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Essential role for a heterogeneous nuclear ribonucleoprotein (hnRNP) in oogenesis: hrp40 is absent from the germ line in the dorsoventral mutant squid[1]

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ABSTRACT **The *Drosophila melanogaster* hrp40 proteins are abundant nuclear pre-mRNA-binding proteins that are similar to the heterogeneous nuclear ribonucleoprotein (hnRNP) A/B proteins of vertebrates. Recently, hrp40 has been shown to be encoded by the squid gene, which is required for dorsoventral axis formation during oogenesis. Eggs and embryos from homozygous squid[1] mothers are severely dorsalized, and complete deletion of the squid gene results in lethality. Here we have examined the expression and localization of hrp40 in wild-type and squid[1] mutant ovaries. Using a monoclonal antibody specific for hrp40, the same isoforms of hrp40 are detected in both wild-type and squid[1] ovaries, but the amount of hrp40 is reduced in squid[1] ovaries. Furthermore, immunolocalization of hrp40 in wild-type egg chambers shows that hrp40 is present in the nurse cells, oocyte, and follicle cells. In contrast, in squid[1] mutant egg chambers, hrp40 is absent from the germ-line-derived nurse cells and oocyte, but it is detected in the somatic follicle cells. The absence of hrp40 from the germ-line-derived cells of developing egg chambers is likely to lead to the striking dorsalized phenotype of squid[1] eggs. In addition, dramatic stage-specific changes in the cellular localization of hrp40 are seen; the protein found in the nurse cell nuclei during early stages of oogenesis migrates to the cytoplasm at later stages. These findings reveal dynamic patterns of expression and localization of hnRNP proteins during development and provide evidence for an essential role for hnRNP proteins.**

Heterogeneous nuclear ribonucleoproteins (hnRNPs), the abundant nuclear RNA-binding proteins that associate with heterogeneous nuclear RNA (or pre-mRNA) as it is transcribed, participate in multiple steps of mRNA biogenesis (1). To facilitate the genetic analysis of hnRNP protein function, the major hnRNP proteins of *Drosophila melanogaster*, which are similar in overall primary structure to the vertebrate hnRNP A/B proteins, have recently been characterized (2–4). Members of this highly conserved family of RNA-binding proteins have a modular primary structure consisting of two RNP consensus (RNP-CS) RNA-binding domains and a carboxyl-terminal glycine-rich domain, or "2x-RBD-Gly." Several *D. melanogaster* proteins with homology to the RNP-CS family of RNA-binding proteins are required for diverse processes including sex determination (5–7), vision and behavior (8–11), and spermatogenesis (12), but the phenotype associated with a mutation in a gene encoding an hnRNP protein has not been known. However, one of the major *D. melanogaster* hnRNP proteins, hrp40, was recently found to be encoded by the squid gene, which was identified during a screen of female sterile mutants (13). The first mutant allele of the squid gene identified in this screen, squid[1], is a *P*-element insertion in the 5′ untranslated region of the squid gene. Females homozygous for the squid[1] allele, which specifically disrupts oogenesis, lay dorsalized eggs that do not survive to adulthood. However, in more severe alleles of squid, such as squid^{ix50}, individuals homozygous for deletions in the first exon of the squid gene die as larvae, indicating that hrp40 plays an essential function in nonovarian tissue also (13). Thus, hrp40 provides an example of an essential function for a typical abundant hnRNP protein, and the squid mutants provide an opportunity to examine the function of these pre-mRNA binding proteins *in vivo*. A comparison of the transcripts produced from the squid gene in wild-type and squid[1] ovaries showed that a subset of ovary-specific transcripts are absent in mutant ovaries (13). However, the expression of the hrp40 proteins was uncharacterized. Here, to gain insight into the role(s) of hrp40 during the establishment of embryonic polarity, we have examined the expression and localization of this protein during oogenesis in wild-type and squid[1] ovaries. We find that hrp40 is present in the nurse cells and oocyte and follicle cells of wild-type egg chambers and that it has a dynamic pattern of distribution. In contrast, hrp40 is present in the follicle cells but is absent from the nurse cells and oocyte of squid[1] egg chambers, providing evidence for an essential role for hrp40 in the germ line during oogenesis.

