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Soma-germline asymmetry in the distributions of embryonic RNAs in *Caenorhabditis elegans*

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SUMMARY

Early embryogenesis in *Caenorhabditis elegans* is characterized by a series of unequal cleavages that mark the stepwise separation of somatic and germ lineages. We have developed an in situ hybridization protocol to examine the localization of specific maternal and embryonically transcribed messenger RNAs during these early cleavages. We detected three classes of maternal RNAs: RNAs that are maintained in all cells, RNAs that are maintained in germline cells but are lost from somatic cells, and a population of RNAs that are associated with the germline-specific P granules. We observed embryonically transcribed RNAs in somatic cells as early as the 4-cell stage. These transcripts were not detected in germline cells. These observations suggest that mechanisms which distinguish between soma and germline cause asymmetries in mRNA stability and transcription within the first few cleavages of *C. elegans* embryogenesis.

Key words: maternal RNAs, embryonically transcribed RNAs, P granules, embryogenesis, in situ hybridization, *Caenorhabditis elegans*

INTRODUCTION

The distinction between soma and germline is essential to the development of multicellular organisms (e.g. Denis and Lacroix, 1993). In the nematode *Caenorhabditis elegans*, the separation of somatic and germ lineages occurs during the first four cleavages (Fig. 1; Sulston et al., 1983). The zygote, P_0, can be considered the first germline cell (e.g. Schierenberg and Strome, 1992). The first cleavage divides P_0 into a larger somatic blastomere AB and a smaller germline cell P_1. The AB blastomere divides equally to generate somatic descendants that divide in synchrony (AB lineage). In contrast, the P_1 germline cell divides unequally to give rise to the somatic blastomere EMS and the germline cell P_2. Unequal germline cell divisions continue until the five somatic founder lineages (AB, MS, E, C and D) and the primordial germ cell P_4 are formed (24-cell stage, Fig. 1).

The fates of somatic blastomeres are established by mechanisms involving both cell-cell interactions and the asymmetric activity of maternally encoded components (reviewed in Wood and Edgar, 1994). The mechanisms that distinguish somatic cells from germline cells are less understood. Cytoplasm centrifugation and extrusion experiments have suggested that a posteriorly located 'germline factor' is sufficient for certain aspects of germline identity in nematodes (Boveri, 1910; Schierenberg, 1988). Direct evidence for asymmetrically localized factors first came from the analysis of the germline-specific P granules. P granules are cytoplasmic particles of unknown composition which can be visualized by immunofluorescence (Strome and Wood, 1982) or by electron microscopy (Wolf et al., 1983). P granules are uniformly distributed at the time of fertilization, but become localized to the posterior region of the 1-cell embryo prior to the first cleavage. During this and subsequent divisions, the P granules remain associated with germline cells (Strome and Wood, 1982; Fig. 1). Although this behavior suggests that P granules may function in some aspect of germline development, their function is not yet known. Nevertheless, the existence of such asymmetrically localized particles suggests that the distinction between soma and germline may depend on asymmetric partitioning of germline and/or soma-specifying components.

In this study, we have investigated the consequences of early asymmetry on the distribution of maternal and embryonically transcribed mRNAs. In particular, we have asked two questions. (1) Do specific maternal mRNAs exhibit asymmetric distributions in the early embryo? (2) When do asymmetries in embryonic transcription first appear? To address these questions, we have developed a high resolution in situ hybridization protocol to visualize mRNAs in whole-mount embryos. We have used this protocol to determine the expression patterns of 21 genes and to examine the general distribution of poly(A)$^{+}$ RNAs during embryogenesis. We find that several maternal and embryonically transcribed mRNAs exhibit asymmetric distributions during early embryonic cleavages.

MATERIALS AND METHODS

Strains

We used wild-type *C. elegans* (N2) and the following mutant strains.

SP 756: *mnDf90 / mnC1 [dpy-10(e128) unc-52(e444)]* II. *mnDf90* deletes the gene *cey-1* (Sigurdson et al, 1984; Jantsch-Plunger, 1993);

mnC1 [dpy-10(e128) unc-52(e444)] is a balancer chromosome for LGII (Herman, 1978).

