Requirement for a Noncoding RNA in Drosophila Polar Granules for Germ Cell Establishment

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In Drosophila embryos, germ cell formation is induced by specialized cytoplasm at the posterior of the egg, the pole plasm. Pole plasm contains polar granules, organelles in which maternally produced molecules required for germ cell formation are assembled. An untranslatable RNA, called Polar granule component (Pgc), was identified and found to be localized in polar granules. Most pole cells in embryos produced by transgenic females expressing antisense Pgc RNA failed to complete migration and to populate the embryonic gonads, and females that developed from these embryos often had agamic ovaries. These results support an essential role for Pgc RNA in germ line development.

Fig. 1. Distribution of Pgc RNA during oogenesis and embryogenesis. (A) Germarium through stage 6; Pgc RNA is expressed from germarium region 2B and localized in the posterior region of the oocyte. (B) Stage 7 and (C) stage 9 egg chambers showing Pgc RNA localization to the anterior, close to the oocyte–nurse cell border. (D) Stage 11 egg chamber with Pgc RNA enriched at the posterior pole plasm of the oocyte. No detectable signal in somatic follicle cells was observed at any stage of oogenesis. (E) Cleavage stage embryo in which the Pgc RNA is highly concentrated in pole plasm. (F) Cellular blastoderm embryo and (G) stage 10 embryo with Pgc RNA incorporated into pole cells. (H) Cleavage embryo hybridized with sense Pgc probe as a control. (I and J) In situ hybridization examined at the electron microscopic level reveals that Pgc RNA is localized in polar granules in (I) the pole plasm of cleavage embryos and (J) the pole cells at the syncytial blastoderm stage. The embryo in (I) was embedded, thin-sectioned, and hybridized with a double-stranded DIG-labeled Pgc RNA probe after sectioning (23); the embryo in (J) was hybridized before embedding. In both cases the Pgc probe hybridized over the entire polar granule. Bar, 200 nm; M, mitochondrion; pg, polar granule.
the pole plasm and in the pole cells of syncytial blastoderm embryos (Fig. 1, I and J). Within the pole granules the distribution of Pgc RNA differs from that of mtlrRNA. mtlrRNA is concentrated on the surface of polar granules, frequently at the boundaries between polar granules and mitochondria of early-cleavage embryos; after pole cell formation, mtlrRNA signal is undetectable on polar granules (13). In contrast, a Pgc probe hybridized throughout the entire pole granule, and signals were detected even on polar granules in pole cells.

We cloned more than 30 Pgc cDNAs (14) that hybridize to a major transcript of 0.7 kb and a minor transcript of 1.3 kb; the expression level of the larger transcript was less than 1% of that of the smaller. Pgc is expressed only in female germ cells. Both transcripts were detected in RNA prepared from fertile adult females, ovaries, and early-stage embryos; however, the transcripts were undetectable in RNA prepared from late-stage embryos, larvae, and pupae and from adult males and sterile females from osk56/ok301 mothers, which produce embryos that fail to form pole cells at 25°C (15). Sequence analysis of the cDNAs and corresponding genomic DNA indicates that both transcripts are derived from the same