MATERIALS AND METHODS

Fly Stocks. Oregon-R flies were used as wild type. The genotype of the squid[1] mutant stock used here was *ry*[*sqd*[1]*ry*[+]]*cv–c sbd* balanced with TM3 (13).

Immunostaining of Ovaries. Immunostaining of ovaries was performed as described by Xue and Cooley (14) using a 1:1000 dilution of monoclonal antibody (mAb) 8D2 in PBT buffer (PBS containing 0.5% bovine serum albumin BSA/0.3% Triton X-100). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cappel) diluted 1:500 in PBT buffer was used as a secondary antibody, and optical sections were obtained with a model MRC600 laser scanning confocal microscope (Bio-Rad). Immunolocalization experiments were repeated several times, and ovaries stained in parallel with nonspecific mouse mAbs were included to provide a reference for the level of background staining each time. Images were photographed on Kodak T-Max 100 film.

Gel Electrophoresis and Immunoblotting. Ovaries dissected in PBS from 3-day-old females were transferred to isofocusing sample buffer (49% urea/1.71% Nonidet P-40/1.71% Bio-Lyte 3/10 ampholytes) and sonicated three times for 5 sec each using a microtip fitted on a Heat Systems/

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Abbreviations: hnRNP, heterogeneous nuclear ribonucleoprotein; mAb, monoclonal antibody; DM, dorsalizing morphogen.

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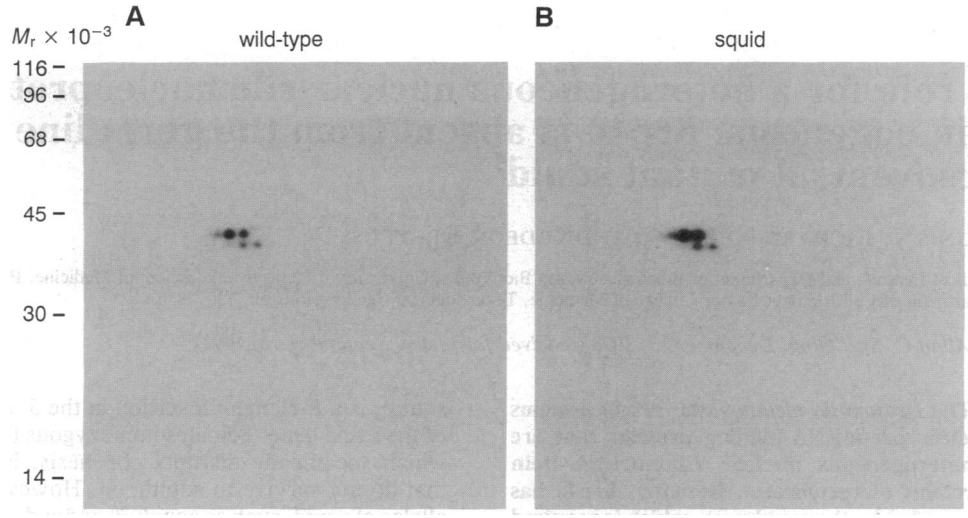

FIG. 1. Expression of hrp40 proteins in wild-type and squid mutant ovaries. Total proteins from wild-type (*A*) and squid[1] (*B*) ovaries were simultaneously resolved by two-dimensional gel electrophoresis, transferred to nitrocellulose, and probed with the anti-hrp40 mAb 8D2. Molecular weight markers are indicated on the left.

Ultrasonics W-380 sonicator. Dithiothreitol was added to 20 mM, and proteins (corresponding to eight ovaries per gel) were separated by two-dimensional nonequilibrium pH-gradient electrophoresis as described by O'Farrell *et al.* (15). The first dimension was separated using a pH 3–10 ampholine gradient at 400 V for 4 hr. The second dimension was separated by SDS/PAGE as described previously (16) using 12.5% acrylamide in the separating gel. Immunoblotting was done as described previously (17) using a 1:1000 dilution of mAb 8D2.