PD 9593: *mDf4 / DnT1 [unc-?(n754) let-?]* (IV; V). *mDf4* deletes *ama-1* (*ama-1* encodes the large subunit of RNA polymerase II; Bird and Riddle, 1989). *DnT1 [unc-?(n754) let-?]* is a reciprocal translocation (LG IV-V) which carries a dominant Unc mutation and a recessive lethal mutation (Ferguson and Horvitz, 1985).

BW 163: *ctDf1 / DnT1 [unc-?(n754) let-?]* (IV; V). *ctDf1* deletes *dpy-30* (Hsu and Meyer, 1994; Manser and Wood, 1990).

MT 5734: *nDf41 / DnT1[unc-?(n754) let-?]* (IV; V): *nDf41* deletes *skn-1* (Bowerman et al., 1992, 1993).

KK 300: *par-4 (it57ts). par-4 (it57ts)* is a temperature-sensitive maternal effect mutation which causes embryos to divide abnormally at 25°C (Morton et al, 1992).

PD 182: *glp-1(e2141ts); ccIs182. ccIs182* is a chromosomally integrated transgenic array containing the *glp-1* gene (Yochem and Greenwald, 1989), and a *glp-1::lacZ* translational fusion (pPD26.99, A. F. unpublished results).

PD 9556: *ccIs9556. ccIs9556* is a chromosomally integrated transgenic array containing *rol-6(su1006dom)* (Mello et al., 1991) and a *pes-10::lacZ* translational fusion (pGS15.24; G. S. unpublished results).

Clones

The following clones were used to generate gene-specific probes.

cm12b2 (*act-1*), cm04g2 (*ncc-1*; cdc2 related), cm04h4 (tubulin α-related), cm01d4 (tubulin β-related), cm12d4 (EF1α-related), cm01h11 (HSP70A-related), cm04g7 (HSP90-related), cm14g5 (histone H1-related) (Waterston et al., 1992);

pBSZ (*lacZ;* Nipam Patel, personal communication);

pPCA (*crf-2;* Ann Sluder, personal communication);

pUZ1200 (*hlh-1*; Krause et al., 1990);

SK-/b7 (*lin-19;* Edward Kipreos and Edward Hedgecock, personal communication);

JP600 and JP601 (*skn-1*; Bowerman et al., 1992);

MC334 (*cey-1*) and MC422 (*cey-2*) (cey: C. elegans Y box homolog; Jantsch-Plunger, 1993);

pDH38 (*dpy-30*; Dave Hsu and Barbara Meyer, personal communication; Hsu, 1992);

pDB33 (*ama-1*; Bird and Riddle, 1989);

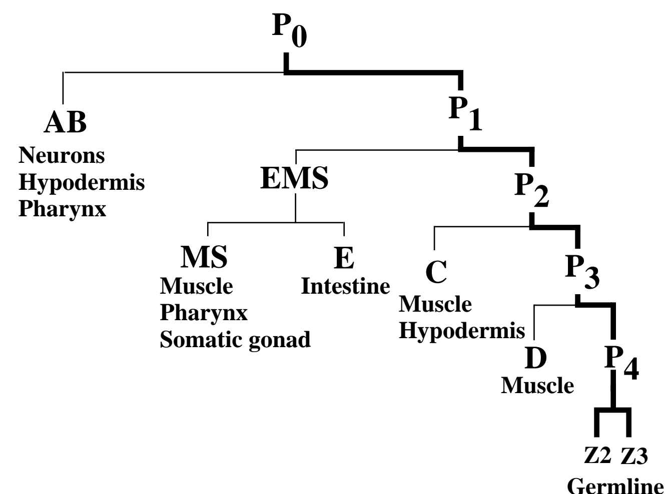

Fig. 1. Early cell lineage. P_0 is the zygote and can be thought of as the first germ cell. Unequal cleavages of the germline cells (P_0-P_3) lead to five somatic founder cells (AB, MS, E, C, D) and the germline progenitor cell P_4. Thickened lines indicate the segregation of P granules.

pCeIF-1800 (*CeIF*, eIF4-A-related; Roussell and Bennett, 1992);

pPD19.60 (*unc-54*), pPD53.02 (*glp-1*), pPD33.24 (RP21C-related) and pGS17.09 (*pes-10;* Seydoux and Fire, unpublished data; pes: pattern expression site).

