egg chamber in panel J and the posterior accumulation of OSK, marked with an arrow, in the stage 10 oocyte shown in panel K.

BIC-C protein. Indeed, only two phenotypically strong alleles, *Bic-C*^{RU35} and *Bic-C*^{AB79}, produce normal levels of protein. Both of these alleles contain mutations in the KH domains (Fig. 4A). *Bic-C*^{RU35} changes a conserved glycine in the third KH domain to an arginine, and *Bic-C*^{AB79} deletes 159 nucleotides of coding region in KH domains 2 and 3 and the first 120 nucleotides of intron 6, including the donor splice site (31). We produced cDNA from this allele by RT-PCR and determined its sequence to learn how this mutant RNA is spliced. This analysis predicted that *Bic-C*^{AB79} would produce a shorter protein with the last 17 amino acids of KH domain 2 and the first 35 amino acids of KH domain 3 removed. Western blot analysis confirmed that *Bic-C*^{AB79} produces a shorter protein, consistent with this prediction. The phenotypic severity of these alleles underlines the importance of the KH domains for *Bic-C* function.

Another strong allele, *Bic-C*^{IIF34}, expresses low levels of a truncated protein. Sequence analysis of this allele revealed a mutation causing a frameshift after amino acid 574, which leads to a stop codon, consistent with the size of the truncated protein. It is not clear whether the phenotypic severity of this allele implies an important function for the C-terminal region of the protein, which includes the SAM domain, or results from the greatly reduced levels of protein expression.

***oskar* translation is misregulated in *Bic-C*$^{-}$ oocytes.** We previously reported that *osk* mRNA is ectopically localized in part to the anterior of eggs produced by *Bic-C*/+ or *Bic-C*/*Bic-C* females (31). However, a substantial amount of *osk* mRNA still localizes normally to the oocyte posterior, even in homozygous *Bic-C* oocytes (31) (Fig. 5A to D). To determine whether OSK protein expression is affected in *Bic-C* mutants, we used immunohistochemistry to analyze OSK protein expression in ovaries from *Bic-C*/*Bic-C* females. In wild-type oocytes, translation of *osk* is repressed until posterior localization of the RNA at stage 9, resulting in restriction of OSK protein to the posterior tip of the oocyte (23) (Fig. 5J and K). In contrast, in *Bic-C*$^{-}$ ovaries we found that *osk* is prematurely translated, beginning in stages 7 and 8 (Fig. 5E to I). Through stages 7 to 10, OSK protein remains diffuse and is most concentrated near the center of the oocyte. Similar results were obtained with ovaries from *Bic-C*^{AA4}/*Bic-C*^{AA4}, *Bic-C*^{AA4}/*Df*(2L)*RA5*, *Bic-C*^{AB79}/*Df*(2L)*RA5*, and *Bic-C*^{RU35}/*Df*(2L)*RA5* flies (Fig. 5; data not shown), indicating that the RNA-binding activity of BIC-C is necessary for the correct repression of *osk* translation. These results suggest that BIC-C may function directly to regulate the translation of target RNAs such as *osk*.

Surprisingly, despite its precocious translation in *Bic-C* mutants and the substantial posterior concentration of its RNA

(31), OSK does not accumulate at the posterior pole as late as stage 10 (Fig. 5H and I). It is possible that pole plasm-specific activation of *osk* translation is also compromised by *Bic-C* mutations. However, as many developmental defects become apparent in *Bic-C* egg chambers beyond stage 9 (31), and oogenesis fails to progress beyond stage 10, we cannot be certain that this failure to activate *osk* translation is a specific consequence of *Bic-C* mutations.

DISCUSSION

***osk* is a potential target RNA for BIC-C.** Using a poly(U)-Sepharose binding assay and cell extracts expressing various forms of BIC-C, we have shown that BIC-C is an RNA-binding protein. We believe this activity is required for *Bic-C* function in vivo, based on the strong phenotype seen in alleles with mutations in the KH domains. *Bic-C*^{RU35} substitutes an arginine for a conserved glycine in the third KH domain (31), a change that is predicted to destabilize the domain by placing a charged residue into the hydrophobic core (36). We have shown that this mutation weakens RNA binding in vitro. Since *Bic-C*^{RU35} produces high levels of protein, its strong phenotype in vivo can be correlated with the RNA-binding defect we observed in vitro. Similarly, *Bic-C*^{AB79} produces high levels of a protein with 53 amino acids from KH domains 2 and 3 deleted and has a strong phenotype (31). While we infer a direct in vivo association between BIC-C and RNA from our results, we cannot exclude the possibility that the observed RNA-binding activity of BIC-C requires the presence of another protein or proteins in the Cos cell extracts.