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**Fig. 2.** (A) Genomic organization around Pgc. A total of 4.8 kb of genomic sequence containing Pgc has been deposited in GenBank under accession number U66411. Sequence specific to the minor 1.3-kb transcript region is delineated by a striped box. The gp150 gene (16) ends about 800 base pairs (bp) upstream from the Pgc transcription initiation site. A putative type III alcohol dehydrogenase gene (T3dh; BLAST scores 4.6 × 10−12 with Bacillus methanolicus C1 methanol dehydrogenase (24); 6.9 × 10−43 with a partial human cDNA clone [GenBank accession number H78978]) is transcribed from the opposite strand of sequences overlapping the Pgc intron and a portion of the exon specific to the 1.3-kb Pgc transcript (striped box). T3dh is transcribed in 12- to 24-hour embryos and larvae. (B) Nucleotide sequence of the 0.7-kb cDNA of Pgc (sequences corresponding to both the smaller and larger transcripts have been deposited in GenBank under accession numbers U66409 and U66410, respectively). A putative polyadenylation signal [AAATA, frequently used in Drosophila genes that are expressed in ovaries (25)] is indicated by double underlining. (C) Alignment of the potential translational start site for the longest ORF in the 0.7-kb Pgc transcript with a consensus sequence derived from actual translational start sites (17). Frequency refers to the percentage of actual start sites, as given in (17), that have the same nucleotide as does the Pgc sequence in the listed position. Rank refers to how frequent a particular nucleotide found in Pgc is in actual start sites; a value of 1 means the most common, a value of 4 means the least common. (D) Codon usage table for a 46-amino-acid (AA) ORF (nucleotides 117 to 254) whose AUG codon is in a favorable context for translation. For all amino acids encoded by more than one codon and present in the ORF, the expected percentage (%) in Drosophila ORFs, as computed from published tables (26), is compared with the actual distribution of codons (Ω) in the Pgc ORF. Although some amino acids (notably Ser, Asp, Glu, and Cys) are encoded favorably, many others (such as Arg, Phe, Ala, Pro, Thr, and Gly) diverge substantially from Drosophila codon usage. The longest ORF in the minor 1.3-kb Pgc transcript extends for 92 codons; this ORF largely overlaps the T3dh coding sequence on the opposite strand and also has poor Drosophila codon usage.
gene (Fig. 2A). Pgc maps to a gene-rich area of chromosome region 58D, with the 3’ end of the gp150 gene (16) less than 1 kb proximal to the 5’ end of Pgc. A putative type III alcohol dehydrogenase gene (T3dh), transcribed from the opposite strand, is nested in the Pgc intron and overlaps a portion of the exon specific to the minor 1.3-kb Pgc transcript (Fig. 2A). For the following reasons we conclude that Pgc encodes an untranslatable RNA. In the major 0.7-kb transcript, the longest open reading frame (ORF) (nucleotides 480 to 692; Fig. 2B) would encode a polypeptide of 71 amino acids, but its AUG codon is in an extremely poor context for translation initiation (17) (Fig. 2C). A shorter 46-codon ORF (nucleotides 117 to 254) begins with an AUG in a good translation initiation context, but it has poor Drosophila codon usage (Fig. 2D). No highly homologous [probability of a chance match, P(N), < 10⁻⁴] sequences were obtained in BLAST searches of the nonredundant nucleic acid sequence database when any ORFs or the nucleotide sequences of either Pgc transcript were analyzed.

We examined embryos produced by mothers homozygous for various posterior-group mutations to determine the effects of such mutations on Pgc RNA localization. Embryos from osk, vas, and tud homozygous females failed to localize Pgc RNA in pole plasm (Fig. 3, A to C), and Pgc RNA is undetectable at the cellular blastoderm stage in these embryos. In contrast, nos embryos localized Pgc RNA normally and incorporated it into pole cells (Fig. 3D). Ectopic Pgc RNA localization to the anterior was observed (Fig. 3E) in embryos from females carrying the osk-bcd3'UTR transgene (5). In embryos from either Bicaudal-C or Bicaudal-D females, Pgc RNA was mislocalized to the anterior in a diffuse manner (Fig. 3, F and G), as has been reported for other pole plasm RNAs (4).

To produce flies with reduced Pgc function, we made transgenic lines carrying a hybrid gene in which antisense Pgc is expressed under the control of the hsp70 promoter (18). To eliminate nonspecific deleterious effects on subsequent embryonic development, which we observed when even wild-type flies were heat shocked during mid- to late oogenesis, in subsequent experiments we analyzed the effect of antisense Pgc expression on pole cell development by comparing embryos from females carrying two copies of the hsp70-AS-Pgc transgene (2×AS-Pgc embryos) cultured at constant temperature (25°C) without heat shocking. As judged by in situ hybridization with a strand-specific Pgc probe, the amount of localized Pgc RNA was greatly reduced in 2×AS-Pgc embryos (Fig. 4, A and B). Although Pgc is expressed in female germ cells throughout oogenesis, we did not observe any defect in oogenesis in females expressing antisense Pgc.

We analyzed the spatial distributions of several RNAs and proteins that are localized in pole plasm in 2×AS-Pgc embryos. The posterior concentration of all pole plasm components analyzed appeared to be essentially normal in these embryos at the cleavage stage (Fig. 4, C and D); however, in postblastodermal development, localized nos, gcl, and VAS signals were reduced in intensity (Fig. 4, E to K). Furthermore, we observed defects in pole cell migration in the 2×AS-Pgc embryos. In wild-type embryos, an average of 28 pole cells complete migration and associate with mesodermal tissue during stage 14 to form the two embryonic gonads (6) (Fig. 4, I, K, and M). In 2×AS-Pgc embryos, the ability of pole cells to complete migration and colonize the gonad is dramatically impaired (Fig. 4, J, L, and N). Three of four 2×AS-Pgc lines, with substantially reduced Pgc RNA concentrations, showed a slight reduction from 34 to 25 and 27 in the number of VAS-positive migrating pole cells at stage 14 (Table 1). In subsequent development, many pole cells died or failed to migrate into the embryonic gonads; at stage 14 the median pole cell number was four to five
per gonad in the three 2×AS-Pgc lines (Table 1). To confirm these effects on adult fertility, we examined the gonads of adult females that developed from 2×AS-Pgc embryos. Most embryos from these lines hatched and completed development, but, consistent with the failure of pole cells to colonize the embryonic gonads, up to 53% of adult ovaries were agametic (Table 1). These defects in germ cell proliferation correlate with a specific decrease in the amount of Pgc RNA (Table 1).

