Establishment of the Germ Cell Lineage of *Drosophila* and Has Activities Which Are Dependent and Independent of Its Localization to the Nuclear Envelope

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The germ cell precursors of *Drosophila* (pole cells) are specified by maternally supplied germ plasm localized to the posterior pole of the egg. One component of the germ plasm, *germ cell-less* (*gcl*) mRNA, encodes a novel protein which specifically localizes to the nuclear envelope of the pole cell nuclei. In addition to its maternal expression, *gcl* is zygotically expressed through embryonic development. In this report, we have characterized a null allele of *germ cell-less* to determine its absolute requirement during development. We have found that *gcl* activity is required only for the establishment of the germ cell lineage. Most embryos lacking maternal *gcl* activity fail to establish a germline. No other developmental defects were detected. Examination of germline development in these mutant embryos revealed that *gcl* activity is required for proper pole bud formation, pole cell formation, and pole cell survival. Using this null mutant we have also assayed the activity of forms of Gcl protein with altered subcellular distribution and found that localization to the nuclear envelope is crucial for promoting pole cell formation, but not necessary to initiate and form proper pole buds. These results indicate that *gcl* acts in at least two different ways during the establishment of the germ cell lineage. © 1999 Academic Press

Key Words: pole cell; germline; myristoylation; germ cell specification; germ plasm; primordial germ cell.

INTRODUCTION

In organisms ranging from insects to amphibians the formation of the germline is dependent on the presence of a differentiated cytoplasm, referred to as "germ plasm," which is localized to the region of the embryo where the germ cell precursors form (Beams and Kessel, 1974; Eddy, 1975). Experiments in *Drosophila* have demonstrated that its germ plasm contains all of the determinants required to specify germ cell fate (Illmensee and Mahowald, 1974; Ephrussi and Lehmann, 1992).

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Early in *Drosophila* development, formation of the germ cell precursors, or "pole cells," is initiated when a small subset of the syncytial nuclei migrate into the germ plasm and induce the formation of "pole buds." These visible protrusions of the plasma membrane engulf the germ plasm components and later pinch off to form the pole cells. As soon as they form, pole cells are restricted to germline fate (Hay *et al.,* 1988; Lasko *et al.,* 1990; Technau and Campos-Ortega, 1986). They are also transcriptionally silent relative to the soma during the first several hours of embryogenesis (Zalokar, 1976; Seydoux, 1997). Later during gastrulation, the pole cells undergo a patterned migration to the embryonic gonad (Williamson and Lehmann, 1996).

In the past decade, several components of the germ plasm required for pole cell formation and later germline development have been identified (for review see Williamson and Lehmann, 1996; Rongo *et al.,* 1997). However, to date, only

the germ plasm components encoded by *germ cell-less* and mtlrRNA (mitochondrial large ribosomal RNA) have been implicated as having a role in germline development at the time pole cells form (Jongens *et al.,* 1992, 1994; Kobayashi and Okada, 1989; Iida and Kobayashi, 1998).

Maternal *germ cell-less* mRNA is concentrated in the germ plasm. The resultant Germ cell-less protein specifically associates with the nuclei which enter the germ plasm and become incorporated into the pole cells (Jongens *et al.,* 1992). The function of Gcl protein is unknown. It is a novel protein which localizes to the nucleoplasmic surface of the nuclear envelope, with a concentration near nuclear pores (Jongens *et al.,* 1994). The importance of this subcellular localization for *germ cell-less* function is unknown.

Evidence that *gcl* activity is important for pole cell formation comes from antisense, overexpression, and ectopic localization studies. The selective reduction of maternal *gcl* mRNA levels, by expressing antisense gcl RNA during oogenesis, leads to a reduction in pole cell formation (Jongens *et al.,* 1992). Overexpression of maternal *gcl* mRNA results in the transient formation of additional pole cells, and its ectopic localization leads to the ectopic initiation of events similar to those seen during normal pole cell formation (Jongens *et al.,* 1994). The results from these analyses demonstrate that *germ cell-less* is both required for and capable of initiating some of the events of pole cell formation. However, its absolute role in development has been unclear as the antisense approach did not remove all of the maternally contributed *gcl* mRNA, nor did it affect *gcl*'s zygotic expression (Jongens *et al.,* 1992; T.A.J., unpublished observation).

In this study, we have characterized a null allele of *germ cell-less* to investigate its absolute requirement during development. We have found that *gcl* activity is required only for the establishment of the germ cell lineage. The processes of pole bud formation, pole cell formation, and pole cell survival are affected in embryos that lack maternal *germ cell-less* activity. Also in this study, we have used the *gcl* null mutant to examine the importance of Gcl's subcellular localization for function. We find that both cytoplasmic and nucleoplasmic forms of Germ cell-less protein can rescue pole bud formation, but localization to the nuclear envelope is essential for pole cell formation. These results suggest that *gcl* has at least two different activities required for proper germline development.

MATERIALS AND METHODS

Immunocytochemistry and in situ hybridizations. Antibody stainings and whole-mount *in situ* hybridizations were performed as previously described (Jongens *et al.,* 1992). Embryos and tissue culture cells were visualized on a Leica DMR microscope using DIC optics and epifluorescence with image capture using a Hamamatsu color CCD camera or with a Leica scanning laser confocal microscope.

Pole cell counts and analysis of pole bud formation. Pole cell counts were performed on fixed embryos stained with anti-Vasa antibody as previously described (Jongens *et al.,* 1992). Analysis of pole bud formation was performed on both fixed and living embryos as previously described (Jongens *et al.,* 1992, 1994).

Northern analysis. For comparison of *gcl* mRNA levels between the control and the transformant lines, RNA was prepared from 0- to 1-h embryos and analyzed by Northern analysis and quantitated as described previously (Jongens *et al.,* 1992).

