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The *germ cell-less* Gene Product: A Posteriorly Localized Component Necessary for Germ Cell Development in Drosophila

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Summary

The first cell fate specification process in the Drosophila embryo, formation of the germline precursors, requires posteriorly localized germ plasm. We have cloned a gene, *germ cell-less* (*gcl*), required for germline formation. Posterior localization of the *gcl* messenger RNA (mRNA) requires the function of those genes essential for the localization of both *nanos* RNA, which specifies the abdomen, and the germ cell determinants. Mothers with reduced *gcl* function give rise to sterile adult progeny that lack germ cells. In embryos with reduced maternal *gcl* product, the germ cell precursors fail to form properly. Consistent with this phenotype, gcl protein specifically associates with those nuclei that later become the nuclei of the germ cell precursors. These observations suggest that *gcl* functions in the germ cell specification pathway.

Introduction

The asymmetric distribution of cytoplasmic components within the egg is a mechanism for early cell fate specification in vertebrates and invertebrates (for review see Beams and Kessel, 1974; Davidson, 1986). In particular, formation of germ cell precursors in a number of species ranging from insects to amphibians depends on the localization of the germ plasm, which is often cytologically distinct (Beams and Kessel, 1974; Davidson, 1986). In Drosophila, the cytologically defined pole plasm or functionally defined germ plasm is localized at the posterior pole of the egg and is necessary for the formation of pole cells, the precursors of germ cells. The germ plasm is also sufficient for pole cell formation; its placement at the anterior pole results in the formation of ectopic pole cells that can give rise to functional germ cells (Illmensee and Mahowald, 1974; Niki, 1986). Thus, all of the components required to specify germline development are present in the germ plasm.

The formation of the germ plasm occurs during oogenesis (Mahowald, 1962; Mahowald and Kambysellis, 1980). After four incomplete divisions of a cystoblast, 1 of the 16 progeny cells becomes the oocyte (King, 1970). The other 15 cells, the nurse cells, synthesize and supply materials to the oocyte through ring canals at the anterior end of the developing oocyte (Mahowald and Kambysellis, 1980). The components of the germ plasm, as well as determinants for abdominal tissues, must then be localized to the posterior pole of the egg. Because the synthesis, transport, and localization of both determinants depend solely on the maternal genome, several groups of investigators have searched for maternal-effect mutations that disrupt these activities. Mutations that disrupt only the germline determinants would be grandchildless, as mutant mothers would produce progeny that fail to form germ cells and thus are sterile.

Maternal-effect mutations that disrupt formation of both the germ cell lineage and the abdomen have been identified from genetic screens (*tudor*, Boswell and Mahowald, 1985; *oskar*, Lehmann and Nüsslein-Volhard, 1986; *staufen*, *vasa*, and *valois*, Schüpbach and Wieschaus, 1986; *cappucino* and *spire*, Manseau and Schüpbach, 1989). Besides these, two other members of the posterior group mutants, *nanos* and *pumilio*, affect abdomen formation but do not have any direct effect on the germ plasm; although the embryos fail to form an abdomen, they do form pole cells that are capable of producing functional germ cells (Lehmann and Nüsslein-Volhard, 1987, 1991). *nanos* has been shown to encode the determinant for posterior axis formation (Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991), and *pumilio* is required to deliver *nanos* gene activity to the abdominal region of the embryo (Lehmann and Nüsslein-Volhard, 1987). Thus, determinants for posterior axis specification are separable from determinants for germ cell formation. Although mutations have been found that affect the germ cell lineage, they do not appear to affect the germline determinants per se; some mutations delay nuclear migration so that the nuclei fail to reach the posterior pole while the determinants are active (Mahowald et al., 1979; Niki, 1984), whereas other mutations affect the maintenance but not the formation of pole cells (Engstrom et al., 1982; Oliver et al., 1987). To our knowledge, specific determinants for the germ cell lineage have not been isolated previously.

Several genes in the posterior group appear to function in a stepwise manner for the posterior localization of *nanos* messenger RNA (mRNA) and the yet unidentified germ cell determinant(s). *oskar* mRNA and the staufen protein are the earliest identified components to be localized to the posterior pole of the developing egg (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). This localization requires maternal gene function of *oskar*, *staufen*, *cappucino*, and *spire*. At a later stage of oogenesis, the vasa protein, a polar granule component, is localized to the posterior pole (Lasko and Ashburner, 1990; Hay et al., 1988a, 1990). The functions of *cappucino*, *spire*, *oskar*, and *staufen* are required for the initial localization of vasa, whereas the activities of *tudor* and *valois* appear to be necessary for the maintenance of vasa localization during early embryogenesis (Lasko and Ashburner, 1990; Hay et al., 1990). All of these genes, as well as *vasa*, are

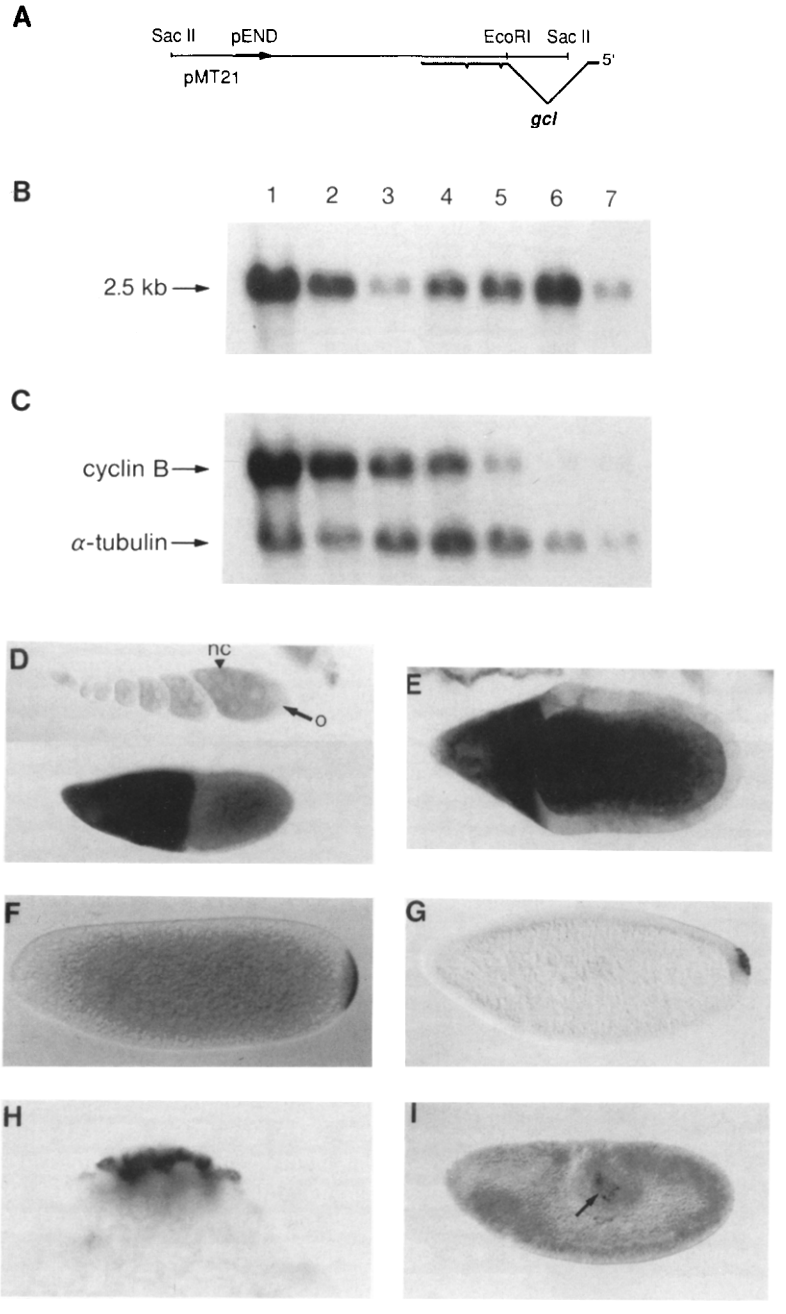

Figure 1. Physical Map of *gcl*, Its Temporal Expression Pattern, and Its Expression Pattern during Oogenesis and Early Embryogenesis

(A) The *gcl* transcript is localized to one side of the P element insertion in the line A5 2nd 10. Bacterial sequences within pMT21 allowed for the rescue of neighboring genomic sequences contained in this SacII fragment (Bier et al., 1989). The 3′ end of the transcript is 5.5 kb from the P element end (pEND, shown by a closed arrow). Sequence comparison of the cDNA with the genomic sequences revealed three introns: the first was of an undetermined size and the second and third were rather small, 54 and 69 bp, respectively.

(B) Northern analysis of *gcl* expression in ovarian and embryonic RNA. Ten micrograms of poly(A)$^+$ RNA was run in each lane of a formaldehyde–agarose gel, transferred to a nylon membrane, and probed with the full-length cDNA (10B-1). A transcript of ~2.5 kb was detected in RNA from ovaries and all embryonic stages. The embryo samples in each lane are as follows: lane 1, ovarian; lane 2, 0–3 hr; lane 3, 3–6 hr; lane 4, 6–9 hr; lane 5, 9–12 hr; lane 6, 12–16 hr; and lane 7, 16–23 hr.

(C) The same Northern blot was reprobed with α-tubulin cDNA and cyclin B cDNA to provide a reference for the amount of RNA loaded per lane. The developmental profile of cyclin B is used as a marker in later experiments (note a similar decrease in its levels and that of *gcl* between the ovarian and 0–3 hr samples). The variation of α-tubulin in lanes 6 and 7 reflects a drop in its levels, which has been noted previously (Kalfayan and Wensink, 1982).

(D) In an ovariole containing egg chambers at different developmental stages, *gcl* RNA is first detected in the nurse cells (nc) at stage 8 of oogenesis (all stages of oogenesis in this text are given according to King, 1970). The slight staining in the oocyte (o) is not reproducibly seen. The level of RNA increases until stage 10, as in the egg chamber below.

(E) An egg chamber at stage 11 of oogenesis. At stage 10 the nurse cells rapidly dump their contents into the developing oocyte. At stage 11 this continues; *gcl* RNA is detected at high levels in the degenerating nurse cells as well as in the oocyte. Note that *gcl* RNA is not localized in the oocyte.

(F) An early cleavage-stage embryo showing a concentration of *gcl* RNA at the posterior pole, in the region referred to as the pole plasm.

(G) A preblastoderm embryo after the pole cells have formed. Note that *gcl* RNA is incorporated into the pole cells.

(H) An embryo at the pole bud stage photographed at higher magnification to show some of the *gcl* RNA in a punctate, perinuclear pattern in the pole buds.