RESULTS AND DISCUSSION

Three major isoforms of hrp40 are detected in *D. melanogaster* embryos and in Schneider's 2 (S2) cells (3). These isoforms, generated by alternative splicing, differ only slightly from one another in their carboxyl-terminal glycine-rich domains and share a common epitope recognized by mAb 8D2 (2, 3, 13). Northern blotting analysis indicated that a complex set of at least six transcripts encoding the three major hrp40 isoforms is produced in wild-type ovaries, while in squid[1] ovaries four of these transcripts are much less abundant (13). However, the level of hrp40 protein expression and its localization in ovaries was not previously known. To compare the hrp40 protein isoforms produced in wild-type and squid[1] ovaries, and to verify the specificity of mAb 8D2 in this tissue, two-dimensional immunoblots of total protein isolated from wild-type and squid[1] ovaries were probed (Fig. 1). When resolved by two-dimensional SDS/PAGE, each of the three major isoforms appears as two or more spots, the more acidic isoforms arising from phosphorylation (M. J. Matunis and G.D., unpublished work). The same isoforms of hrp40 that are detected in embryos are specifically detected in wild-type ovaries (Fig. 1*A*) and in squid[1] ovaries (Fig. 1*B*). However, the amount of hrp40 in squid[1] ovaries is about half that found in the same number of wild-type ovaries; the immunoblot in Fig. 1*B* was exposed twice as long as that in Fig. 1*A* in order to visualize all of the isoforms.

Since hrp40 proteins were detected in both wild-type and squid[1] ovaries by immunoblotting, it was of interest to compare the subcellular distribution of hrp40 during oogenesis by indirect immunofluorescence in wild-type and squid[1] egg chambers. In *Drosophila*, oogenesis is divided into 14 stages, based on the morphological changes that occur as the oocyte develops from a cyst containing 16 interconnected germ line cells (reviewed in refs. 18 and 19). Fifteen of these cells develop into nurse cells that provide the 16th cell, the oocyte, with RNA and protein. The surrounding somatic follicle cells secrete the chorion onto the surface of the oocyte. The nurse cells and follicle cells become highly polyploid and are transcriptionally active, while the oocyte nucleus remains diploid and is relatively quiescent transcriptionally. A comparison of the localization of hrp40 in wild-type and squid[1] stage 10 egg chambers by indirect immunofluorescence reveals that its distribution differs strikingly (Fig. 2). In wild-type egg chambers, hrp40 is detected at high levels in the nurse cells and follicle cells at stage 10 (Fig. 2*A*). However, in squid[1] egg chambers, hrp40 is conspicuously absent from the nurse cells at stage 10, but the follicle cell staining remains (Fig. 2*B*). A closer examination of the localization of hrp40 in squid[1] egg chambers using confocal microscopy reveals that hrp40 is largely absent from the germ-line-derived nurse cells and oocyte throughout oogenesis, and is detected primarily in the somatic follicle cells

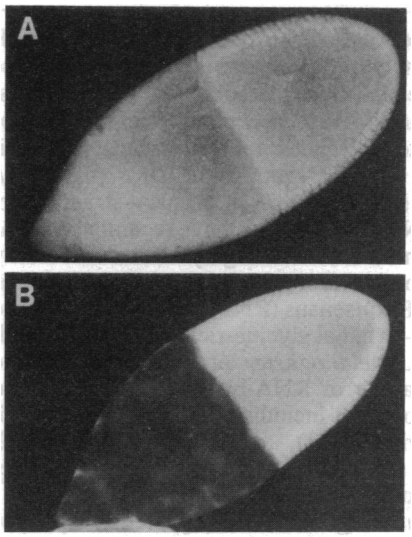

FIG. 2. Comparison of the localization of hrp40 in wild-type and squid egg chambers. Egg chambers from wild-type (*A*) and squid[1] (*B*) females were immunostained with the anti-hrp40 mAb 8D2 and visualized by standard epifluorescence microscopy. Stage 10 egg chambers are shown. The anterior of the egg chambers (which contains the nurse cells) is toward the lower left, posterior toward the upper right.