Construction of the Gcl(-nls) and Gcl(-myr) forms of Gcl and their analysis in Drosophila S2 cells and embryos. To express *gcl* and mutant forms thereof in S2 cells the following constructs were made. An *Eco*RI (filled in)/*Xba*I fragment of pBSgcl containing most of the wild-type *gcl* cDNA was cloned into pmtaL (Johansen *et al.,* 1989) cut with *Eco*RV/*Xba*I. The 3′UTR sequences of *gcl* were removed with a *Pml*I/*Stu*I digest. This construct, pmtaLgcl, contains the *gcl* ORF downstream of the metallothionein promoter. To mutate the putative NLS (aa 19–24), site-directed mutagenesis was performed on pBSgcl with the oligo 5′GAATCAGTGCTGAGCTTCAGCTTCAGTCGATTGC3′ using a Muta-Gene Phagemid *in vitro* mutagenesis kit (Bio-Rad). The mutagenized sequences were transferred to pmtaLgcl as an *Nco*I/*Sap*I fragment, forming pmtaLgcl(-nls). To mutate the putative myristoylation site the primers 5′TCAGCCATGGCTCAAATAGTGGGATCCATCG3′ and 5′CAGAGCCATGACAGCCACATCC3′ were used to PCR amplify the N-terminus of Gcl. The PCR product was sequenced and then digested with *Nco*I/*Sap*I and ligated into pmtaLgcl digest with the same, forming pmtaLgcl(-myr).

To determine the subcellular localization of the mutant forms of Gcl, the above-described constructs were transfected into S2 cells with lipofectin (Life Technologies) according to the manufacturer's instructions. Twenty-four hours after the transfection CuSO$_4$ was added to a final concentration of 200 μM and the cells were incubated for an additional 24 h. The cells were fixed and stained as described in Fehon *et al.* (1990).

To maternally introduce the mislocalized forms of Gcl into the germ plasm of *gcl*$^\Delta$ embryos, the above-described mutations were introduced into the P element vector phg, which contains the entire *germ cell-less* cDNA downstream of the hsp83 promoter (Jongens *et al.,* 1994). The *Bam*HI/*Not*I (filled in) fragment of pBSgcl(-nls), containing +238 to 2465 of *gcl,* was ligated into phg (Jongens *et al.,* 1994) digested with *Bam*HI/*Xho*I(filled in) to make phg(-nls). phg was digested with *Bam*HI/*Nco*I, and the oligos 5′CATGGCTCAAATAGTGG3′ and 5′GATCCCACTATTTGAGC3′ were annealed and ligated in to form phg(-myr). P-element-mediated transformation was performed as described by Spradling (1986) to obtain stable transgenic lines.

RESULTS

germ cell-less Is Required Solely during the Establishment of the Germ Cell Lineage

Since the previous analysis of *germ cell-less* indicated that it plays an important role in the establishment of the germ cell lineage, we wanted to further explore its function in this process by obtaining and analyzing bona fide *gcl* mutants. As all of our mutagenesis-based screens failed, we turned to a P-element-based approach to obtain *germ cell-less* mutants. From this approach, we have isolated a null allele of *gcl* that has allowed us to examine its absolute role during development. This null allele was constructed using a small deletion (*rev390*) which removes the entire coding region of *gcl* and the adjacent gene, *cmp44E* (Fig. 1A).
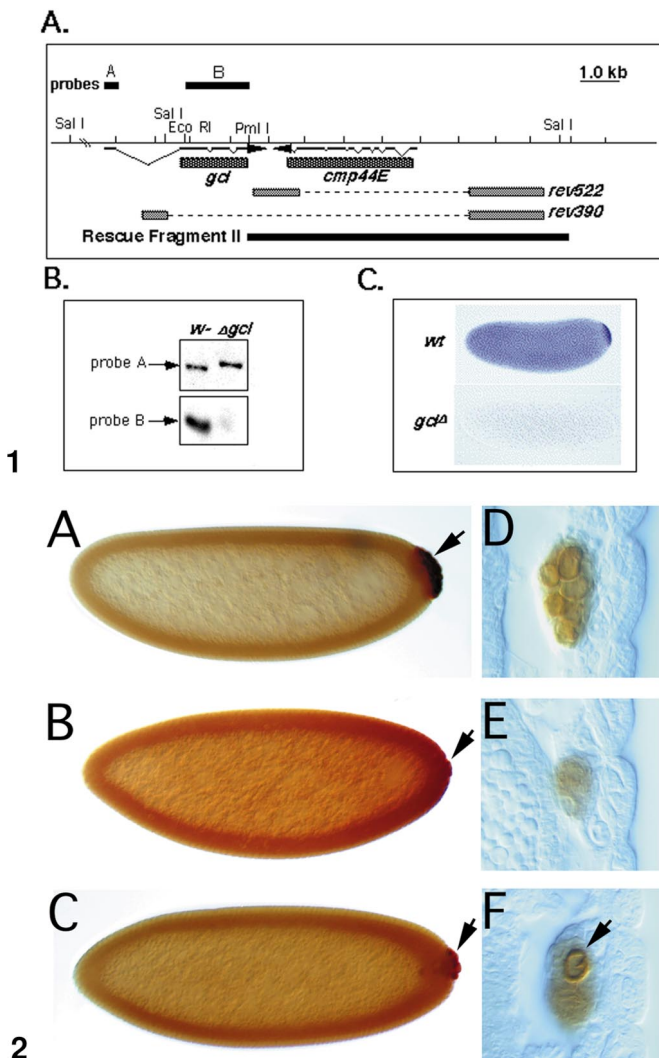
Previous analysis of *cmp44E* revealed that it is an essential gene and that a null allele (*rev522*) could be rescued by the stable introduction of an 8.5-kb genomic fragment containing the *cmp44E* gene (rescue fragment II, Fig. 1A; Faulkner *et al.,* 1998). Thus the introduction of rescue fragment II into the *rev390* background bypassed the developmental blocks due to the loss of *cmp44E* activity and allowed examination of the *gcl* null phenotype.