Our results suggest that the untranslatable Pgc RNA has an essential role in the differentiation of pole cells into functional, proliferative germ cells. In contrast to gcl, which is thought to be primarily required for pole cell formation (9, 11), reduction of the Pgc RNA concentration has only a modest effect on initial pole cell formation. However, between stages 12 and 14, pole cells in 2×AS-Pgc embryos are severely compromised in their ability to migrate into the gonads and develop into functional germline stem cells. We believe that the effects we observed of antisense Pgc expression on germ cell establishment result from a specific interference with endogenous Pgc function for the following reasons: bicaudal and osk RNAs were normally localized in cleavage embryos from all of the hsp70-AS-Pgc lines (19), and 2×AS-Pgc eggs hatched at high efficiency and developed into viable morphologically normal adults (Table 1). We hypothesize that reduction of the Pgc RNA concentration in the antisenes lines leads to reduced stability of polar granules after their initial formation because Pgc RNA is an integral component of polar granules and the concentrations of various pole plasm components are reduced in postblastodermal pole cells of 2×AS-Pgc embryos. No abdnomal defects were observed in 2×AS-Pgc embryos; however, because our results are based on a reduction of localized Pgc RNA concentrations, we cannot exclude a role for Pgc in abdominal specification. Null mutations may reveal additional functions for Pgc.

In both Drosophila and Xenopus, germ plasm can induce germ cell fate (1, 20). In addition, specific components of germ plasm appear to be conserved between these two evolutionary divergent animals (8, 21). A group of untranslated RNAs, called XSLR, are localized in Xenopus germ plasm and are required for anchoring of Vg1 RNA to the vegetal cortex of the oocyte (22). Although the exact role, if any, of XSLR in germ cell establishment is unclear, our results suggest that, like the XSLR, Pgc RNA functions in the maintenance of germ plasm integrity. Further analysis of the composition and role of Drosophila polar granules will be of relevance to understanding the molecular basis of germ cell determination in both invertebrates and vertebrates.

### Table 1. Correlation between Pgc RNA amount and numbers of functional pole cells in progeny from females carrying two copies of the hsp70-AS-Pgc transgene. Relative Pgc RNA amount was determined by densitometric quantitation of Northern (RNA) hybridizations of a strand-specific probe to polyadenylated RNA from ovaries of the indicated lines. The filter was rehybridized with a probe for the ribosomal protein gene Pps714a (30) for loading control. Hatch rate, pole cell numbers, and ovary phenotype were scored for progeny from females of the indicated lines mated with w– males. Agametic ovaries were frequently observed in w– progeny from females carrying one copy of hsp70-AS-Pgc mated with w– males, indicating that the agametic ovary phenotype was caused by maternally supplied antisense Pgc RNA.

<table>
<thead>
<tr>
<th>Line</th>
<th>Pgc RNA amount (%)</th>
<th>Hatching rate percent (n)</th>
<th>Number of pole cells/embryo</th>
<th>Distribution of pole cell number in gonads of stage 14 embryos</th>
<th>Adult ovaries</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>With eggs/Agametic</td>
<td></td>
</tr>
<tr>
<td>w–</td>
<td>100</td>
<td>95.8 (409)</td>
<td>34.2 ± 6.2 (44)</td>
<td>100</td>
<td>310</td>
</tr>
<tr>
<td>AS19</td>
<td>63</td>
<td>76.5 (562)</td>
<td>32.8 ± 5.5 (36)</td>
<td>175 2 1 1 0 39 26 39 19</td>
<td>586</td>
</tr>
<tr>
<td>AS26</td>
<td>2</td>
<td>94.9 (196)</td>
<td>27.4 ± 6.2 (55)</td>
<td>94 8 39 35 30 24 18 19 19</td>
<td>266</td>
</tr>
<tr>
<td>AS55</td>
<td>12</td>
<td>93.4 (455)</td>
<td>25.7 ± 6.5 (45)</td>
<td>103 35 27 30 24 18 19 19</td>
<td>275</td>
</tr>
<tr>
<td>AS80</td>
<td>40</td>
<td>82.9 (316)</td>
<td>25.0 ± 6.3 (25)</td>
<td>99 20 18 23 20 32 30 410 410</td>
<td>112</td>
</tr>
</tbody>
</table>