Both of the *Bic-C* mutations that affect the KH domains also lead to premature translation of *osk* mRNA in oocyte stages 7 and 8, as does a *Bic-C* mutation (*Bic-C*^{AA4}) that is a protein null. A role for BIC-C in repressing *osk* translation could explain the mechanistic basis of the bicaudal phenotype observed at low penetrance in *Bic-C* heterozygotes. Restriction of OSK to the posterior pole of the developing oocyte is critical to embryonic anterior-posterior patterning. The premature *osk* translation observed in *Bic-C* mutants results in a diffuse distribution of OSK in the oocyte and thus may be directly responsible for the generation of bicaudal embryos. Females carrying an *osk* transgene with mutated BREs (*oskBRE*$^{-}$) also show premature translation of *osk*. Bruno protein is a translational repressor of *osk*, which prevents *osk* translation from occurring until *osk* RNA reaches the posterior pole, where Bruno-mediated repression is relieved (23, 51). The similarity between the *oskBRE*$^{-}$ and *Bic-C*$^{-}$ results suggests that BIC-C could act as a specific translational repressor like, and perhaps in coordination with, Bruno. As *Bic-C*$^{-}$ oocytes do not complete development and are never fertilized, and as maternal mutations in *Bic-C* also affect cellularization of the embryo (31), we cannot directly determine what embryonic patterning defects would result from homozygous *Bic-C*$^{-}$ oocytes.

The BIC-C protein binds ribohomopolymers differentially in vitro, a binding characteristic shared by several other KH proteins. However, despite clear differences in binding homopolymers, only two KH proteins, Nova-1 and Sam68, have been shown to bind a specific RNA sequence with high affinity (4, 27). In both cases the KH domains are necessary for high-affinity binding. Using the poly(U)-Sepharose assay with a number of candidate RNAs, including *osk*, as competitors, we have so far been unable to identify specific substrates for BIC-C (data not shown). Although it is possible that we have not yet tested the correct substrate RNA or that a modification of BIC-C not produced in Cos cells is required for specificity, it is more likely that BIC-C binds specifically to RNA only in the presence of cofactors. Identifying proteins that BIC-C interacts with will therefore be of great interest in the future. Intriguingly, BIC-C contains a SAM domain, a conserved domain which is believed to represent a protein binding domain and contains a site of tyrosine phosphorylation likely to serve as an SH2 domain binding site (40). We found that the strong allele *Bic-C*^{IIF34} produces a truncated protein with this region removed, suggesting that it may be important for *Bic-C* function, although we cannot rule out the possibility that the severity of this allele results from the reduced protein level also observed.

BIC-C is sensitive to small changes in expression. We found that the severity of the *Bic-C* alleles can be correlated with the amount of protein they produce, since strong alleles produce either no protein or proteins with identified lesions, while weak alleles produce levels of protein comparable to those of wild type. Moreover, for four weak alleles (*Bic-C*^{QL53}, *Bic-C*^{AR72}, *Bic-C*^{PX1}, and *Bic-C*^{AA29}), we did not identify any sequence changes in the coding region, suggesting that small differences in protein expression have phenotypic consequences. A fifth weak allele, *Bic-C*^{PE37}, does contain a point mutation in the coding region (S674G), but this mutation may destabilize the protein as the allele produces reduced levels of protein (Fig. 4B). These results indicate that the level of BIC-C is critical during oogenesis, an observation that is consistent with the haplo insufficiency of the locus (35).

***Bic-C* RNA may be translationally regulated.** Our finding that careful regulation of the level of BIC-C protein is critical during oogenesis would be consistent with translational regulation of *Bic-C*, and several lines of evidence support this hypothesis. First, the *Bic-C* RNA is localized to the oocyte as early as germarium region 2A (31), but the protein is not detectable until later stages (stages 3 and 4), consistent with translational repression of the *Bic-C* RNA. We also have observed a modest decrease in BIC-C protein levels in *vas*-null ovaries (46a). *vas* is a member of the DEAD family of RNA helicases, with similarity to the translation initiation factor eIF4A, and *vas* function is required for efficient translation of *osk*, *nos*, and *grk* (15, 32, 46, 47). Finally, preliminary results indicate that the *Bic-C*^{AA4} allele, which does not produce protein, contains a point mutation in the 5′ UTR of *Bic-C*. This mutation could potentially affect a translational regulatory element, and experiments are currently under way to determine whether 5′ UTR sequences are required to regulate *Bic-C* translation.

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