The introduction of a single copy of rescue fragment II fully rescued the lethality of the *rev390* deletion, indicating that *germ cell-less* function was not required for viability. The resulting homozygous *rev390* flies lacked any visible phenotypes. In addition, the morphology of the testis and ovaries and the egg-laying rates of the females appeared normal. Thus zygotic *gcl* expression is not required for germline development or germ cell proliferation.

To confirm that the homozygous *rev390* flies were null for *gcl,* Southern hybridization was performed on genomic DNA prepared from these flies and whole-mount *in situ* hybridization was performed on embryos from homozygous *rev390* females. As expected, no *germ cell-less* signal was detected on the Southern blot or in the embryos when a probe specific for the *gcl* coding region was used (Figs. 1B and 1C).

Since our analysis failed to reveal a developmental requirement for the zygotic expression of *gcl,* we focused our attention on the role of its maternal expression. To do this, we examined development in embryos lacking maternal *germ cell-less* contribution, i.e., embryos from mothers that were homozygous for *rev390* and contained one copy of rescue fragment II. We refer to these progeny as *gcl*$^{\Delta}$ (*gcl* null) embryos and adults.

To determine if maternal *gcl* was required for viability, we compared the hatch rates of *gcl*$^{\Delta}$ embryos to those of control embryos. We found the hatch rates of both types of embryos to be identical. This was true even when the embryos lacked both maternal and zygotic *gcl* activity. Thus maternal *gcl* is not required for embryonic patterning or viability, leaving the establishment of the germline as the only possible developmental process requiring *gcl* function.

FIG. 1. Construction of a *germ cell-less* null mutant. (A) The *germ cell-less* and *cmp44E* genomic region. The genomic structures and relative positions of *gcl* and the 2.9-kb transcript of *cmp44E* are indicated as thick black lines with introns denoted as thinner black lines. The arrowhead at the 3′ end of each gene indicates the direction of transcription. The dark-shaded boxes below indicate the coding region for each gene. Two small deficiencies obtained by imprecise excision of a P element (*rev522* and *rev390;* Faulkner *et al.,* 1998) are indicated with dotted lines. The hatched box at each end indicates the potential range of the end points as determined by Southern analysis. In *rev522* the deletion removes most, if not all, of the *cmp44E* coding region while the deletion in *rev390* removes the entire coding region of both *gcl* and *cmp44E.* Genomic rescue fragment II used in this study is shown as a very thick solid black line. Relevant restriction sites used for Southern analysis and construction of genomic rescue fragment II are indicated. (B) Southern analysis of genomic DNA prepared from control (*w*[1118]) and *gcl* null flies. The genomic DNA was digested with *Eco*RI and *Pml*I and then probed with both probes A and B (see A). Sequences containing the coding region of *gcl* are not detected in the *gcl* null flies. (C) Whole-mount *in situ* hybridization of control and *gcl*$^{\Delta}$ embryos with a probe specific for *germ cell-less* mRNA. The wild-type distribution of *gcl* mRNA is seen in the control embryo but none is detected in the *gcl*$^{\Delta}$ embryo.

FIG. 2. Decreased pole cell formation in the *gcl*$^{\Delta}$ embryos. Blastoderm-staged (A) control and (B and C) *gcl*$^{\Delta}$ embryos stained with anti-Vasa antibody. Although an average of 24 pole cells are formed in the control embryos, no or few pole cells are formed in the *gcl*$^{\Delta}$ embryos. Arrows indicate the position at which pole cells form at the blastoderm stage. (D–F) Individual gonads of control and *gcl*$^{\Delta}$ stage 14 embryos stained with anti-Vasa antibody. (D) A normal complement of pole cells is found in the host embryonic gonad, whereas no or a few pole cells are found in the *gcl*$^{\Delta}$ embryonic gonads (E and F).
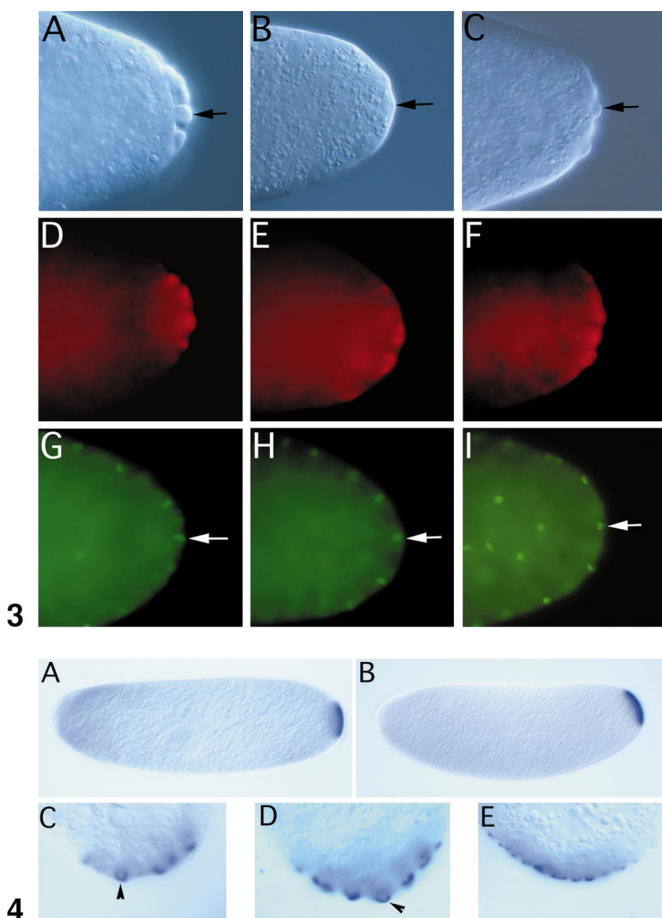
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FIG. 3. Pole bud formation is affected in the *gcl*$^\Delta$ embryos. Control and *gcl*$^\Delta$ embryos were fixed and stained with anti-Vasa antibody followed with a rhodamine-labeled secondary antibody to label the germ plasm and Høescht stain to label the DNA. The formation of pole buds was visualized with DIC optics and the germ plasm and nuclei were visualized with epifluorescence. The images in the left column (A, D, and G) are taken from a control (*w*^{1118}) embryo and the middle and right columns (B, C, E, F, H, and I) are images from *gcl*$^\Delta$ embryos. All embryos are at prophase of nuclear cycle 10 as identified by nuclear density and the state of chromatin condensation. The DIC images showing the pole buds are shown in top row (A, B, and C). The arrows indicate the position of a representative nuclei in the germ plasm, also see (G, H, and I). The rhodamine signal indicating the position of the germ plasm is in the middle row (D, E, and F) and the positions of nuclei in the germ plasm are shown in the bottom row (G, H, and I). Prominent pole buds, characteristic of wild-type embryos, are shown in (A). Failed and reduced pole bud formation seen in the *gcl*$^\Delta$ embryos are shown in (B) and (C), respectively.