*Pgc RNA amounts normalized to Pps714a RNA amounts and presented relative to the w– control. †Numbers of cells that stained with affinity-purified anti-VAS. **Wild-type stage 14 gonads have an average of 14 pole cells (8).
was digested with Eco RI and Xba I. This fragment was subcloned into the pCOSpeR-hs vector [C. S. Thummel and V. Pirrotta, Drosophila Inf. Serv. 71, 150 (1992)] to generate the hsp70-AS-Pgc transgene. This plasmid was introduced into the germ line of Drosophila with P element-mediated transformation [A. C. Spradling and G. M. Rubin, Science 218, 341 (1982)]. Because pCOSpeR-hs contains the mini-white (w^+) gene, transformed w^+ flies show orange to red eye color.


23. For in situ hybridization, we used procedures previously described [R. Amikura, S. Kobayashi, K. Endo, M. Okada, Dev. Growth Differ. 35, 617 (1993); further details of the postembedding procedure will be reported elsewhere (R. Amikura et al., in preparation)].


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**Evidence for the Conformation of the Pathologic Isoform of the Prion Protein Enciphering and Propagating Prion Diversity**

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The fundamental event in prion diseases seems to be a conformational change in cellular prion protein (PrP^C) whereby it is converted into the pathologic isoform PrP^Sc. In fatal familial insomnia (FFI), the protease-resistant fragment of PrP^Sc after deglycosylation has a size of 19 kilodaltons, whereas that from other inherited and sporadic prion diseases are abnormal conformers of a normal, host-encoded protein designates PrP^C (3, 4). PrP^C has a high α-helical content and is virtually devoid of β-sheets, whereas PrP^Sc has a high β-sheet content (4, 5); thus, the conversion of PrP^C into PrP^Sc involves a profound conformational change. Formation of PrP^Sc is a posttranslational process that does not appear to involve a covalent modification of the protein (6).

The prion diseases are unique in that they may present as inherited and infectious disorders (3, 7). More than 20 different mutations of the human (Hu) PrP gene segregate with dominantly inherited disease; five of these have been genetically linked to familial Creutzfeldt-Jakob disease (fCJD), Gerstmann-Sträussler-Scheinker disease, and fatal familial insomnia (FFI) (8). The most common prion diseases of animals are scrapie of sheep and bovine spongiform encephalopathy; the latter may have been transmitted to people through foods (9).

To extend studies on the transmission of wild-type and mutant prions from sporadic Creutzfeldt-Jakob disease (sCJD) and iCJD patients, respectively, to transgenic mice expressing a chimeric mouse-human PrP gene (Tg(MHu2M) mice) (10, 11), we inoculated these mice with mutant prions from the brains of patients who died of FFI. Transmission of human prions to Tg(MHu2M) mice involves the conversion of chimeric MHu2M PrP^c into MHu2M PrP^Sc through a process that is thought to involve the binding of PrP^Sc to PrP^C as PrP^C undergoes a structural transition (12, 13). A point mutation of the PrP gene at codon 178 [in which an Asp residue at position 178 is mutated to Asn (D178N)] is the cause of FFI, but a Met residue must be encoded at position 129 on the mutant allele for the FFI phenotype to be manifest (14). The same D178N mutation segregates with a subtype of iCJD, but in this case, Val is encoded on the mutant allele at position 129. The D178N mutation is thought to destabilize the structure of PrP^C, resulting in its transformation into PrP^Sc (13, 15). Some investigators have reported transmission of FFI prions to non-Tg and Tg(HuPrP) mice; the incubation times exceeded 400 days, and only a minority of the inoculated Tg(HuPrP) mice expressing both human and mouse PrP^C developed disease (16). These findings with Tg(HuPrP) mice are in accord with earlier studies showing that transmission of human prions to Tg(HuPrP) mice is inhibited by mouse PrP^C, and this inhibition can be abolished by ablation of the mouse PrP gene (Prnp^0/0) (10, 11).

Tg(MHu2M)Prnp^0/0 mice (17) were inoculated intracerebrally with extracts prepared from brain tissue obtained after the death of individuals who died of FFI, iCJD(E200K) (with a mutation in which Glu at position 200 has mutated to Lys), or sCJD. The mice developed signs of experimental prion disease about 200 days after inoculation (Table 1). At the time of writing, inoculation of Tg(MHu2M)Prnp^0/0 mice has resulted in primary passage of prions from at least one brain region from...
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