(I) A stage 9 embryo showing faint staining in the pole cells (indicated with an arrow) that are carried in the posterior midgut primordium at this stage. Also at this stage the first zygotic expression in the somatic cells can be detected.

required for the posterior localization of *nanos* mRNA (R. Lehmann, unpublished data) and the determinants of the germ cell lineage (Boswell and Mahowald, 1985; Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986; Manseau and Schüpbach, 1989).

In this paper we describe the identification and characterization of the gene *germ cell-less* (*gcl*). Maternally generated *gcl* mRNA is localized to the posterior pole of the egg and is required for pole cell formation. Posterior localization of *gcl* mRNA depends on those genes of the posterior group that are required for the formation of both the abdomen and the germ cell lineage. Selective removal of this *gcl* mRNA and the subsequent protein product by the synthesis of antisense RNA during oogenesis results in a failure to develop a germline; the fate of the germ cell lineage appears to be aborted during, or shortly after, the

formation of the pole cells. This mutant effect appears to be specific for germ cells, as eggs with much reduced levels of *gcl* mRNA develop into sterile but otherwise morphologically normal adults. Consistent with the mutant phenotype, the gcl protein distribution is specific for the pole cells. During the later half of the cleavage stages, gcl protein appears in the pole plasm and becomes associated with the nuclei that enter the pole buds. This nuclear association persists in the pole cells up until the pole cells begin to migrate to the embryonic gonad. The genetic dependence of *gcl* mRNA localization, the mutant phenotype created by antisense RNA expression during oogenesis, and the distribution of gcl protein all suggest that the role of maternal *gcl* is in the specification of the germline.

Results

The *gcl* Gene and Its mRNA Expression Pattern

We are interested in identifying genes required for the specification of the germ cell lineage. The gene *gcl* was discovered in a screen for grandchildless mutants and was adjacent to a partially deleted P element at 44E (see Experimental Procedures). This insertion was not associated with a phenotype; however, the flanking genomic DNA hybridized to a transcript localized to the posterior pole in early embryos. Because the localization of this mRNA was consistent with it playing a role in germ cell specification, the function of this gene was investigated. Since mutants in this gene were unavailable and the deleted P element was not useful in the isolation of such mutants, we generated mutants with reduced maternal *gcl* mRNA due to the expression of antisense RNA during oogenesis. Because these mutants failed to form germ cells, this gene is named *germ cell-less*.

Using a full-length cDNA obtained with the genomic DNA at 44E (Figure 1A) as a probe on a Northern blot we detected a single transcript of about 2.5 kb, expressed during oogenesis and embryogenesis (Figure 1B). This RNA was first detected in the nurse cells of stage 8 egg chambers (staging is according to King, 1970) and became abundantly expressed in these cells at stage 10 (Figure 1D). The transcript first entered the oocyte at stage 10 and became posteriorly localized sometime between stage 11 of oogenesis and egg deposition (Figures 1E and 1F). This localization was maintained throughout the syncytial nuclear cleavage stage of embryogenesis, and the *gcl* mRNA was subsequently incorporated into the pole cells as they formed (Figure 1G). Within the forming pole cells, the staining pattern was punctate and perinuclear, suggesting it may be associated with the polar granules (Figure 1H). This pattern is similar to that of the vasa protein, a known polar granule component (Hay et al., 1988a, 1988b), and to that of cyclin B mRNA (Lehner and O'Farrell, 1990; Raff et al., 1990). The *gcl* mRNA remained detectable in the pole cells until about 4 hr of embryogenesis (early gastrulation, Figure 1I). Later during embryogenesis, we observed a dynamic pattern of *gcl* expression in a number of tissues, including the foregut, hindgut, muscle, and a subset of the cells in the central nervous system. Consistent with the in situ hybridization data, the level of the 2.5 kb *gcl* RNA was higher in 0–3 hr embryos than in 3–6 hr embryos, before the appearance of a prominent zygotic expression (Figure 1B).

Reduction of *gcl* Function Due to Antisense *gcl* Expression

Lacking a mutant in the *gcl* gene, we attempted to block *gcl* function by expression of RNA complementary to the *gcl* mRNA. This antisense approach has been used to reduce gene function in several other eukaryotic systems (van der Krol et al., 1988). In cases where mutants exist, the introduction of antisense molecules into wild-type animals has resulted in precise phenocopies (*Krüppel*, Rosenberg et al., 1985; *wingless*, Cabrera et al., 1987; *snail*, Boulay et al., 1987). To introduce antisense *gcl* RNA into the embryo, we took the approach of creating transgenic flies that could produce antisense RNA under the control of the hsp70 (heat shock inducible) promoter (Figure 2A). If the maternally contributed *gcl* mRNA or the resulting protein (see Figure 6) is required for germ cell development, we would expect its removal to result in individuals that lack germ cells and are sterile, giving a grandchildless phenotype.

We found that 6 of the 25 existing transformant lines displayed varying penetrance of a grandchildless phenotype even in the absence of any heat shock treatments. The homozygous mothers produced a percentage of sterile progeny that lacked germ cells (Table 1). The testes of the sterile male progeny were extremely small and contained no sperm. The ovaries of the sterile female progeny were also small, lacking differentiated ovarioles, oocytes, and often the associated tracheols. These phenotypes are similar to those of other grandchildless mutants (Boswell and Mahowald, 1985).

Obtaining a mutant phenotype without heat shock is not unexpected with this construct, as the expression from the hsp70 promoter is often influenced by nearby enhancer elements (K. Blochlinger, unpublished data). This property is similar to that of promoters used for enhancer trapping with P element vectors (Bier et al., 1989; Bellen et al., 1989). Given that one pattern often obtained with enhancer trap lines is germline expression during oogenesis (Grossniklaus et al., 1989), such enhancer trap lines might produce antisense *gcl* RNA without any heat shock induction.

The severity of the mutant phenotype of the transformant lines in the absence of heat shock correlates well with the detection of antisense *gcl* RNA expression in the mother during oogenesis (Figure 2C) and with the reduction of *gcl* mRNA in embryos (Figure 2B and Table 1). No effect on the levels of another pole plasm component, cyclin B mRNA (Whitfield et al., 1989; Lehner and O'Farrell, 1990; Raff et al., 1990), was observed (Figure 2B). A transformant line with normal offspring, 5-27, did not express any detectable amount of antisense RNA and had a normal level of both *gcl* and cyclin B mRNA in embryos when compared with the w^- control line that lacks the antisense *gcl* construct. In contrast, three lines with the grandchildless phenotype (5-110, 5-118A, and 5-108) showed detectable levels of antisense *gcl* RNA expression

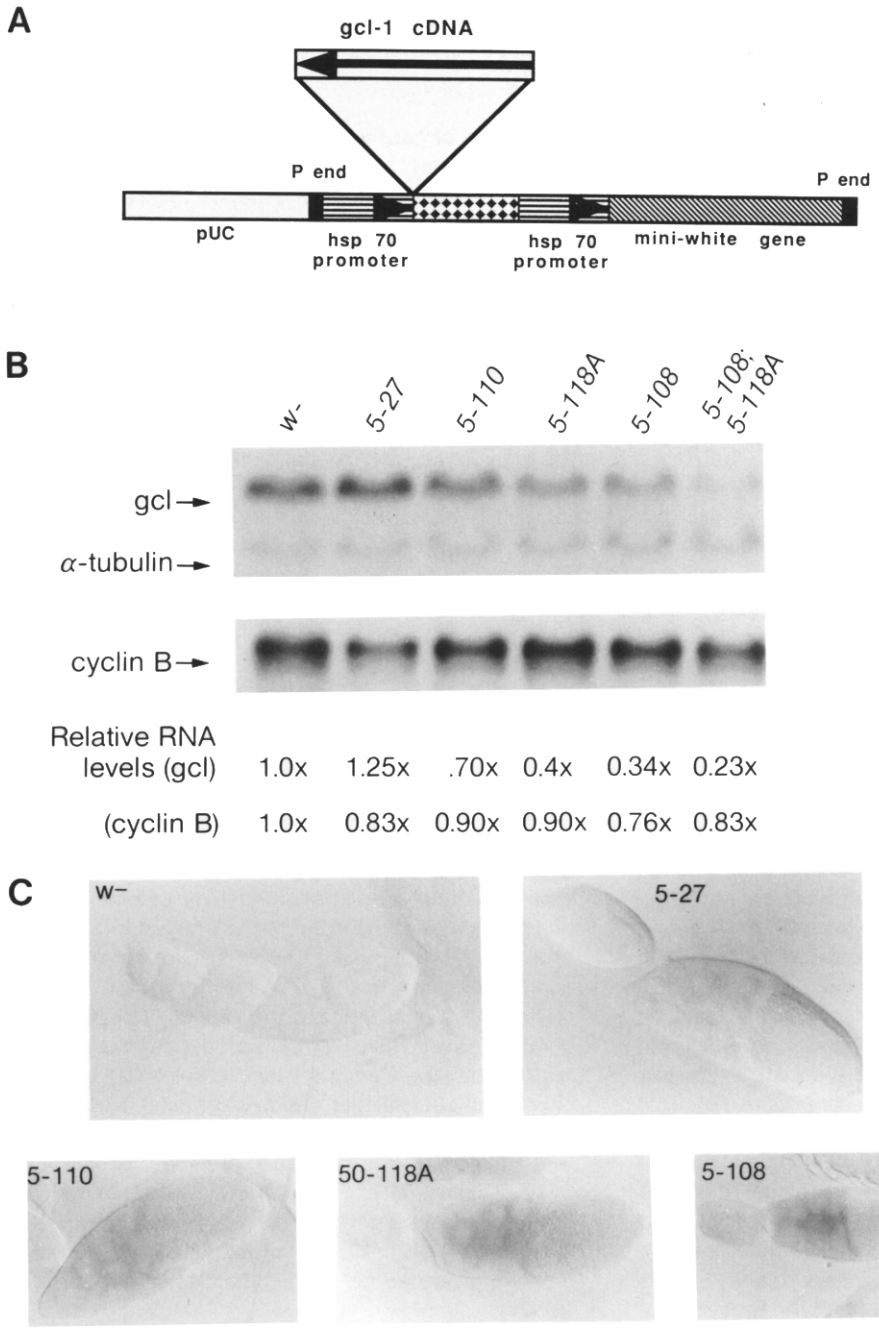

Figure 2. The Antisense *gcl* Construct (pWHIα–*gcl*), Its Expression in the Germline during Oogenesis, and the Effects of This Expression on the Levels of *gcl* mRNA Levels in the Embryo

(A) The pWHIα–*gcl* vector contains sequences necessary for maintenance and selection in bacteria located outside of P element transposon ends. Internal to the P element is the full-length *gcl* cDNA, driven by the *hsp70* promoter in the reverse orientation, and a *white* minigene, driven by its own promoter and a second *hsp70* promoter, to provide an eye color marker for the resulting transformants.