Probe synthesis

cDNA-derived probes

Digoxigenin (DIG)-labeled single-stranded DNA probes were synthesized by multiple cycles of primer extension in the presence of DIG-dUTP, using genomic or cDNA clones as templates (Patel and Goodman, 1992). Probes were used at a concentration of 0.5-2.5 μg/ml in hybridization buffer. Sense orientation control probes were tested only for the following genes: *pes-10*, *cey-1*, *cey-2*, *skn-1* and *ama-1*. No signal was detected with any of these control probes (data not shown).

Oligonucleotide probes

We used the following single stranded oligonucleotide probes: oligo(dT) (30mer), oligo(dA) (30 mer), anti-SL1 (CTCAAACTTGGGTAATTAAACC), anti-SL1 precursor (GCTAACGCCAAATTTCTTTGGG). These probes were end-labeled with terminal transferase and digoxigenin-ddUTP (Boehringer Mannheim) and used at a concentration of 0.5 μg/ml in hybridization buffer.

Permeabilization and fixation

Gravid hermaphrodites and their laid eggs were harvested in water, digested twice for three minutes in hypochlorite solution (1 N NaOH, 10% commercial bleach), and washed in PBS (140 mM NaCl, 3 mM KCl, 4 mM Na_2HPO_4, 1.5 mM KH_2PO_4). Embryos are protected by their egg shells from hypochlorite digestion, while larvae and adults are dissolved by this treatment. The earliest embryos isolated by this method were in the 1-cell stage at the time of pronuclear meeting, approximately 30 minutes after fertilization.

Embryos (approx. 1000) in PBS (10-20 μl) were pipetted onto a 14×14 mm polylysine-coated well (Cel-Line Associates Inc., Cat# 10-2066), overlayed with a glass coverslip and frozen on an aluminum block on dry ice. After cracking off coverslips, the slides were immersed in 100% methanol at −20°C for 5 minutes, placed in 100% methanol at room temperature for 5 minutes and rehydrated at room temperature using the following methanol series: 90% MeOH in H_2O, 70% MeOH in PBS; 50% MeOH in PBS (1 minute washes), followed by two 5 minute washes in PTw (1× PBS, 0.1% Tween 20).

At this point, in some experiments, a Proteinase K digestion step was included: embryos were incubated for 15 minutes in 1 μg/ml or 10 μg/ml of proteinase K in PTw at room temperature. Proteinase K digestion was stopped by incubating for 2 minutes in 2 mg/ml glycine in PTw.

After two 5 minute washes in PTw, the embryos were fixed for 20 minutes in fixative (1× PBS, 0.08 M Hepes (pH 6.9), 1.6 mM $MgSO_4$, 0.8 mM EGTA, 3.7% formaldehyde), and washed twice for 5 minutes in PTw, once for 5 minutes in 2 mg/ml glycine in PTw, and three times for 5 minutes in PTw.

Hybridization using cDNA-derived probes

Hybridization buffer consisted of 50% formamide, 5× SSC, 100 μg/ml autoclaved salmon sperm DNA, 50 μg/ml heparin, 0.1% Tween 20. Fixed embryos on slides were prehybridized in three steps: (1) 10 minutes at room temperature in a 1:1 mix of PTw and hybridization buffer; (2) 10 minutes at room temperature in undiluted hybridization buffer; (3) 1-4 hours at 48°C in previously boiled hybridization buffer.

30 μl of probe was applied and embryos overlayed with a parafilm coverslip, sealed with rubber cement and incubated overnight at 48°C. After hybridization, embryos were washed at 48°C twice with hybridization buffer, twice with 3:2 [hybridization buffer:PTw], twice with 1:4 [hybridization buffer:PTw], and twice in PTw (15 minute washes). Embryos were then washed twice (20 minutes) in PBT (1× PBS, 0.1% BSA, 0.1% Triton X-100) at room temperature.

Hybridization using oligonucleotide probes

Prehybridization and hybridization were performed at 37°C as above with adjusted concentrations of SSC and formamide as follows: oligo(dT) and oligo(dA) probes: 3× SSC; 3% formamide; anti-SL1: 2× SSC, 7.7% formamide; anti-SL1 precursor: 2× SSC, 14.3% formamide. These conditions are predicted to give optimal hybridization for each oligonucleotide (Davis et al., 1986). After hybridization, embryos were washed for 10 minutes in hybridization solution at 37°C, and twice for 10 minutes in TTBS (150 mM NaCl, 50 mM Tris-HCl pH 7.8, 0.1% BSA, 0.1% Tween-20) at room temperature.