(Fig. 3). Very low levels of nuclear staining are seen in the germ-line-derived cells of squid[1] egg chambers during stages 1–6. We estimate that the level of hrp40 in the nuclei of stages 1–6 squid[1] nurse cells in Fig. 3 is about 1/10th that seen in the nuclei of wild-type nurse cells at the corresponding stages (Fig. 4). However, there is no hrp40 detected in the cytoplasm of squid[1] nurse cells or oocytes at any stage, and by stage 7 hrp40 has disappeared entirely from the germ-line-derived cells of squid[1] egg chambers. The absence of hrp40 in the germline of squid[1] egg chambers seen at stage 7 persists through stage 10 (stages 1–9 are shown in Fig. 3). We note that a few of the follicle cells in stages 1–7 squid[1] egg chambers contain very little hrp40. It is possible that these cells produce higher levels of hrp40 at later stages, since this heterogeneity is not apparent after stage 7. The lack of hrp40 staining observed in the nurse cells and oocyte of squid[1] egg chambers is not due to the inability of the mAb to penetrate these egg chambers, since the subset of follicle cells that migrate to a position deep within the stage 9 egg chambers, the border cells, are stained intensely with mAb 8D2 (Fig. 3 arrow). Thus, the dorsalized phenotype observed in squid[1] egg chambers seems to result from the loss of hrp40 in the germ cells rather than in the somatic follicle cells.

In *D. melanogaster* the dorsoventral axis is specified during oogenesis by a complex cascade of events involving at least 18 genes (20). To better understand how hrp40 functions during this process, the localization of hrp40 in stages 1–10 wild-type egg chambers was analyzed in detail by confocal microscopy (Fig. 4). hrp40 is present in all three cell types composing the egg chamber—the nurse cells, the oocyte, and the follicle cells. In the follicle cells, hrp40 is present in both the nucleus and cytoplasm, and it is excluded from the nucleoli throughout oogenesis (Fig. 4, stages 1–10). However, in the nurse cells and the oocyte, the distribution of hrp40 is surprisingly dynamic. In the nurse cells, hrp40 is

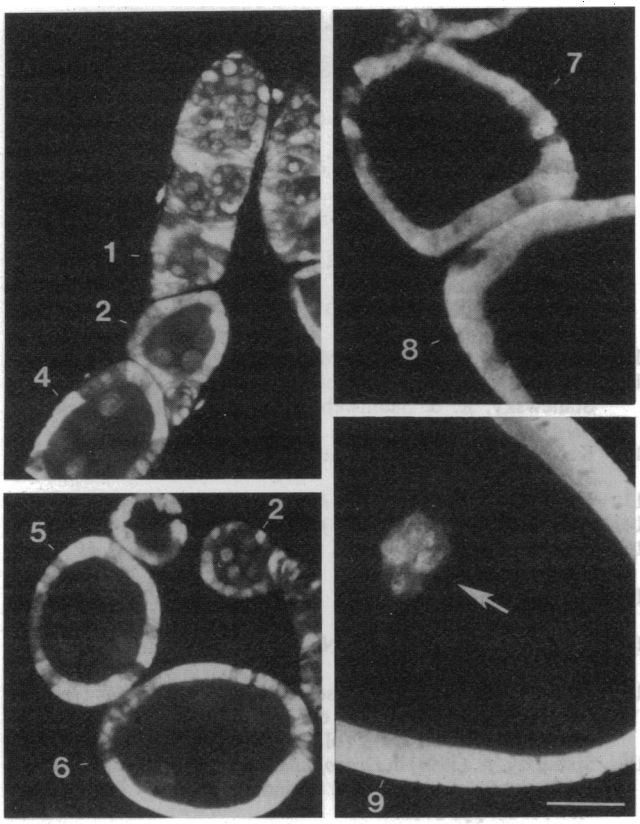

FIG. 3. Localization of hrp40 in squid egg chambers. Confocal micrographs of squid[1] egg chambers stained with the anti-hrp40 mAb 8D2. Stages of selected egg chambers are denoted with numbers. The arrow indicates the somatic follicle cells (border cells) located at the anterior margin of the stage 9 oocyte. (Bar = 25 μm.)