(B) Northern analysis of the transformant lines; 0–1 hr embryos (prepole bud formation) were used to prepare RNA from each of the transformant lines, as well as from the line with the recombinant chromosome containing both of the inserts of lines 5-108 and 5-118A (i.e., 5-108;5-118A). α-tubulin was used to monitor the amount of RNA in each lane. The level of cyclin B mRNA was investigated, since it also has been shown to be a pole plasm component (Whitfield et al., 1989; Lehner and O'Farrell, 1990; Raff et al., 1990). After gel fractionation and transfer to a nylon membrane, the blot was exposed to a single-stranded probe specific for *gcl* mRNA. In addition, the blot was reprobed with α-tubulin– and cyclin B–specific sequences. After exposure to autoradiography, the hybridization was quantitated using a phospho-imager (Molecular Dynamics). The α-tubulin signal was used to normalize the amount of *gcl* and cyclin B mRNA. The relative levels of each of these mRNAs are given for each transformant line analyzed from an average of two Northern experiments. The RNA levels in the w^- (control) line were set at 1.0×.

(C) Expression of antisense *gcl* RNA in the ovarian germline. Whole mount in situ hybridizations were performed on the transformant lines as well as on the control w^- line using a single-stranded probe specific for antisense *gcl* RNA. Antisense *gcl* RNA is detected in the nurse cells of the transformant lines 5-110, 5-118A, and 5-108. No antisense expression was detected in egg chambers from the control line or the transformant line 5-27.

Table 1. Correlation between the Reduction of *gcl* mRNA in Embryos and the Percentage of Agametic Sterile Flies among the Progeny from Homozygous Females, Mated to Wild-Type Males

Line	Percent of Sterile Progeny	Relative *gcl* mRNA Levels
w^-	0.0	1.0
5-27	0.0	1.25
5-110	3.7	0.70
5-118A	22.8	0.40
5-108	52.0	0.34

See text and Experimental Procedures for details. The percentage of sterile progeny is derived from the examination of at least 100 adults for each line. The levels of *gcl* mRNA in early embryos were determined by Northern analysis (see Figure 2B).

in the nurse cells of stage 9 and stage 10 egg chambers (Figure 2C), at the peak of *gcl* expression (see Figure 1). Although we could not detect antisense *gcl* RNA by Northern analysis in these three lines, early during embryogenesis the level of *gcl* mRNA, but not cyclin B mRNA, was reduced. This was also true for embryos from the line with a recombinant chromosome carrying the inserted antisense *gcl* constructs of the two lines with the strongest phenotype (i.e., 5-108;5-118A). The extent of *gcl* mRNA reduction correlated with the sterility frequency of these lines (Table 1 and Figure 2B).

Some detectable *gcl* mRNA remained in the mutant embryos and was localized to the posterior pole, as were the cyclin B mRNA and the vasa protein (Figure 3). Thus, the production of antisense *gcl* RNA during oogenesis, apparently owing to the influence of nearby enhancers, reduced the level of *gcl* mRNA in early embryos but did not affect either its posterior localization or the levels and localization of other posteriorly localized components. This is in contrast with the effects on localization caused by mutations in the posterior group genes that affect pole cell formation (Hay et al., 1990; Lasko and Ashburner, 1990; Raff et al., 1990). Taken together, these results show that the expression of antisense *gcl* RNA during oogenesis causes a reduction of *gcl* mRNA in early embryos, leading to a failure of germ cell formation and a grandchildless mutant phenotype.

Effect of Antisense *gcl* Expression on Pole Cell Number

To determine whether the lack of identifiable germ cells in the sterile adults, the progeny of females with antisense *gcl* expression, resulted from abnormal development of the pole cells, we stained embryos from homozygous females of the transformant lines with antibodies against the vasa protein. The vasa protein is present in pole cells from their formation throughout the rest of their development (Hay et al., 1990; Lasko and Ashburner, 1990). Normally, pole cells form at the posterior pole of the embryo (Figure 4A) and, at the onset of gastrulation, are carried in a pit of somatic cells, which later become the posterior midgut (Figure 4C, stages 6–10; staging is according to Campos-

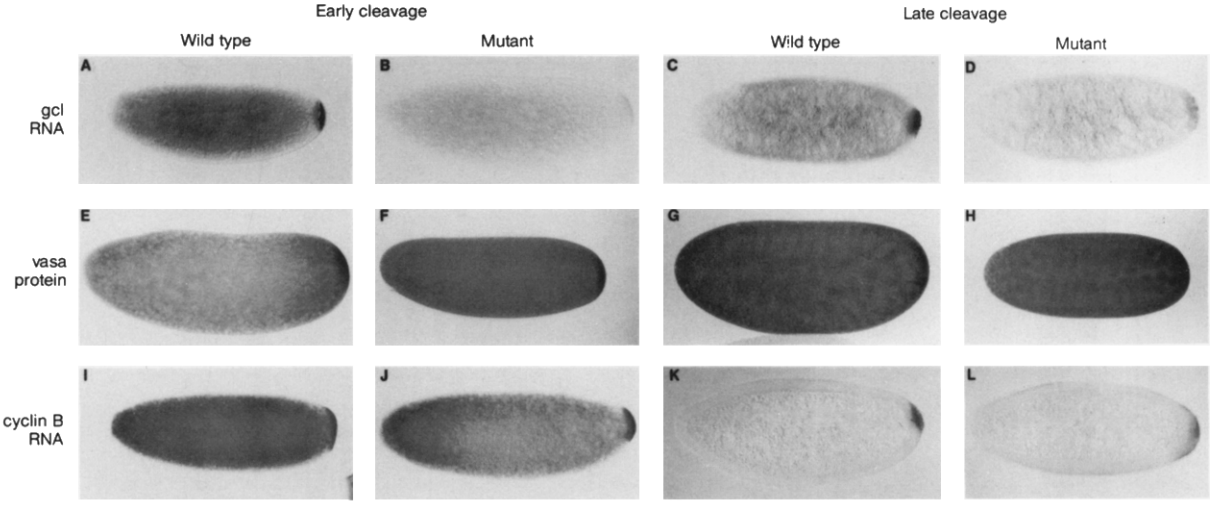

Figure 3. Localization of Pole Plasm Components in Embryos from 5-108;5-118A Mutant Mothers

Whole mount in situ hybridization and antibody stainings were performed in parallel on wild-type (w^-) and mutant embryos from 5-108;5-118A homozygous females.

(Top) The levels of *gcl* mRNA are noticeably reduced in mutant embryos at both the early and late cleavage stages; however, there is still a detectable posterior concentration.

(Middle) In early and late cleavage stages, the level and distribution of vasa protein are indistinguishable between the mutant and wild-type embryos. After completion of pole cell formation, the intensity of vasa staining increases in wild-type embryos, reflecting the reformation of polar granules in pole cells after they break down during the cleavage divisions (Mahowald, 1968). In contrast, the level of vasa staining in mutant embryos appears to decrease during the formation of the pole buds and continues to decrease in those embryos that fail to form pole cells. The remaining vasa staining, however, is still posteriorly localized at the start of somatic cellularization (see Figure 5B).

(Bottom) cyclin B mRNA staining is indistinguishable between mutant and wild-type embryos at the early cleavage stages. The late stage embryos shown are actually blastoderm embryos beyond the stage of pole cell formation. The posterior localization of cyclin B mRNA can still be seen in the mutant embryos, even though pole cells failed to form.

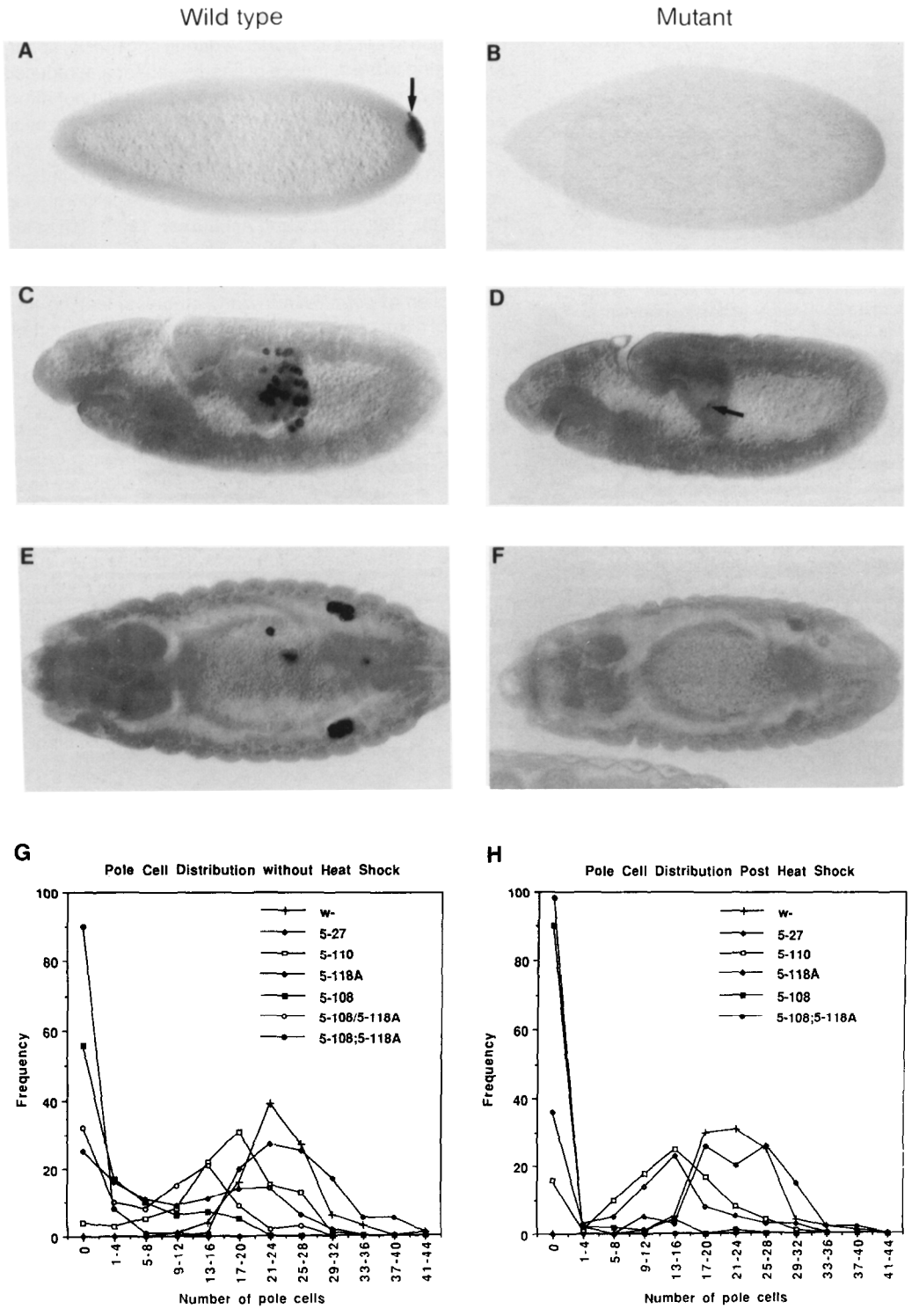

Figure 4. Pole Cell Formation and Migration as Revealed by Anti-vasa Staining of Wild-Type (w^-) and Mutant Embryos from 5-108 Homozygous Females and Effects of Reduction of *gcl* mRNA on Pole Cell Numbers at Stage 14

(A) An early blastoderm wild-type embryo showing a normal number of pole cells (indicated with an arrow) at the posterior pole.