FIG. 4. Localization and maintenance of germ plasm components is unaffected in the *gcl*$^\Delta$ embryos. Whole-mount *in situ* hybridization of control and *gcl*$^\Delta$ embryos using a probe specific for *nanos* mRNA. Early cleavage stage (A) control and (B) *gcl*$^\Delta$ embryos. In late cleavage embryos *nanos* mRNA concentrates around the nuclei, which enter the germ plasm in both (C) control and (D) *gcl*$^\Delta$ embryos. (E) The localization of *nanos* mRNA is maintained even in *gcl*$^\Delta$ embryos, which fail to form pole cells.

Germline development was examined in control and *gcl*$^\Delta$ embryos using anti-Vasa antibody, as this stains the germ cells throughout development (Hay *et al.,* 1988; Lasko and Ashburner, 1990). At the blastoderm stage (stage 5) we found that 48% of the *gcl*$^\Delta$ embryos lack any pole cells and the remainder formed fewer pole cells than control embryos (Figs. 2A–2C; Table 1). At stage 14, the time pole cells should have successfully reached the embryonic gonad (Campos-Ortega and Hartenstein, 1985; Hay *et al.,* 1988; Williamson and Lehmann, 1996); we observed that 67% of the *gcl*$^\Delta$ embryos lacked pole cells and that the pole cell survival rate was much lower in this mutant compared to control embryos (Figs. 2D–2F and Table 1). In our study, 91.0% of the pole cells that initially formed in the control line successfully reached the embryonic gonad (an average of 23.4 pole cells at the blastoderm stage to 21.3 at stage 14, Table 1). In *gcl*$^\Delta$ embryos, only 39% of the pole cells that formed at the blastoderm stage successfully reached the embryonic gonad (an average of 2.8 at the blastoderm stage to 1.1 at stage 14, Table 1). This increased rate of pole cell loss in the *gcl*$^\Delta$ embryos, relative to control embryos, indicates a requirement for *gcl* activity after the blastoderm stage. However, since the percentage of embryos which had at least 1 pole cell at stage 14 (~32%) matched the percentage of fertile *gcl*$^\Delta$ adults (32%, *n* = 354), it appears that *gcl* activity is not required after this stage.

To determine when the increased rate of pole cell loss was occurring, we counted pole cells in *gcl*$^\Delta$ embryos at stages between blastoderm formation and stage 14. We found that by stage 10, the average number of pole cells in the *gcl*$^\Delta$ embryos was close to the average observed at stage 14 (Table 1). Thus the loss of *gcl* activity affects pole cell survival most severely from the time of their formation to stage 10, the time at which they initiate their migration through the posterior midgut primordium. The frequent presence of cellular debris that stained with Vasa antibody in *gcl*$^\Delta$ embryos up until stage 10 suggests that the fate of many of the pole cells formed in these embryos is cell death (not shown).

To verify that the reduction in pole cell formation and survival observed in the *gcl*$^\Delta$ embryos was due to the loss of maternal *gcl* activity, we introduced an hg construct (hg130) into the *gcl* null mutant background. This construct, which provides high levels of maternal *gcl* mRNA to embryos (Jongens *et al.,* 1994), fully rescued the *gcl* null phenotype, as all of the resulting embryos contained wild-type levels of pole cells (Table 1) and all of the resulting progeny were fertile (*n* = 200). Thus the defects observed in the *gcl*$^\Delta$ embryos are due to the loss of maternal *gcl* activity.

Examination of the Earliest Defect of Pole Cell Formation in the *gcl*$^\Delta$ Embryos

To determine the earliest stage at which *germ cell-less* activity was required for germline development, we examined the process of pole cell formation in *gcl*$^\Delta$ embryos. In wild-type embryos, this process begins with the migration

TABLE 1

Reduced Pole Cell Formation and Survival in the gcl^Δ Embryos

Line	Stage	Average number of pole cells	Percentage of embryos with the indicated number of pole cells							
			0	1–5	6–10	11–15	16–20	21–25	26–30	31–35
$w-$	Blastoderm	23.4 ± 0.7	0	0	0	2	28	36	32	2
gcl^Δ	Blastoderm	2.8 ± 0.4	48	33	11	4	3	0	0	0
$w-$	14	21.3 ± 0.4	0	0	0	6	40	32	22	0
gcl^Δ	10	1.3 ± 0.3	62	31	7	0	0	0	0	0
gcl^Δ	14	1.1 ± 0.4	67	26	5	1	0	0	0	0
gcl^Δ+hg130	14	21.5 ± 0.7	0	0	0	6	32	52	10	0

Note. Embryos lacking maternal *germ cell-less* activity have a greatly diminished capacity to form a germline. Pole cell counts of control (w^{1118}), gcl^Δ, and gcl^Δ+hg130 embryos are shown. Average number of pole cells and the SEM, as well as the distribution of pole cell number, are given. Over 100 embryos were counted for each line. (See text and Materials and Methods for details.)