Detection

Alkaline phosphatase (AP)-mediated detection was carried out using an AP-anti-DIG antibody conjugate (Boehringer Mannheim). The AP color reaction was performed in staining solution (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5; 0.1% Tween 20, 1 mM Levamisole) containing 4.5 µl NBT/ml, 3.5 µl X-phosphate/ml and 1 µg DAPI/ml, and stopped by washing twice in PBS. The embryos were mounted in 5-10 µl of a 70% glycerol solution under a glass coverslip.

Fluorescent detection was carried out using rhodamine-labeled or unlabeled anti-DIG antibodies (mouse monoclonal or sheep polyclonal, Boehringer Mannheim) and appropriate fluorescent secondary antibodies, as indicated. Embryos were mounted in 5-10 µl of mounting media (70% glycerol, 1 mg/ml p-phenylenediamine pH 9, 0.02% NaN_3) under a glass coverslip.

Double-labeling using anti-P granule antibody

After the detection step, embryos were incubated for 2 hours at room temperature in a 1:400 dilution of mouse monoclonal OIC1D4 anti-P granule antibody (Strome, 1986) conjugated to 5(and 6)-carboxyfluorescein, succinimidyl ester (FSE; the conjugated antibody was a gift of J. Paulsen and S. Strome). Embryos were washed twice for 5 minutes with TTBS before mounting.

Staging of embryos and blastomere identification

The precise developmental stage of individual embryos was determined by counting DAPI-stained nuclei. Blastomeres ABa, ABp, EMS, C, P_1, P_2 and P_3 were identified by their characteristic positions. P_4, Z2 and Z3 were identified using anti-P granule antibodies. In 26-cell embryos, Ca and Cp were identified based on their position relative to P_4 and on their characteristic mitotic nuclei (they are the only dividing cells at that stage). D, Da and Dp were identified based on position relative to P_4.

RESULTS

Analysis of the embryonic expression pattern of 21 genes

We have developed an in situ hybridization protocol for the detection of RNA in whole-mount *C. elegans* embryos (for a detailed manual see Seydoux and Fire, 1994). This protocol is based on *C. elegans* protocols for the permeabilization and fixation of populations of whole-mount embryos (Albertson, 1984; Krause et al., 1990; Ahnn and Fire, 1994) and on *Drosophila* protocols for in situ hybridization (Tautz and Pfeifle, 1989; Patel and Goodman, 1992 and personal communication). Briefly, mixed-stage embryos are permeabilized by freeze-cracking on slides, fixed with methanol and formaldehyde, and hybridized with a single-stranded DNA probe labeled with digoxigenin-dUTP. Hybridized probe is then visualized using an anti-digoxigenin antibody coupled to either an enzymatic or a fluorescent marker.

We have used this protocol to analyze the embryonic expression of 21 genes. Some of these genes encode genetically characterized regulators of embryonic development (e.g. *glp-1* and *skn-1*; Priess et al., 1987; Bowerman et al., 1992), while others encode ubiquitous proteins (e.g. *ama-1*, encoding the large subunit of RNA polymerase II; Bird and Riddle, 1989). The observed distributions of transcripts derived from these genes are presented in Table 1. We found that the in situ hybridization protocol allows visualization of RNA from the 1-cell stage to the end of embryogenesis (pretzel stage; 554 cells). The specificity of the hybridization was tested by comparing staining obtained from sense and antisense probes derived from the same gene, and from antisense probes derived from different genes: no staining was detected with sense probes (data not shown); while antisense probes derived from different genes gave distinct patterns (Table 1).