(B) An early blastoderm mutant embryo showing faint vasa staining at the posterior pole, with no detectable pole cells present. We observe that the levels of vasa staining rapidly decrease when the pole cells fail to form.

(C) A stage 10 wild-type embryo with the pole cells in the posterior midgut primordium.

(D) A stage 10 mutant embryo with only subcellular vasa staining fragments in the posterior midgut primordium (arrow). We assume the vasa staining fragments are remnants of dying pole cells.

(E) A stage 14 wild-type embryo with pole cells coalesced in both of the embryonic gonads.

(F) A stage 14 mutant embryo with only a remnant of vasa staining in the embryonic gonads. Again, we presume the vasa staining to be a remnant of dying pole cells, in this case pole cells that have reached the gonad but subsequently died.

Table 2. Correlation between the Reduction of *gcl* mRNA Level and the Reduction of Pole Cells

Line	Relative *gcl* mRNA Levels	Without Heat Shock		With Heat Shock	
		Mean of Pole Cells	Percentage without Pole Cells	Mean of Pole Cells	Percentage without Pole Cells
w^-	1.0	23.5 ± 0.45	0.0	22.3 ± 0.44	0.0
5-27	1.25	25.3 ± 0.58	0.0	23.1 ± 0.69	0.0
5-110	0.70	17.0 ± 0.63	4.0	12.3 ± 0.75	18.0
5-118A	0.40	10.6 ± 0.94	25.0	9.4 ± 0.87	37.0
5-108	0.34	3.5 ± 0.56	56.0	1.0 ± 0.36	90.0
5-108/5-118A	ND	8.3 ± 0.76	32.0	ND	ND
5-108; 5-118A	0.23	0.4 ± 0.14	90.0	.17 ± .06	98.0

The percentage of embryos without pole cells in the progeny from homozygous females, mated to wild-type males before and after heat shock (see text and Experimental Procedures for details), as well as the mean number and standard error of pole cells in these embryos, correlates well with the reduction of *gcl* mRNA in the embryos. ND, not determined.

Ortega and Hartenstein, 1985). At the end of germband extension, they pass through the posterior midgut cell layer, migrate posteriorly and dorsally, and by stage 14 coalesce in the embryonic gonad (Figure 4E). The strongest phenotype observed in the antisense lines was the failure to complete pole cell formation, resulting in preblastoderm embryos lacking pole cells (Figure 4B). Analysis of this phenotype will be discussed in more detail below. Because the fraction of embryos that lacked pole cells at the blastoderm stage was smaller than the percentage of sterile adults and because the percentage of embryos lacking any identifiable pole cells increased until about stage 14 of embryogenesis when it then approached the frequency of adult sterility (Figure 4G and Tables 1 and 2), we assume that pole cells die during later stages of embryonic development. Consistent with this hypothesis, we found that many embryos contained pole cells with decreased levels of vasa protein as well as vasa-staining cellular debris (Figure 4D).

Homozygous mothers of the transformant lines 5-110, 5-118A, and 5-108 produced embryos that either lacked pole cells or had fewer than the normal number of pole cells at stage 14 (Figures 4F and 4G and Table 2). A similar reduction is also found in embryos from 5-108/5-118A transheterozygous mothers (Figure 4G and Table 2) or in mothers carrying both 5-108 and 5-118A insertions on the same chromosome balanced over a TM3 balancer chromosome (not shown). The mutant phenotype is further increased in embryos from mothers carrying two copies of both insertions 5-108 and 5-118A (Figure 4G and Table 2). Thus, the degree of the defect in pole cell development correlates with the reduction of *gcl* RNA and is not likely to be due to other mutations in the genetic background.

Heat shock induction of antisense *gcl* expression should further decrease the level of *gcl* mRNA in the embryo and lead to an even stronger phenotype. Indeed, pole cell numbers were further reduced by heat shock induction 8–12 hr prior to egg laying (Figure 4H and Table 2). For example, of the embryos from heat-shocked mothers carrying two copies of the insertion 5-108, 90% had no pole cells, an increase from the 57% obtained without heat shock (Figure 4H and Table 2). The viability of the embryos from all of the transformant lines after the heat shock treatment was identical to that of the control line, and there was no detectable deletion of abdominal segments in the embryos that did not hatch. The enhancement of the mutant phenotype by heat shock provides further evidence for the dependence of the pole cell development on *gcl* mRNA, as well as a nearly fully penetrant phenotype for the subsequent analysis.

The strongest reduction of pole cells was detected in embryos derived from oocytes that were heat shocked at around stage 10 of oogenesis (Mahowald and Kambysellis, 1980). This corresponds to the time when *gcl* expression is at its maximum (see Figure 1). It also coincides with the time when the polyploidy of the nurse cell nuclei is at a maximum and before the nurse cells begin to disintegrate (King, 1970; Mahowald and Kambysellis, 1980), thereby allowing for more antisense RNA expression to be induced.

Analysis of Earliest Defect in Pole Cell Formation

To look for the earliest detectable effects of a reduction of *gcl* mRNA, we examined pole cell formation in the preblastoderm embryos, using our strongest mutant. These were embryos derived from eggs laid 8–12 hr after heat shock of mothers that were homozygous for both 5-108 and 5-118A insertions. Using the anti-vasa antibodies to stain pole cells in fixed embryos, we found that prior to blastoderm formation, 77 of 94 (82%) embryos already lacked identifi-

(G) The number of pole cells was counted in embryos from mothers of the control (w^-) line, mothers homozygous for the insertions of the transformant lines, mothers transheterozygous for 5-108/5-118A, and mothers homozygous for both the 5-108 and the 5-118A inserts, 5-108;5-118A (see Experimental Procedures). The number of pole cells was graphed against the frequency of individuals with those numbers of pole cells. The mean number of pole cells is given for each line in Table 2.

(H) The distribution of pole cell numbers after the mothers were heat shocked (see Experimental Procedures). The mean number of pole cells and the percentage change as a result of the heat shock treatment as shown in Table 2. Transheterozygous 5-108/5-118A mothers were not included in the heat shock experiments.

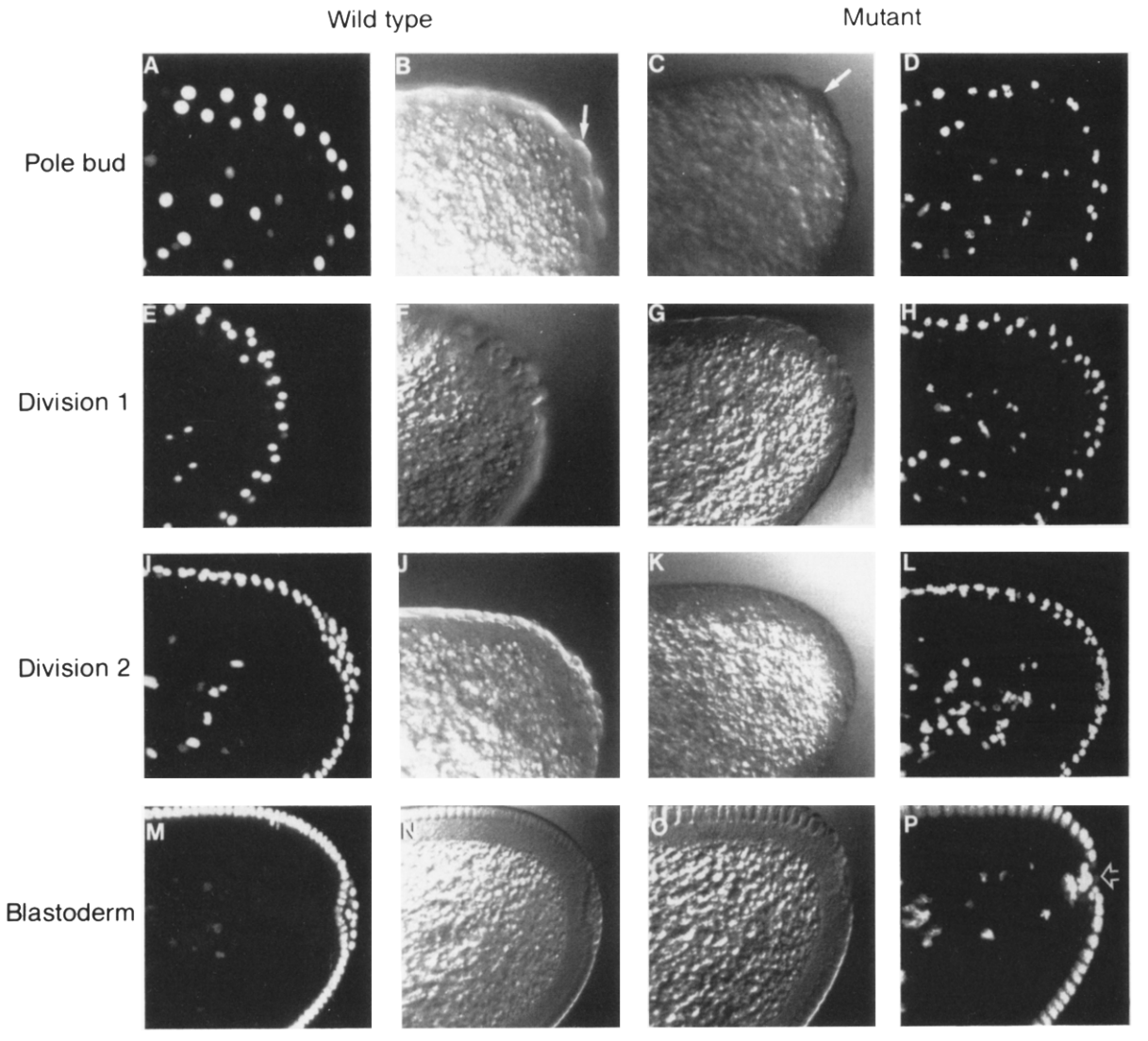

Figure 5. Nuclear Migration and Divisions during Pole Bud and Pole Cell Formation Stages Are Not Affected by Reduction of *gcl* mRNA