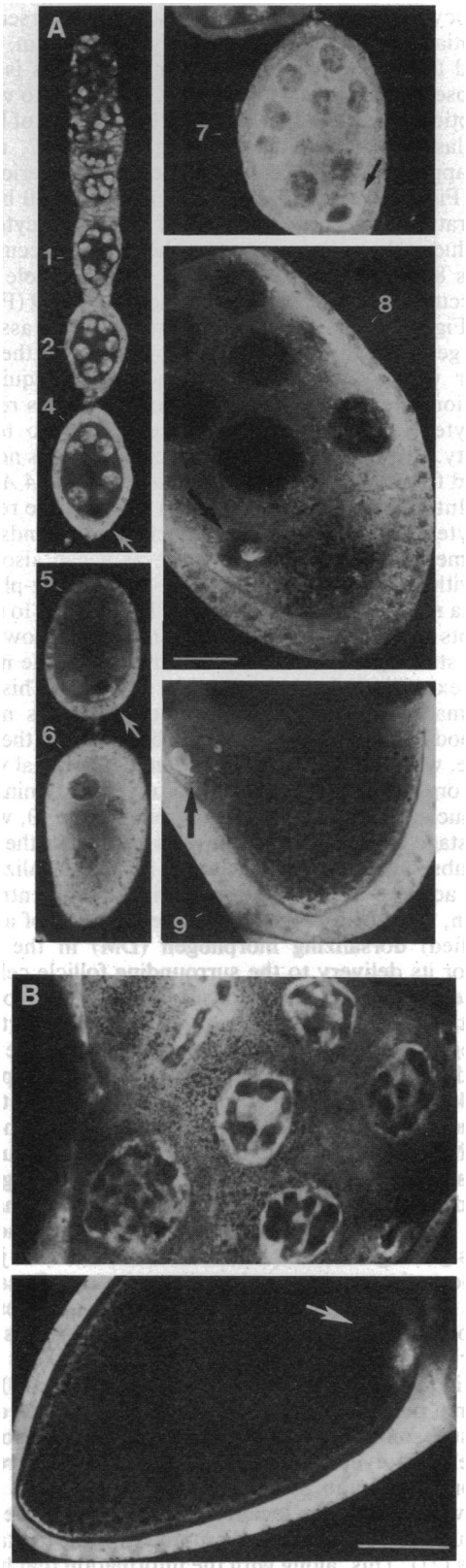

FIG. 4. Localization of hrp40 in wild-type egg chambers. Egg chambers from wild-type females were immunostained with the anti-hrp40 mAb 8D2 and visualized by confocal microscopy. Stages of selected egg chambers are numbered. Arrows indicate the oocyte nucleus. (*A*) Stages 1–9. (Bar = 25 μm.) (*B*) Anterior (*Upper*) and posterior (*Lower*) halves of a stage 10 egg chamber. (Bar = 50 μm.)