of the syncytial embryonic nuclei into the germ plasm, which initiates the formation of pole buds. These pole buds persist for two synchronous nuclear divisions after which they pinch off, forming pole cells (end of nuclear cycle 10; Foe and Alberts, 1983; Warn *et al.,* 1985). By examining the process of pole cell formation in living embryos, we observed that 21.3% ($n = 160$) of the gcl^Δ embryos failed to form detectable pole buds. Most of the remaining gcl^Δ embryos formed pole buds that were less prominent than those formed in wild-type embryos. Reduced pole bud formation was also detected in fixed gcl^Δ embryos stained with Høechst dye to determine nuclear cycle and anti-Vasa antibody to label the position of the germ plasm (Fig. 3). The results of these analyses indicate that *gcl* activity is required for the initiation and formation of proper pole buds. This is earlier than detected with the antisense approach, which placed the earliest requirement for *gcl* activity at the time at which the pole buds pinch off to form pole cells (Jongens *et al.,* 1992).

germ cell-less Is Not Required for the Localization or Maintenance of Germ Plasm Components nor for the Transcriptional Silencing of the Germline

Previous analysis of embryos with reduced levels of *gcl* mRNA suggested that *gcl* does not have a role in organizing or maintaining the normal distribution of germ plasm components (Jongens *et al.,* 1992). To determine if the total removal of *gcl* activity affects the localization or maintenance of germ plasm components, we performed whole-mount *in situ* hybridization on gcl^Δ embryos using probes specific for *nanos, cyclin B,* and *Pgc* mRNAs. All of these mRNAs have been shown to be localized late during oogenesis and therefore would be most likely to reveal defects in germ plasm formation and maintenance (Wang *et al.,* 1994; Nakamura *et al.,* 1996; Dalby and Glover, 1992). No difference in the localization or maintenance of these germ plasm components was detected in the gcl^Δ embryos compared to wild-type embryos (Fig. 4 and not shown). Thus the

failure to form pole cells in the gcl^Δ embryos is not due to defects in germ plasm integrity.

The germ plasm components that normally would have been incorporated into the pole cells are degraded by the time the cellular blastoderm forms in the gcl^Δ embryos. The fate of the pole cell-destined nuclei, as well as that of the somatic-destined nuclei inappropriately exposed to germ plasm components due to the failure of pole cell formation, is not known. However, compared to wild-type embryos, we often observe an increased number of nuclei falling into the center of the embryo at the posterior pole (Jongens *et al.,* 1994) (not shown). This could be due to the selective removal of the nuclei which should have been incorporated into the pole cells or to a random elimination of the excess nuclei present at the posterior pole, due to the failure of pole cell formation. Nonetheless since no posterior defects are observed in the gcl^Δ embryos, whichever nuclei are incorporated into the posterior somatic cells become properly fated.

One striking property of pole cells is that they are transcriptionally silent relative to the somatic cells during the blastoderm and early gastrulation stages (Zalokar, 1976; Seydoux and Dunn, 1997). This transcriptional silence correlates with the absence of anti-H5 antibody staining, which recognizes phosphorylated amino acid residues on the C-terminal tail of actively transcribing RNA polymerase II (Seydoux and Dunn, 1997). We did not detect a difference in the level of anti-H5 staining in the pole cell nuclei of wild-type vs gcl^Δ embryos (not shown), thus it appears that *gcl* activity is not required for transcriptional silencing in the germ cell precursors.

Localization of Gcl Protein to the Nuclear Envelope Is Required for Pole Cell, but Not Pole Bud, Formation

Germ cell-less protein has a striking subcellular distribution in pole cells. Immuno-EM analysis revealed that it is localized to the nucleoplasmic surface of the nuclear enve-

lope in close proximity to nuclear pores (Jongens *et al.,* 1994). The importance of this localization for *germ cell-less* function is unclear. From the time syncytial nuclei enter the germ plasm to the time at which the pole buds pinch off to form the pole cells, Gcl protein is both present in the cytoplasm as well as localized to the nuclear envelope. It is only after pole cell formation that the vast majority of Gcl protein is associated with the nuclear envelope (Jongens *et al.,* 1992). Thus it has not been clear whether pole cell formation is dependent on the localization of Gcl protein to the nuclear envelope.

To examine the importance of its localization for function, we first identified precise mutations which altered the subcellular distribution of Gcl. We have found that when ectopically expressed in *Drosophila* S2 cells, Germ cell-less protein properly localizes to the nuclear envelope (Figs. 5B–5D). Using this assay system, we previously identified a nuclear localization sequence (NLS), which when mutated leads to a cytoplasmic form of Gcl (Dockendorff *et al.,* submitted) (Figs. 5A and 5E–5G). In addition, we have identified a single amino acid residue that is essential for the association of Gcl with the nuclear envelope. The N-terminus of Gcl contains a myristoylation consensus sequence (Fig. 5A). In this consensus sequence, the first glycine (G_2) is critical for the addition of a myristoyl group, a posttranslational lipid modification used to localize proteins to membranes (Towler *et al.,* 1988; Grand, 1989). By changing G_2 to alanine, we created a form of Gcl protein which enters the nucleus but fails to localize to the nuclear envelope, resulting in a nucleoplasmic distribution (Figs. 5H–5J).

To assay the importance of Gcl localization for function, the mislocalized forms of Gcl were tested for the ability to rescue germline formation in the gcl^Δ embryos. The above-described point mutations (Fig. 5A and Materials and Methods) were introduced into the hg construct. The construct containing the mutated NLS is referred to as hg(-NLS) and the one containing the mutated myristoylation site is referred to as hg(-myr). Several transformant lines of each mutant construct and of the hg construct were obtained in the *gcl* null background. Lines expressing similar levels of wild-type or mutant forms of *gcl* mRNA were identified by Northern analysis and used for further analyses. They are hg96(wt), hg(-NLS)49, and hg(-myr)31 (Fig. 6A).