Wild-type (w^-) and mutant embryos from 5-108;5-118A homozygous mothers were fixed and stained with a monoclonal antibody against histones (Chemicon) that cross-react with Drosophila histones. Fluorescein isothiocyanide–conjugated secondary antibodies were used in conjunction with confocal microscopy to visualize the nuclei. Nomarski images were also taken using transmitted light with confocal microscopy. The left two columns are images from wild-type embryos, with a representative sample of the nuclei at the periphery of the embryo shown in the first column ([A], [E], [I], and [M]) and the Nomarski image of the same embryo shown in the second column ([B], [F], [J], and [N]). The third and fourth columns are representative embryos from homozygous 5-108;5-118A mothers: Nomarski images are in the third column ([C], [G], [K], and [O]), and a representative sample of the nuclei is in the fourth column ([D], [H], [L], and [P]). The stages are denoted for each row: namely, pole bud stage prior to pole bud divisions (Pole bud), completion of the first pole bud division (Division 1), completion of the second division (Division 2), and the stage at which the pole cells form (Blastoderm). The pole buds for both the wild-type and mutant embryos are indicated with an arrow in (B) and (C), respectively. Note that after the second pole bud division, pole cells are not present in the mutant embryo (compare [J] and [K]). Only in the mutant embryos at the blastoderm stage did we begin to detect the falling in of nuclei at the posterior pole (arrow in [P]). This is frequently seen in mutant embryos, only at the posterior pole, from the start of cellularization to its completion. In the adjacent Nomarski image (O), there is no evidence of nuclei being surrounded by cellular membranes.

able pole cells. To determine more precisely the stage at which pole cell development becomes defective, we used Nomarski optics and followed the morphological changes in living embryos during the late cleavage stages up until pole cell formation (see Experimental Procedures). In all of the embryos observed, pole buds formed prior to the time when the somatic nuclei reached the surface of the embryo and subsequently divided twice. In 38 of 50 (76%) embryos, however, the pole buds regressed, apparently just after the completion of the second division, instead of forming pole cells. Finally, we analyzed the nuclear migration and division patterns in fixed embryos after fluorescently labeling the nuclei with antibodies against histones and found that the migration of the nuclei was unaffected (Figures 5A, 5B, 5C, and 5D). Nuclei reached the periphery first at the posterior pole. During the subsequent nuclear

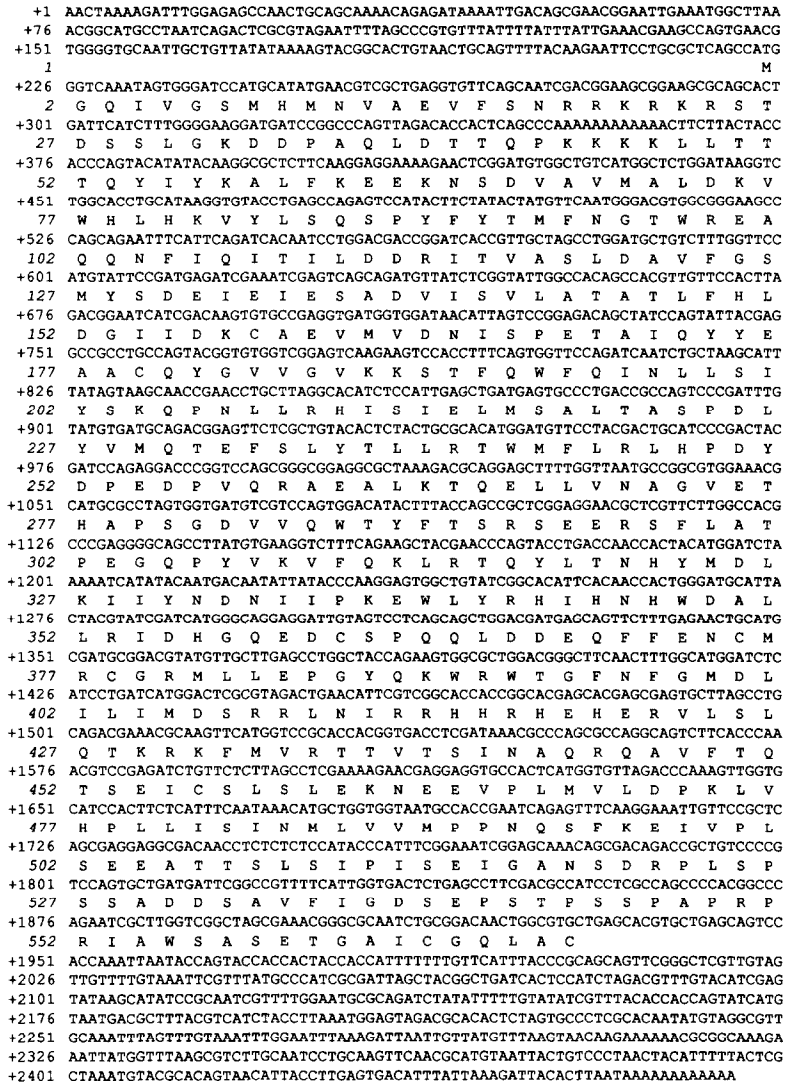

Figure 6. The Nucleotide Sequence and Corresponding Amino Acid Sequence of *gcl*

The genomic sequence corresponding to the coding strand of the *gcl* cDNA is shown, with the amino acid sequence of the longest open reading frame shown below. The first methionine in this open reading frame closely matches the consensus for efficient initiation of translation (Cavenar, 1987). Starting from this methionine, the open reading frame codes for a 569 aa protein. Two independent genomic clones were sequenced and found to be identical. Some differences with the cDNA were observed. The 5′ leader of the cDNA contained an insertion of TAA at position +210. The coding region contained several conservative changes (+288 G to A [Arg], +324 T to C [Asp], +336 A to G [Leu], and +1122 G to C [Ala]) as well as the following nonconservative changes: +302 A to G (Asp to Gly), an insertion of an A at +352, which we presume is a cloning artifact, since it occurred in a stretch of poly(A); and a small frame shift due to an insertion of an A at +1695 with a compensating deletion of an A at +1766. Comparison of the genomic and cDNA sequences also revealed three introns at the following positions: +203, +359, and +1303. Splice donor and acceptor consensus sequences were found for all three introns.

divisions, the nuclei at the posterior pole appeared to divide in synchrony with the rest of the somatic nuclei (Figures 5E–5L) as in wild-type embryos (Foe and Alberts, 1983). At the end of cycle 10, however, embryos were found without pole cells or pole buds (Figures 5K and 5L). The nuclei originally in the pole buds apparently remained at the periphery until the somatic cells began to cellularize (start of nuclear cycle 14). At this point, several degenerate nuclei were found in the center of the embryo (Figures 5O and 5P), which we assume had previously been located at the cortex. This appearance of degenerate nuclei falling into the center of the blastoderm differs from the previously described occasional pole cells intermingling with the somatic cells at the posterior pole (Underwood et al., 1980). Specifically, there were no detectable cell membranes surrounding these nuclei (Figure 5O), and the nuclei were found below the level of the blastoderm. These studies thus reveal that pole cell development in the mutant embryos is affected at the stage of pole cell formation, and those pole cells that form initially are subsequently lost.

The *gcl* Gene Encodes a Novel Product

The 2470 bp *gcl* cDNA codes for a protein of 569 aa (Figure 6) with a predicted molecular size of 65 kd. The noncoding sequences include a 222 bp 5′ leader and a 540 bp 3′ untranslated region. The encoded protein is slightly acidic, with a pK_a of 5.85. No distinguishing structural features or homologies using the BLASTP search program (Altschul et al., 1990) within the GenBank or Swisspro libraries were found.

Distribution of the gcl Protein during Early Embryogenesis

Consistent with the maternal mRNA distribution and the phenotype of the antisense mutants, gcl protein is concentrated in the pole plasm and pole cells as they form (Figure 7). During the early cleavage stages, no obvious gcl protein was detected (data not shown). In the mid to late cleavage stages, however, a concentration of gcl protein was detected at the posterior pole (Figure 7A). As nuclei migrated to the posterior pole, gcl protein became associated with those that migrated through the pole plasm (Figure

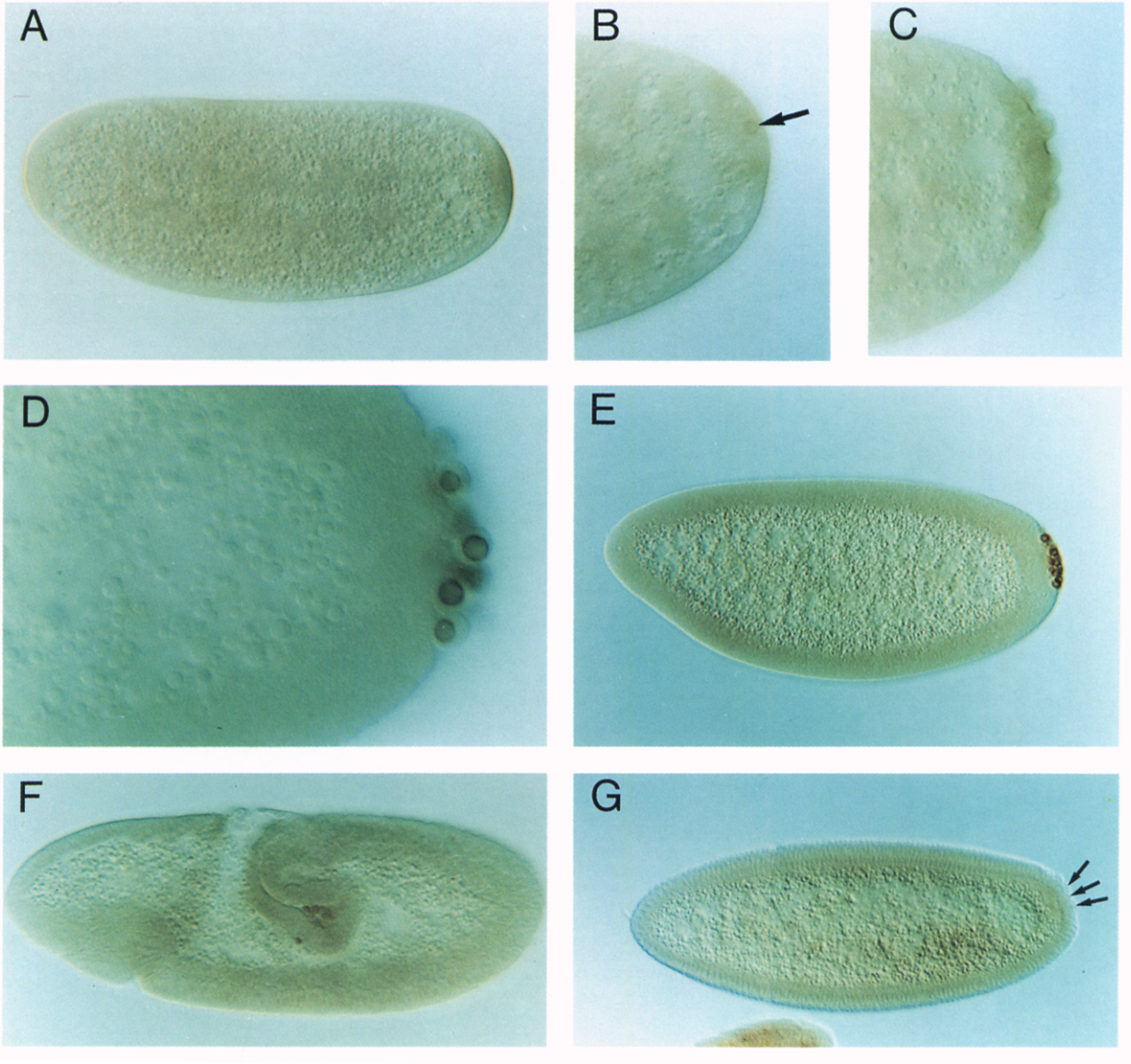

Figure 7. The Distribution of gcl Protein during Early Embryogenesis

Polyclonal antibodies raised to a trpE–gcl fusion protein were used to detect gcl protein in whole-mount embryos.