initially restricted to the nuclei (Fig. 4*A*, stages 1–4), but it later becomes cytoplasmic (Fig. 4*A*, stages 5–9), and it is both nucleoplasmic and cytoplasmic at stage 10 (Fig. 4*B Upper*). In the oocyte, the pattern of accumulation is reversed; hrp40 preferentially accumulates in the oocyte cytoplasm, but it is excluded from the oocyte nucleus initially. This is consistently observed at stage 4 (although it is difficult to visualize in the optical section in Fig. 4*A*). The localization of hrp40 in the ooplasm remains similar during stages 4–7, and the protein appears to be concentrated near the posterior of the oocyte (Fig. 4*A*). As vitellogenesis ensues, hrp40 becomes concentrated at the cortical regions of the oocyte cytoplasm. This includes the anterior cortex, where hrp40 accumulates at stages 8 and 9 (Fig. 4*A*), and the posterior pole, where hrp40 accumulates at stages 8 (data not shown), 9 (Fig. 4*A*), and 10 (Fig. 4*B*). This suggests that hrp40 may be associated with the germ plasm, the specialized cytoplasm at the oocyte posterior whose assembly during oogenesis requires the localization of specific RNAs and proteins to this region of the oocyte (19). Further studies are needed to test this possibility. Beginning at stage 8, however, hrp40 is no longer restricted from entering the oocyte nucleus (Fig. 4 *A* and *B*, arrow). Intense hrp40 staining is seen in a discrete region of the oocyte nucleus, which most likely corresponds to the karyosome, or condensed chromatin, as it can also be detected with DNA stains such as 4′,6-diamidino-z-phenylindole (data not shown), suggesting that hrp40 binds to nascent transcripts synthesized in the oocyte nucleus. A lower level of hrp40 staining is seen throughout the rest of the nucleus, with the exception of a dark spherical structure. This sphere is presumably the endobody, a structure that is not well understood but has been suggested to be related to the sphere organelle, which has been described in the germinal vesicles of many organisms (19). The pattern of hrp40 staining in the oocyte nucleus at stage 8 persists through stage 10, which is the last stage examined here (the chorion covers the oocyte during subsequent stages, precluding immunolocalization).

hrp40 acts at an early stage during dorsoventral axis formation, perhaps by regulating the expression of a (as yet unidentified) dorsalizing morphogen (DM) in the oocyte nucleus or its delivery to the surrounding follicle cells (13). Our observations here are consistent with both of these possibilities. The accumulation of hrp40 in the oocyte cytoplasm beginning at stage 4, and its presence near the oocyte nucleus during stages 8–10, is consistent with the hypothesis that hrp40 functions in the cytoplasm by localizing the DM mRNA near the oocyte nucleus. Upon translation, the localized mRNA would produce a gradient of protein, such that only those follicle cells closest to the source of the gradient become dorsalized. In this scenario, in squid[1] egg chambers the DM mRNA would be free to diffuse throughout the oocyte, signaling all of the follicle cells, rather than just the subset near the oocyte nucleus, to adopt a dorsal fate. Epistatic interactions between squid and other genes required for dorsoventral axis formation support this model (13). For example, squid acts upstream of gurken, a gene required in the germ line for dorsoventral polarity (13). Since we have not performed mosaic analysis, the question of germ line versus somatic requirement for squid has not been definitively resolved here. However, gurken has recently been cloned and the striking distribution of the gurken mRNA, which becomes localized to the dorsal side of the oocyte nucleus at stage 8, is disrupted in squid mutant egg chambers (21). This, along with the information that hrp40 is an avid RNA-binding protein with a pattern of distribution overlapping that of the gurken mRNA, strongly suggests that hrp40 interacts directly with the gurken mRNA to effect its localization in the germ line. However, since hrp40 accumulates in the oocyte nucleus during stages 8–10, an alternative model in which hrp40 suppresses the level of functional DM mRNA by regulating aspects of nuclear pre-mRNA metabolism (such as alternative splicing, RNA stability, polyadenylylation, and export) cannot be ruled out completely. Thus, in squid[1] egg chambers an excess of functional DM could be produced, causing all of the follicle cells to adopt a dorsal fate. In support of a role for hnRNP proteins during alternative splicing, a mammalian 2x-RBD-Gly protein, hnRNP A1, can regulate alternative splicing *in vitro* (22, 23). The complex pattern of hrp40 localization seen here represents all three major isoforms of hrp40 and is consistent with both of the models discussed above. However, transcripts encoding different isoforms of hrp40 are differentially produced in ovaries and in somatic tissues, suggesting that the individual isoforms may be functionally distinct (13). Determining whether the individual protein isoforms of hrp40 are functionally distinct, and whether they are differentially localized, should help to determine the specific roles of hrp40 during oogenesis. It is also clear that *Drosophila* hnRNP proteins can be both nuclear and cytoplasmic proteins and can have essential roles during development. We anticipate that other hnRNP proteins will be found to share these characteristics, both in *Drosophila* and in other systems.

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