If either mislocalized form of Gcl protein were fully functional, our expectation was that it would be able to rescue pole cell formation as effectively as wild-type *gcl.* To determine the activity of cytoplasmic and nucleoplasmic forms of *gcl,* pole cell counts were performed on blastoderm-staged embryos from the lines hg96, hg(-NLS)49, and hg(-myr)31**.** While the average number of pole cells formed in the hg96(wt) embryos was significantly increased over that of gcl^Δ embryos, no increase was observed in the hg(-NLS)49 or hg(-myr)31 embryos (Table 2).

To ensure that the failure to detect activity from the mislocalized forms of Gcl protein was not due to diminished protein stability, we stained hg 96(wt), hg(-NLS)49,

and hg(-myr)31 embryos with anti-Gcl antibody. At the mid-cleavage stage, all three forms of Gcl protein were faintly detected in the germ plasm (Figs. 6B–6D). In hg96 embryos, the wild-type form of Gcl protein could be detected on the nuclear envelopes of the pole cell nuclei (Figs. 6E–6G). Similarly, the nucleoplasmic form of Gcl was detected at significant levels in the nucleoplasm of the few pole cells which formed in the hg(-myr)31 embryos (Figs. 6H–6J). Thus this form of Gcl is stable and remains localized to the germ plasm. The cytoplasmic form of Gcl in the hg(-NLS) embryos, however, was not detected in the few pole cells which formed (not shown), thus it is possible that this form of Gcl is not stable in the embryo or it diffuses out of the germ plasm prior to pole cell formation. Nonetheless, our results indicate that Gcl protein must be localized to the nuclear envelope to promote pole cell formation.

Since pole bud formation is also affected in the gcl^Δ embryos, we examined whether the mislocalized forms of Gcl could rescue this process. Pole bud formation was observed in both fixed and living embryos from the hg 96, hg(-NLS) 49, and hg(-myr) 31 lines. We found that all three forms of Gcl were capable of rescuing pole bud formation. All of the resulting embryos had prominent pole buds (Fig. 7). Thus Germ cell-less protein does not need to be localized to the nuclear envelope or the nucleus to promote pole bud formation.

DISCUSSION

We have obtained and analyzed a *germ cell-less* null mutant to determine the absolute requirement of *germ cell-less* activity during development. Although *gcl* encodes a germ plasm component and is expressed in several tissues throughout development, we detected a requirement only for its maternal expression. Embryos that lack maternal *germ cell-less* activity form either no or fewer pole cells than control embryos. Most of the resulting adults are sterile, with no other developmental defects being observed. This effect on germline formation is not due to a decrease in germ plasm integrity or due to a failure to establish transcriptional repression in the early pole cells. Analysis of the *germ cell-less* null mutant phenotype, in combination with assaying the ability of mislocalized forms of Gcl protein to rescue this mutant, has revealed that *gcl* is required up to three times during the establishment of the germ cell lineage and acts in at least two different ways. Furthermore, our results confirm the previous suggestion, inferred from antisense studies, that maternal *gcl* is required only for the establishment of the germ cell lineage (Jongens *et al.,* 1992).

The Requirement for germ cell-less Activity Multiple Times during the Establishment of the Germ Cell Lineage

gcl activity is initially required at or prior to the time of pole bud formation, as pole buds failed to appear or had poor
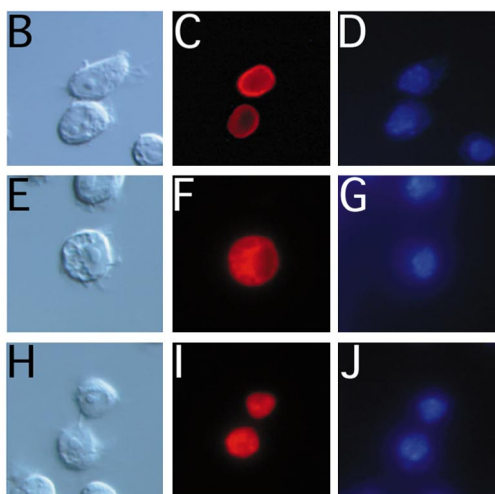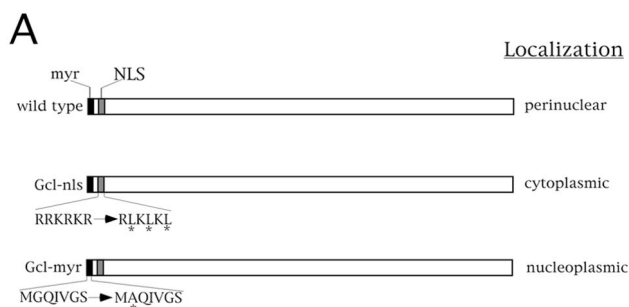
morphology in the *gcl*^Δ embryos. Previously we observed that the overexpression and ectopic localization of *gcl* led to extra and ectopic pole bud formation, respectively (Jongens *et al.,* 1994). Taken together these results indicate that *gcl* is both necessary and sufficient to induce pole bud formation although, since some level of pole bud formation is observed in the *gcl*^Δ embryos, there is clearly another activity in the germ plasm capable of initiating this process. The failure to form proper pole buds is probably the major cause of sterility in the *gcl*^Δ progeny, as pole cell formation is presumably dependent on proper pole bud formation. It is clear, however that this is not the only reason that a germline fails to form in the *gcl*^Δ progeny.