(A) An embryo in the midcleavage stages with a detectable concentration of gcl protein in the pole plasm.

(B) The posterior of an embryo at a slightly later stage, where a nucleus has entered the pole plasm (arrow) and is associated with gcl protein.

(C) The posterior pole of a pole bud stage embryo, showing the gcl protein associated with the pole bud nuclei and a slight but detectable level of diffuse staining within the pole bud.

(D) The pole bud nuclei after the first division (shown at higher magnification), revealing gcl protein associated with the nuclear membrane and present diffusely throughout the nucleus.

(E) An embryo after pole cell formation, showing the specificity of gcl protein distribution to the germline precursors.

(F) A stage 10 embryo, showing detectable levels of gcl protein in the pole cells.

(G) A blastoderm stage embryo from a 5-108 homozygous mother, with a few pole cells but no obvious gcl staining.

7B). At the pole bud stage, gcl protein appears to be associated with the plasma membrane, in addition to the diffuse cytoplasmic staining and staining of the pole bud nuclei (Figure 7C). After the first pole bud division, the diffuse cytoplasmic staining was greatly reduced, while the intensity of the nuclear staining increased. The gcl protein concentrated at the nuclear membrane with a low level of diffuse staining within the nucleus (Figure 7D). The level of gcl protein peaked at the time the pole cells formed and remained detectable in the pole cells until just prior to the time at which they migrated through the posterior midgut primordium (Figures 7E and 7F). During this interval of development, gcl protein was detected primarily in pole cells, except for occasional and very transient staining in posterior blastoderm cells adjacent to the pole cells. Later during embryogenesis, the gcl protein pattern roughly correlated with the zygotic expression pattern of the mRNA (see above). In the somatic cells in which it was detected,

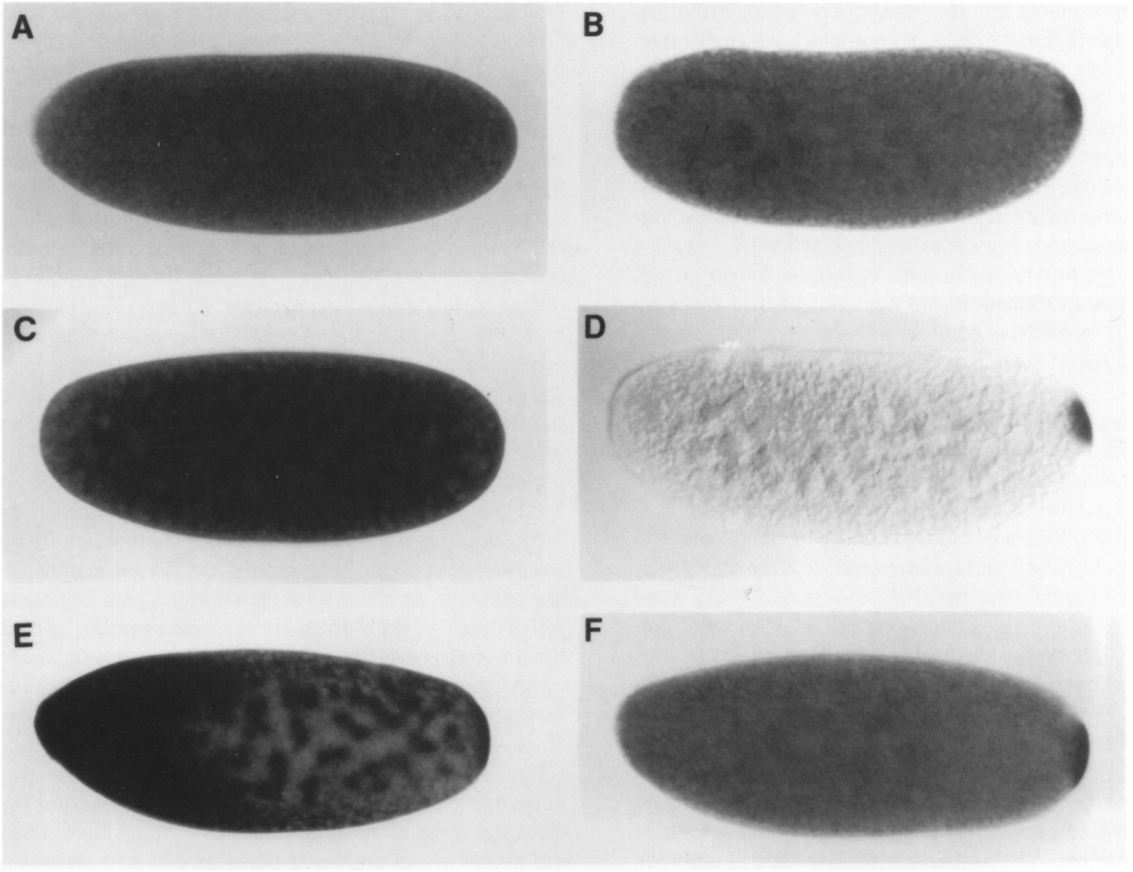

Figure 8. The Genetic Dependence of *gcl* RNA Localization to the Posterior Pole

Shown are representative whole-mount embryos that have been probed for *gcl* mRNA ([A], [B], [C], [D], and [F]) or cyclin B mRNA (E). All of the embryos tested were derived from homozygous mutant mothers from the various maternal-effect posterior group mutants and wild-type fathers.

(A) An early cleavage stage embryo from a $stau^{D3}/stau^{D3}$ mother. No localized concentration of *gcl* mRNA was ever detected in these embryos or in those from $capp^{G7}/capp^{G7}$, $spir^{PJ56}/spir^{PJ56}$, osk^{336}/osk^{336}, or vas^{PD}/vas^{PD} mothers.

(B and C) An early cleavage stage embryo from a tud^{IWC8}/tud^{IWC8} mother. Note the small amount of *gcl* mRNA concentrated at the posterior pole. Such localization was occasionally seen in these embryos, as well as in those from val^{RB71}/val^{RB71} mothers. This localization, however, was abnormal in appearance and limited to the early cleavage stages; it was not detectable in the later cleavage stages as shown in (C), which is also an embryo from a tud^{IWC8}/tud^{IWC8} mother.

(D) A late cleavage stage embryo from a nos^{L7}/nos^{L7} mother, showing normal *gcl* mRNA localization. Normal localization was also observed in embryos from pum^{680}/pum^{680} mothers. (E) and (F) are embryos from transheterozygous $BicD^{EIII48}/BicD^{7134}$ mothers.

(E) An embryo with cyclin B mRNA, showing obvious mislocalization to the anterior pole (compare with cyclin B distribution in Figure 4). This was detected in essentially every embryo from the transheterozygous mothers. We, however, did not detect any obvious mislocalization in embryos from $BicD^{EIII48}/+$ mothers, as has previously been documented (Raff et al., 1990).

(F) An embryo without any detectable *gcl* mislocalization to the anterior pole. Mislocalization of a minute amount of *gcl* mRNA was occasionally seen in embryos, suggesting that *gcl* mRNA localization is affected by the *BicD* mutation but is clearly qualitatively different from the effects seen with cyclin B and *nanos* mRNA.

gcl protein was also associated with the nuclei (data not shown).

As expected from the reduction of *gcl* mRNA, the level of gcl protein was reduced in embryos from mothers that expressed antisense *gcl* RNA in their ovaries. In all of the embryos from 5-108 or 5-108;5-118A homozygous mothers, noticeably lower levels of gcl protein were detected. An example of such a mutant embryo is shown in Figure 7G, which has a few pole cells but little or no detectable gcl protein. Thus, the reduction of mRNA levels has a direct effect on gcl protein levels.

Genetic Dependence of the Localization of *gcl* RNA

All of the mutants that affect pole cell formation and lack identifiable polar granules failed to localize *gcl* mRNA properly (Figure 8). In early cleavage stage embryos of *cappucino*, *spire*, *oskar*, *staufen*, and *vasa* mutants, posterior localization was never detected (Figure 8A). In early cleavage stage embryos from *valois* and *tudor* mutants, occasionally small amounts of localized *gcl* mRNA were detected, but this localization was always lost in the later cleavage stage embryos (Figures 8B and 8C). The poste-

rior group mutants that do not affect pole cell formation, namely *nanos* and *pumilio*, did not affect the localization of *gcl* mRNA (Figure 8D).

Finally, the maternal-effect mutation *Bicaudal D* (*BicD*) causes mirror-image duplication of the abdomen due to ectopic *nanos* localization to the anterior pole (Wharton and Struhl, 1989; Suter et al., 1989; Ephrussi et al., 1991), but no abnormality in pole cell formation, indicating that the mutation does not cause mislocalization of at least a subset of the pole cell determinants. Indeed, the *gcl* mRNA was properly localized to the posterior pole of embryos from *BicD* mothers, in contrast with the anterior mislocalization of cyclin B and *nanos* mRNA (Figures 8E and 8F; Ephrussi et al., 1991). The embryo shown in Figure 8F represents the mRNA distribution pattern most commonly seen. Occasionally, embryos with a subtle hint of *gcl* mRNA mislocalization to the anterior were observed. This failure of *gcl* mRNA to mislocalize to the anterior pole is similar to the failure of vasa protein to mislocalize to the anterior pole in *BicD* mutant embryos (Wharton and Struhl, 1989; Lasko and Ashburner, 1990; Hay et al., 1990). Taken together, the effects of mutations of the posterior group genes on *gcl* mRNA localization are consistent with their effects on germ cell determinants.

Discussion

We have identified a new component of the pole plasm (*gcl* mRNA and protein) that is required for the formation of the germline. The earliest defect detected in embryos with reduced *gcl* mRNA levels is the failure to complete the formation of pole cells. The pole cells that form in these embryos tend to die en route to or after they reach the embryonic gonad. We have elucidated the distribution of gcl protein during early embryogenesis and shown it to be specifically associated with the nuclei that become fated to the germ cell lineage. We have also demonstrated the dependence of the posterior localization of *gcl* mRNA on the seven posterior group genes previously implicated in localizing the germ cell determinants. All of our observations are consistent with the proposal that the maternal *gcl* gene product is a component of the pole plasm necessary for and probably specific to the germline specification pathway.

The Role of *gcl* Function in the Germline

The *gcl* gene product is required for the specification of pole cells, precursors of the germline. Reduction of *gcl* mRNA results in the failure of pole cell formation or the subsequent loss of pole cells. This loss of the germline precursors occurs without any apparent defects in the machinery for posterior localization, as we observe normal localization of both cyclin B mRNA and vasa protein. Also, the specification of abdominal development appears to be unaffected, as the viability of these embryos is essentially 100%, with no detectable deletion of abdominal segments in the occasional nonhatching embryos. However, these results are based on the analysis of embryos with reduced *gcl* function (some *gcl* mRNA is still present). Such grandchildless phenotypes have been observed for weak alleles of *vasa*, *oskar*, and *tudor* (Boswell and Mahowald, 1985; Lehmann and Nüsslein-Volhard, 1986; Lasko and Ashburner, 1990), all of which are required for posterior localization of *nanos* mRNA and the germline determinants. It remains possible that additional roles of the maternally contributed *gcl* mRNA may be revealed with null mutants, but clearly *gcl* function is required for germ cell formation.

Though at present the phenotype of a maternal *gcl* null mutation is unknown, the distribution of maternally encoded gcl protein in the early embryo strongly supports a role in germline fate specification. As soon as pole cells form, they are fated to the germ cell lineage (Underwood et al., 1980; Technau and Campos-Ortega, 1986). Prior to this time, as soon as nuclei migrate into the pole plasm, gcl protein becomes associated with those nuclei that enter the pole buds that form the germline precursors. gcl protein remains associated with the pole cell nuclei beyond the stage at which pole cells form. Thoughout the time period of pole cell formation, the presence of the maternally encoded gcl protein is rather specific to the germline, as it is only detected in the pole cell nuclei with a relatively transient presence in some posterior blastoderm cells near the pole cells. Later in development, zygotic expression of gcl protein is detected in other cell types, and thus *gcl* may serve additional functions at other times in development.

The phenotypic analysis of the *gcl* mutant embryos suggests that the earliest defect occurs during the last nuclear cleavage cycle prior to the formation of pole cells. Thus, the action of *gcl* precedes those of *ovo* and *agametic*. Whereas the zygotic activity of *ovo* is necessary for pole cell maintenance after the onset of gastrulation in female embryos (Oliver et al., 1987), maternal *agametic* function is required for the reinitiation of cell divisions after pole cells reach the embryonic gonad (Engstrom et al., 1982). The *gcl* mutant phenotype cannot be due to delayed migration of nuclei into the pole plasm, as has been found for some of the grandchildless mutants (*gs* [Drosophila subobsura], Mahowald et al., 1979; *gs*(1)N26, Niki, 1984), because reduction of *gcl* mRNA does not affect nuclear migration or division, including the divisions of the pole buds. A more likely explanation for the *gcl* mutant phenotype is that *gcl* function is required early in the specification or formation of pole cells; in the absence of a sufficient amount of *gcl* mRNA and protein, the nuclei that reach the posterior pole of the embryo at the appropriate time either cannot form pole cells at all or form pole cells that are inviable.

What is the fate of the nuclei in those pole buds that fail to form pole cells? We have found that many nuclei at the posterior pole of the mutant embryos fall into the yolk at the beginning of somatic cellularization. If these are the nuclei in the pole buds, it is interesting to speculate why they do not become part of the somatic blastoderm. If *gcl* is required to specify the germ cell lineage, a lack of sufficient levels of *gcl* activity could cause a partial loss of the commitment to the pole cell fate. Those nuclei in the pole buds that fail to form pole cells may be unable to take on the fate of somatic cells. At the moment, we cannot distinguish between this possibility and an alternative ex-

planation that, simply because too many nuclei are present at the posterior pole when cellularization is initiated, some of the nuclei are physically forced into the center of the embryo. Given that those pole cells that form in the mutant embryos become lost later in development, it seems reasonable to suppose that reduction of *gcl* mRNA leads to an incomplete or total loss of the commitment to pole cell fate of not only the pole cells but also the nuclei in the pole buds that fail to form pole cells.

Localization of *gcl* RNA

The localization of *gcl* RNA requires the subset of posterior group genes that are necessary for the formation of abdomen and pole cells. No localization is ever detected in the posterior pole of early embryos from mothers mutant for *cappucino*, *spire*, *oskar*, *staufen*, or *vasa*, consistent with recent findings that show that these genes act in the posterior localization pathway prior to the time at which *gcl* mRNA is introduced into the oocyte. The *cappucino* and *spire* gene functions are required for the localization of the known posteriorly localized molecules (St Johnston et al., 1991; Ephrussi et al., 1991; Kim-Ha et al., 1991; Hay et al., 1990; Lasko and Ashburner, 1990; Raff et al., 1990). *oskar* RNA and staufen protein begin to be localized to the posterior at stage 8 of oogenesis, with *oskar* RNA requiring *staufen* function for initial localization and staufen protein and *oskar* RNA requiring *oskar* function for maintenance of their localization (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). *cappucino*, *spire*, *oskar*, and *staufen* are required for the initial localization of the vasa protein, which begins at the end of stage 9 (Lasko and Ashburner, 1990; Hay et al., 1988a, 1990). The majority of *gcl* mRNA does not enter the oocyte until stage 10 and is not yet concentrated at the posterior at stage 11. This observation, along with our demonstration that *gcl* is required for pole cell formation, is also consistent with the report that the posterior pole plasm is not competent for the induction of pole cell formation until stage 13 of oogenesis (Illmensee et al., 1976).

valois and *tudor* appear to be required for the maintenance of the localization of vasa protein and cyclin B mRNA. In early *valois* and *tudor* embryos, vasa protein is localized normally and cyclin B mRNA is sometimes properly localized, but the localization is subsequently diminished or abolished (Lasko and Ashburner, 1990; Hay et al., 1990; Raff et al., 1990). In comparison, *gcl* mRNA appears to be more dependent on the function of these genes for its localization. In the youngest embryos examined, the posterior localization of *gcl* mRNA never appears normal in these mutants, though occasionally embryos are found that have a detectable amount of localized RNA. This observation suggests that either *gcl* mRNA requires the function of *tudor* and *valois* for its initial localization, with the small amount of localization seen being a result of hypomorphic alleles, or that maintenance of *gcl* mRNA localization is more sensitive to mutations in *tudor* and *valois* than the localization of vasa protein and cyclin B mRNA. We cannot distinguish between these two possibilities at the moment, but we are currently studying the distribution of *gcl* mRNA during oogenesis to determine when it is localized and how the localization is affected in these two mutants.

Besides the subtle differences in the localization of *gcl* mRNA and cyclin B mRNA described above, a more pronounced difference is seen in embryos from *BicD* mothers. These embryos develop two abdomens, though no pole cells are formed at the anterior end (Mohler and Wieschaus, 1986). The formation of two abdomens has been shown to be a result of ectopic localization of the posterior determinant *nanos* (Ephrussi et al., 1991; Wang and Lehmann, 1991). The lack of pole cells in the anterior pole suggests that the pole cell determinant(s) or a subset thereof is not mislocalized to the anterior pole. In *BicD* mutant embryos, *nanos* mRNA (Ephrussi et al., 1991) and cyclin B mRNA are clearly mislocalized to the anterior pole, whereas *gcl* mRNA is not. Thus, cyclin B and *nanos* mRNA mislocalization is similar to that of *oskar* mRNA in *BicD* embryos (Ephrussi et al., 1991), whereas *gcl* mRNA, like staufen and vasa proteins (Hay et al., 1990; Lasko and Ashburner, 1990; St Johnston et al., 1991), is unaffected. One possible explanation is that *gcl* mRNA is more directly dependent on *vasa* and *staufen* function for its localization, whereas localization of cyclin B and *nanos* mRNA is more dependent on *oskar* function.

The results presented in this paper suggest that the *gcl* gene is required for the specification of the germline in Drosophila. It is possible that *gcl* serves additional functions, which can be better characterized once null mutations of *gcl* become available. At the early stages of embryogenesis, however, the gcl protein is found exclusively in nuclei of pole buds and pole cells and only occasionally and transiently in neighboring somatic cells of the cellular blastoderm. Even in those cases where the maternal antisense *gcl* RNA expression greatly reduced the *gcl* mRNA in early embryos and abolished any detectable staining for gcl protein, the embryos develop into sterile but otherwise morphologically normal adult flies, suggesting that *gcl* is not involved in the specification of the posterior axis. The posterior localization of *gcl* mRNA, the genetic dependence of *gcl* mRNA localization on all of the genes known to be required for the localization of germ cell determining activity, and the concentration of gcl protein specifically into the forming pole buds all are consistent with the hypothesis that *gcl* is an important component of the machinery required to specify the germ cell fate. Whether *gcl* is the only component localized to the pole plasm for the specification of the germline is not known. Further analysis of the role of *gcl* in pole cell formation, as well as more extensive genetic investigation into the requirements for germ cell determination, will reveal whether additional localized components are required.

Experimental Procedures

Fly Stocks

Stocks used in the grandchildless screen are described in Bier et al. (1989). The hosts for pWHIα–gcl, w^-, TM3, and *Sb* are described in Lindsley and Grell (1968). The following stocks were used in the localization study: $cappucino^{G7}$ and $spire^{PJ56}$ (Manseau and Schüpbach, 1989), $oskar^{336}$ (Lehmann and Nüsslein-Volhard, 1986), $staufen^{D3}$(St Johnston et al., 1991), $tudor^{WC3}$ (Boswell and Mahowald, 1985), $vasa^{PD}$ and $valois^{RB71}$ (Schüpbach and Wieschaus, 1986), $nanos^{L7}$ (Lehmann

and Nüsslein-Volhard, 1991), *pumilio*[680] (Lehmann and Nüsslein-Volhard, 1987), and *BicD*[7134]/*BicD*[EIII48] (Mohler and Wieschaus, 1986).