Attempts to rescue the *germ cell-less* null phenotype with mislocalized forms of Gcl protein revealed that *gcl* is required in two distinct ways for efficient pole cell forma-

FIG. 5. Identification of mutant forms of Gcl protein which fail to localize properly within the cell. (A) Mutations introduced into the *gcl* coding region to create cytoplasmic and nucleoplasmic forms of Gcl protein. (B–J) Wild-type and mutant forms of Gcl protein were ectopically expressed in *Drosophila* S2 tissue culture cells. The distribution of the Gcl protein was determined by fixing the cells and staining them with α-Gcl antibody followed with a rhodamine-coupled secondary antibody, as well as staining with Høechst dye to label the DNA. (B, E, and H) DIC images of a field of transfected S2 cells. (D, G, and J) Epifluorescence images showing the position of the nuclei labeled with the Høechst dye. (C, F, and I) Epifluorescent images showing the distribution of the ectopically expressed Gcl protein. (B, C, and D) S2 cells transfected with wild-type Gcl protein, which localizes to the nuclear envelope. Amino acids 19–24 of Gcl encode a nuclear localization sequence (NLS) (Dockendorff *et al.,* submitted). (E, F, and G) S2 cells transfected with a form of Gcl (Gcl-NLS) which localizes to the cytoplasmic compartment of the cell due to mutation of the NLS; see (A). Amino acids 1–7 of Gcl encode a putative myristoylation consensus. (H, I, and J) S2 cells transfected with a form of Gcl (Gcl(-myr)) which localizes to the nucleoplasm due to mutation of the putative site of myristoylation; see (A).
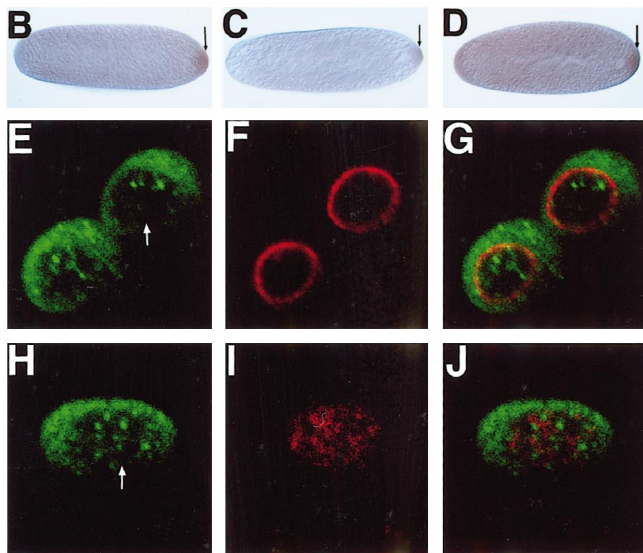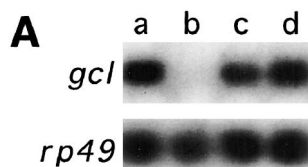
FIG. 6. Expression of wild-type and mislocalized forms of Gcl protein in the *gcl*^Δ embryos. (A) Northern analysis of total RNA prepared from 0- to 1-h embryos from the following lines: Lane a, hg96; b, *gcl*^Δ; c, hg(-NLS)49; and d, hg(-myr)31. The Northern blot was first probed with the coding region of *gcl* (top) and then *rp49*(bottom) for a loading control. Quantitation indicates that the mutant forms of *gcl* are expressed at levels that are at least 80% of wild-type *gcl* mRNA present in hg96 embryos. (B–D) Mid-cleavage-staged embryos stained with anti-Gcl antibody. The arrow indicates Gcl protein which can be detected in the germ plasm of (B) hg96, (C) hg(-NLS)49, and (D) hg(-myr)31 embryos. (E–J) Confocal images of pole cells stained with anti-Vasa antibody (green, E and H) and anti-Gcl antibody (red, F and I). The merged images are shown in (G and J). (E–G) The distribution of Gcl protein in the pole cells of an hg96 embryo shows the normal perinuclear distribution. (H–J) The distribution of Gcl protein in the pole cell of an hg(-myr)31 embryo shows diffuse nucleoplasmic staining. Note that the positions of the nuclei can be seen as the region of the cell with reduced Vasa protein (arrows in E and H).

TABLE 2
Mislocalized Forms of Gcl Fail to Rescue Pole Cell Formation

Line	Stage	Average number of pole cells	Percentage of embryos with the indicated number of pole cells							
			0	1–5	6–10	11–15	16–20	21–25	26–30	31–35
hg96	Blastoderm	23.1 ± 1.3	0	0	2	12	20	26	28	12
hg(-NLS)49	Blastoderm	3.3 ± 0.4	45	29	16	10	0	0	0	0
hg(-myr)31	Blastoderm	2.9 ± 0.3	40	38	18	4	0	0	0	0

Note. Mislocalized forms of Gcl fail to promote pole cell formation. Pole cell counts of gcl^{Δ} embryos with equivalent maternal contribution of wild-type (hg96), cytoplasmic (hg(-NLS)49), or nucleoplasmic (hg(-myr)31) forms of Gcl protein are shown. Average number of pole cells and the SEM, as well as the distribution of pole cell number, are given. Over 100 embryos were counted for each line. (See text and Materials and Methods for details.)

tion to occur. Although pole bud formation can occur when Gcl protein is restricted to the cytoplasm or nucleoplasm, efficient pole cell formation requires that Gcl protein localizes to the nuclear envelope. This indicates that the dramatic reduction in the number of pole cells formed in the gcl^{Δ} embryos is due to the loss of *gcl* activity at two distinct times, once for pole bud formation and a second time for efficient pole cell formation. At this point, we do not know if pole bud formation and pole cell formation require different activities encoded by *gcl* or if the two processes have unique subcellular localization requirements for the same activity.

Analysis of germ cell precursor development in the gcl^{Δ} embryos revealed that *gcl* activity may also be required after pole cell formation. During gastrulation the pole cells undergo a patterned migration to the embryonic gonad (see Williamson and Lehmann, 1996, for review). Previous studies have noted that during this migratory phase some of the pole cells die or migrate aberrantly, reporting that only 70% of the pole cells successfully reach the embryonic gonad (Technau and Campos-Ortega, 1986; Hay *et al.,* 1988). Although we found that our host line had a slightly higher success rate (91%), only 39% of the pole cells formed in the gcl^{Δ} embryos successfully reached the embryonic gonad. Although this reduction in pole cell survival may be due to pole cells being poorly formed or being poorly determined at the time of formation, it is also possible that it is due to a requirement for *gcl* activity after pole cell formation. It is interesting to note with respect to this last possibility that Gcl protein is detected in the pole cells up until stage 10 (Jongens *et al.,* 1992). The presence of Gcl protein up until this time may be important for keeping pole cells directed toward germ cell fate.