Grandchildless Screen

The screen for grandchildless mutants took advantage of an ongoing P element enhancer trap screen in the lab. Detailed description of the screen and vector used are described in Bier et al. (1989). In brief, in a genetic background of *yw*, homozygous females containing a P–lacW construct (containing P element ends, a P transposase–lacZ fusion, mini-*white* gene, ampicillin resistance, and bacterial origin of replication) on the X chromosome were crossed to males containing a Δ2-3 P element (constitutive transposase source; Robertson et al., 1988) on an *Sb*-marked third chromosome. Resultant *Sb* males were crossed to *CyO* females. In the following generation, w^+ males, which could only result from transposition of the X-linked P element to an autosomal chromosome, were isolated and used to establish independent lines. For the grandchildless screen, mated homozygous females, selected by their darker eye color from the established stocks, were allowed to lay eggs in fresh vials. The resulting progeny, if any, were transferred to fresh vials and tested for fertility. Sterile progeny indicated that a line was grandchildless. Two such stocks, A5 2nd 10 and A2 2nd 27, were obtained in a screen of approximately 4000 lines. These lines were retested by mating virgin homozygous females to wild-type males, to ensure that the sterility phenotype was due to maternal effect.

Analysis of Transcripts Adjacent to the P Elements in the Grandchildless Mutants

Genomic sequences flanking P elements were cloned via plasmid rescue following the protocol of Pirrotta (1986) and used to probe wild-type embryos to identify transcripts flanking the P elements. Genomic DNA isolated by plasmid rescue from both A5 2nd 10 and A2 2nd 27 were used as probes for whole-mount in situ analysis, and both identified transcripts were localized to the pole plasm. A 8.0 kb SacII DNA fragment from A2 2nd 27 identified the cyclin B gene at 59A. A SacII genomic fragment from A5 2nd 10 identified *gcl* at 44E. The levels of expression of both transcripts were normal in both lines.

Genetic Analysis of Grandchildless Mutants

The two mutants isolated in this screen are A2 2nd 27 and A5 2nd 10. Both lines contained P–lacW on the second chromosome. Complementation analysis was carried out with each other as well as with previously identified posterior group mutants on the second chromosome, namely, *tudor*, *valois*, *vasa*, and *staufen*. Both lines failed to complement each other as well as *tudor* alleles.

Meiotic recombination was also done to confirm that the grandchildless phenotype was due to a mutation at the *tudor* locus. Homozygous males from each line were crossed to females of a line containing a multiply marked second chromosome over *CyO*. The markers used were *Star* (2-1.3), *Sternopleural* (2-22.0), *Tuft* (2-53.2), *narrow-wing*[dom] (2-83), and *Pin* (2-107.3). The resultant females containing the multiply marked second chromosome over either A2 2nd 27 or A5 2nd 10 were mated to males of the corresponding grandchildless line. Females with a recombinant chromosome over the original P-containing chromosome were mated to wild-type males and the resulting progeny tested for sterility. The grandchildless mutation for A2 2nd 27 mapped to 2-99, while that for A5 2nd 10 mapped distal to *narrow-wing*, which is distal to 44E. The *tudor* locus has previously been mapped to 2-97 (Boswell and Mahowald, 1985). Thus, both the complementation and recombinational mapping are consistent with both of these lines containing weak *tudor* alleles.

Isolation of *gcl* cDNA

The SacII genomic DNA fragment was used as a probe to isolate cDNAs from an ovarian λZAP expression library according to the Stratagene protocol (Stratagene, San Diego, California). The cDNAs obtained were used as probes for in situ hybridization to salivary chromosomes to confirm that they corresponded to the 44E region. Primer extension, performed according to the protocol of McKnight and Kingsbury (1982), indicated that the longest cDNA was not full length. The remaining 5′ region was cloned using a rapid amplification of cDNA ends (RACE) polymerase chain reaction (Frohman, 1990) using the following *gcl*-specific primers: primer 1, 5′-AAGATGAACCAGTGCTGCGC-3′; primer 2, 5′-TTTCGCTTCCGTCGATTGCTG-3′ for the RACE-specific amplification. Total RNA was prepared using the hot phenol method (Jowett, 1986). *gcl*-specific cDNA was prepared using primer 1 according to the primer extension protocol of McKnight and Kingsbury (1982), with the following modification of the annealing reaction. Ten micrograms of total RNA prepared from hand-dissected whole ovaries was denatured with 50 fmol of primer in 10 μl of hybridization buffer (1.25 mM KCl in TE) at 95°C for 2 min and then annealed at 50°C for 15 min. After the extension reaction, the excess primers were removed with a Centricon 100 spin filter, washing two times with 1 ml of 0.2 × TE; the retained liquid was concentrated with a speed vac to 10 μl. The tailing and first and second round of amplification were done as in Frohman (1990). The 5′ end of *gcl* was subcloned into pBluescript KS(+) using the SmaI site polylinker. This fragment was then used to probe an ovarian λZAP (Stratagene) library, from which a full-length cDNA (10B-1) was obtained.

We have sequenced both strands of two independent genomic clones, one from a λDASH library, containing all of the corresponding *gcl* sequence except for the region from +1 to +205, and one complete genomic clone from a λFix library, as well as the full-length cDNA (10B-1). Although the genomic clones had identical sequences, there were differences when compared with the 10B-1 sequence; these differences are noted in the legend to Figure 8. Comparison of the genomic and cDNA sequences also revealed three introns, all of which had consensus splice donor and acceptor sequences. The sizes of the introns are discussed in the legend to Figure 1A, and the positions are noted in the legend to Figure 8.

Construction of pWHIα–*gcl* and Subsequent Transformation

The full-length *gcl* cDNA was excised from pBluescript KS(+) using the SmaI and EcoRV sites and subcloned into the unique KpnI site of pWHI. pWHIα–*gcl* was coinjected into w^- host embryos according to the procedure of Rubin and Spradling (1982), with the modification of using a constitutive source of transposase activity as described in Spradling (1986).

Production of Polyclonal Anti-gcl Rabbit Serum

The trpE–*gcl* construct for the expression of the fusion protein was generated as follows; the *gcl* cDNA was cut with NcoI (at the initial methionine) and treated with Klenow and, subsequently, with XbaI. The NcoI–XbaI fragment was then subcloned into the trpE fusion vector pATH1 (Dieckmann and Tzagaloff [1985]) cut with EcoRI, cut with Klenow fragment, then recut with XbaI. The fusion protein was induced in Escherichia coli and isolated for immunization as described by Hay et al. (1990).

Immunocytochemistry and In Situ Hybridization

The procedure for antibody staining of whole-mount embryos was described previously (Bodmer and Jan, 1987). Affinity-purified anti-vasa antibodies were prepared as described by Hay et al. (1988b) and used at a concentration of 1:1000. The anti-gcl antibody was preabsorbed at a concentration of 1:100 with an equal volume of 3–6 hr embryos, then used at a concentration of 1:1000 to 1:2000. The secondary horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) was used at a concentration of 1:100, after preabsorption to an equal volume of embryos of the same stage to be stained.

In situ hybridizations to RNA in whole-mount embryos were performed as described by Tautz and Pfeifle (1989). Minor modifications were used on ovaries that were prepared en masse according to Mahowald et al. (1983), with the following modification (Therkauf and Hawley, 1992). Well-fed flies 3–5 days old were anesthetized with CO_2 and then transferred to an Osterizer blender (Pulsematic 12 model) containing 250 ml of modified Robb's buffer (55 mM sodium acetate, 40 mM potassium acetate, 100 mM sucrose, 10 mM glucose, 1.2 mM magnesium chloride, 1.0 mM calcium chloride, and 100 mM HEPES [pH 7.4]). The flies were blended three times, for 2 s each time, at the lowest setting. The homogenate was filtered through a 1 mm nylon mesh, with the retained material being reblended and filtered as above. The filtrates were pooled, and the egg chambers were allowed to settle for 5 min at 1 × g, after which most of the supernatant was aspirated away. The settled egg chambers were resuspended in 200 ml of modified Robb's buffer and then filtered through a 250 μm nylon mesh, and the egg chambers were settled again at 1 × g for 5 min. The superna-

tant was removed by aspiration, and the egg chambers were transferred in a minimum volume of Robb's buffer to a 15 ml falcon tube and resettled. As much of the Robb's buffer was removed as possible before the fixative (4% paraformaldehyde [EM grade, Ted Pella, Incorporated], 0.1% dimethylsulfoxide in phosphate-buffered saline [PBS]) was added. The egg chambers were fixed for 1 hr at 22°C with constant rotation, followed with three rinses with PBS of 15 min each. Egg chambers were either dehydrated with an ethanol series to 100% and stored at −20°C or immediately prepared for in situ hybridization. Rehydrated or freshly prepared egg chambers were treated with 50 μg/ml proteinase K in PBS containing 1 mM EDTA for 1 hr at 22°C. The protease treatment was stopped according to Tautz and Pfeifle (1989), and then for the remainder of the procedure the egg chambers were treated as though they were embryos.

Pole Cell Counts and Analysis of Living Embryos

Pole cell number in whole-mount embryos were recorded in stage 14 embryos using a Nikon Optiphot microscope equipped with Nomarski optics. A Nikon Lucida camera was used to outline each labeled cell. Counts were made from these drawings. Observations of pole bud formation and subsequent pole cell formation were made by collecting embryos for 1 hr time periods, in the range of 8–12 hr after heat shock of the adult females, dechorionating them, mounting the embryos on a slide with heptane glue, covering it with halocarbon 3S oil (Voltalef), and observing the posterior pole using Nomarski optics.

Heat Shock Experiments

All heat shock experiments were done using 3- to 5-day-old females mated with a 2- to 3-fold excess of males. The flies were kept in minicages and fed yeast twice daily on grape juice agar plates. The heat shocks consisted of placing the minicages in a 37°C incubator for 45 min. Heat shocks were limited to this time period because longer treatments resulted in embryos that were defective in gastrulation and a reduction in the number of pole cells in the control line was observed. After the heat shock, the flies were maintained at 25°C, whereby egg collections were made every 4 hr. The egg collections were aged for the appropriate amount of time and then fixed either for antibody stainings or for in situ hybridization.

Northern Analysis

For the developmental analysis, timed collections from large cages of Oregon R flies were made, and total RNA was prepared using the hot phenol method (Jowett, 1986). Poly(A)$^+$ RNA was selected using oligo(dT)–cellulose type 2 (Collaborative Research), and 10 μg was loaded per lane. For analysis of *gcl* RNA levels in the antisense mutants, total RNA was prepared according to Chomczynski and Sacchi (1987), and 10 μg was loaded per lane. The gels and subsequent transfer and probing were done according to Vaessin et al. (1987). Single-stranded probes were made from cDNA clones in pBluescript KS(−) in opposite orientations, which were obtained in the screen for cDNA from the λZAP library.

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