Most, if not all, of the pole cells which successfully reach the embryonic gonad in the gcl^{Δ} embryos appear to develop into functional germ cells. The percentage of embryos which have one pole cell or more at stage 14 matches the percentage of fertile gcl^{Δ} progeny. An interesting observation with respect to this point is that upon examination of the gcl^{Δ} females, the minimum number of ovarioles observed in a single ovary was 14 ($n = 200$). Since the germarium, at the tip of each ovariole, contains a minimum of two germline stem cells (Wieschaus and Szabad, 1979), our results show that a single pole cell entering the embryonic gonad can give rise to a minimum of 28 germline stem cells. Technau and Campos-Ortega (1986) had previously shown that a mechanism which limits the maximum number of pole cells that reach the embryonic gonad exists. Our result, in combination with those of Technau and Campos-Ortega (1986), indicates that mechanisms exist to regulate both the minimum and the maximum number of germ cell precursors in the gonad.

The Mechanisms by Which gcl May Initiate the Germ–Cell–Specification Pathway

The analysis of germline formation in the gcl^{Δ} embryos has revealed that *gcl* is required at the time of pole bud formation. We have found that mutant forms of Gcl that are restricted to the nucleoplasm or cytoplasm can rescue pole bud formation in gcl^{Δ} embryos. Thus, the most likely scenario is that Gcl acts in the cytoplasm to promote pole
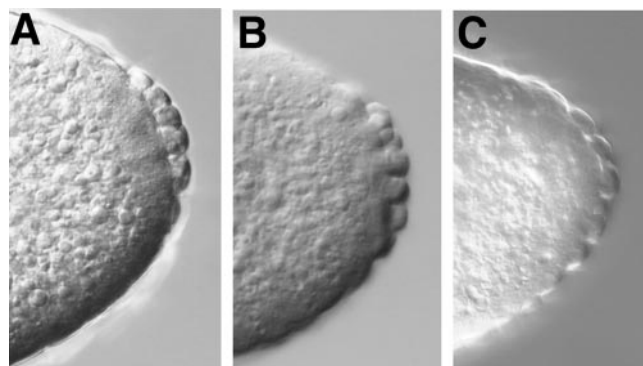

FIG. 7. Rescue of pole bud formation with the expression of mislocalized forms of Gcl protein. (A) hg96, (B) hg(-NLS)49, and (C) hg(-myr)31 embryos showing dramatically increased pole bud formation relative to that seen in the gcl^{Δ} embryos (see Fig. 3).

bud formation, prior to its entry into the pole bud nuclei. At this point we do not know how *gcl* activity affects the cytoskeletal reorganization required for this process (Warn *et al.,* 1985; Planques *et al.,* 1991).

Our results show that Gcl protein must localize to the nuclear envelope for efficient pole cell formation to occur. Previous characterization of the subcellular distribution of Gcl protein revealed that it is mostly localized to the nucleoplasmic surface of the nuclear envelope (Jongens *et al.,* 1994). This distribution precludes it from having a direct role in the cytokinesis event required to form the pole cells and indicates that *gcl* activity may act through some intracellular signaling pathway. An interesting point with respect to this possibility is the apparent myristoylation modification (see Results) required to localize Gcl protein to the nuclear envelope. This N-terminal protein modification is commonly found on components of intracellular signaling pathways which are membrane bound (Towler *et al.,* 1988; Grand, 1989).

The dependence of Gcl protein localization to the nuclear envelope on a myristoylation modification draws into question a previously proposed model whereby Gcl protein localizes to the nuclear envelope through an interaction with the nucleoplasmic surface of the nuclear pore complex (NPC) (Jongens *et al.,* 1994). Clearly, the combination of an NLS and a myristoylation site present in the *germ cell-less* sequence should be sufficient to localize Gcl protein to the nucleoplasmic surface of the nuclear envelope. Therefore the localization of Gcl protein probably occurs independent of an association with the NPC.

The germ cell-less Null Phenotype Compared to the Antisense Phenotype

Given the previous results obtained through antisense, overexpression, and ectopic-expression studies of *gcl,* we expected to find that the germ cell lineage was affected in embryos lacking maternal *gcl* activity. However, it was surprising to find that *gcl* was not absolutely required for this process. We were led to this expectation for two reasons. One is that Gcl protein is found on all of the pole cell nuclei (Jongens *et al.,* 1992). Second, using antisense methodology to reduce maternal *gcl* mRNA levels, we obtained sterility rates that were as high as 90%, even when some maternal *gcl* mRNA was still detected in the embryo (Jongens *et al.,* 1992). Thus the expectation was that if all of the maternal contribution of *gcl* mRNA was removed, all of the progeny would be sterile. This was clearly not the case as we found that roughly 30% of the *gcl*$^\Delta$ progeny could form a functional germline. Therefore, a stronger effect was observed with the antisense approach compared to the null mutant. One possibility for this difference is the existence of another gene with similar activity and a high degree of sequence similarity to *gcl* that is also affected by antisense *gcl* RNA expression. To investigate this possibility we performed low-stringency Southern analysis on *Drosophila* genomic DNA, but failed to identify a *germ cell-less* homo-

logue (not shown). We also introduced a transgene which provided high levels of antisense *gcl* RNA expression into the *gcl* null background, but failed to detect an enhancement of the phenotype. Thus at this time we cannot explain the higher sterility rate obtained in the antisense experiments. Nonetheless a fairly accurate requirement of *gcl* activity was uncovered by the antisense approach.

The failure to identify a *gcl* homologue leaves unanswered the reason for the incomplete penetrance of the *gcl* null mutant. It is conceivable that some *gcl*-like activity is provided by a homologue whose sequence divergence prevents detection with low-stringency hybridization approaches or by a gene with no similarity to *germ cell-less.* This redundant *germ cell-less*-like activity observed during pole cell formation could also be present later in development and mask the requirement for *gcl* zygotic activity.

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