Distinguishing between maternal and embryonically transcribed RNAs

In our survey of RNA distribution patterns, we found some RNAs that were present from the 1-cell stage, and others that appeared de novo at later stages of development (Table 1). The earliest RNAs are likely to be maternal (transcribed by the maternal genome and incorporated in the egg during oogenesis), while RNAs appearing de novo are likely to be embryonically transcribed. To distinguish between the contributions of maternal and embryonic transcription for specific genes, we made use of strains heterozygous for chromosomal deletions that remove relevant loci (Table 2). The *cey-1* gene provides a particularly good example of both maternal and embryonically transcribed RNAs. Deficiency *mnDf90* has been shown to remove the *cey-1* gene and flanking markers (Sigurdson et al., 1984; Jantsch-Plunger, 1993). Deficiency heterozygotes (*mnDf90/+*) were allowed to self-fertilize to generate a population consisting of 75% of embryos that have one or two copies of the *cey-1* gene (*mnDf90/+* or *+/+*), and 25% of embryos that have no copy of *cey-1* (*mDf90/mDf90*). Examining this population for the presence of the *cey-1* RNA, we found that 99% of 1- to 4-cell-stage embryos were positive for *cey-1* RNA, indicating that this early RNA is independent of embryonic transcription (Table 2). In contrast, only 75% of 200- to 550-cell-stage embryos derived from *mnDf90/+* hermaphrodites were positive for *cey-1* RNA, indicating that *cey-1* RNA present at this stage depends on embryonic transcription of the *cey-1* gene. (Wild-type populations of embryos are 100% positive for *cey-1* staining, Table 2). These data are consistent with the hypothesis that RNAs present at the 1-cell stage are maternal, while RNAs arising later in development are embryonically transcribed.

Maternal RNAs

All of the maternal RNAs detected in our survey (14/14) were evenly distributed in the 1-cell embryo. After the onset of cell division, however, we observed two distinct distribution patterns. A subset of maternal RNAs (8/14) continued to show a uniform distribution in all cells. We refer to these RNAs as class I maternal RNAs.

A second class of maternal RNAs in our survey (6/14 ; designated class II maternal RNAs) showed distinctive asymmetry between somatic and germline blastomeres following the onset of cell division: at each P cell division, these RNAs were segregated to both daughters, but appeared to be subsequently lost from the somatic daughter and/or its progeny, while being

maintained in the P daughter. The most rapid rate of loss was observed for the *cey-2* RNA, which by the late 2-cell stage was detected at a lower level in the somatic AB blastomere compared to the germline P_1 blastomere (Fig. 2). Slower rates of somatic loss were observed for the other RNAs (Fig. 2). In all cases, these maternal RNAs appeared absent after the 24-cell stage from all somatic lineages, but could still be detected in P_4 (Fig. 2).

We used appropriate deficiency chromosomes to confirm that the observed distributions of class I and class II RNAs

Table 1. Embryonic expression pattern of 21 genes

Gene*		Stage	Pattern of RNA distribution†
(A)	Genes producing class I maternal RNAs		
	ncc-1	1- to 550-cell	ubiquitous (decreasing intensity with age)
	Tubulin α	1- to 550-cell	ubiquitous (decreasing intensity with age)
	(cm04h4)		
	dpy-30	1- to 550-cell	ubiquitous
		bean to pretzel	faint (ubiquitous?) staining
	Tubulin β	1- to 550-cell	ubiquitous (decreasing intensity with age)
	(cm01d4)	bean to pretzel	many unidentified cells
	ama-1	1- to 550-cell	ubiquitous
		bean to pretzel	occasional faint staining in Z2, Z3 and gut
	EF1a	1- to 550-cell	ubiquitous
	(cm12d4)	bean to pretzel	stronger staining in gut
	HSP90	1- to 8-cell	ubiquitous with occasional stronger staining in P_2 or P_3
	(cm04g7)	10-cell to bean	ubiquitous
		comma to pretzel	gut, Z2 and Z3, and unidentified anterior cells
	act-1	1- to 26-cell	ubiquitous
		28- to 100-cell	decreasing levels in somatic cells, constant level in P4
		100-cell to pretzel	Z2 and Z3
		$1\frac{1}{2}$ fold to pretzel	Z2 and Z3, and de novo expression in body muscle
(B)	Genes producing class II maternal RNAs‡		
	CeIF	1- to 28-cell	maintained in germ lineage, progressively lost in soma
	(pCeIF-1800)	28- to 550-cell	ubiquitous
		bean to comma	stronger staining in gut
	cey-1	1- to 30-cell	maintained in germ lineage, progressively lost in soma
		30-cell	2 then 4 posterior cells
		100- to 550-cell	ubiquitous
		bean to pretzel	muscle and other
	skn-1	1- to 28-cell	maintained in germ lineage, progressively lost in soma
		comma to pretzel	gut
	glp-1	1- to 40-cell	maintained in germ lineage, progressively lost in soma
		40- to 60-cell	2 then 4 anterior cells
		bean to pretzel	Z2 and Z3§
	HSP70A	1- to 15-cell	maintained in germ lineage, progressively lost in soma
	(cm01h11)	bean	ubiquitous, with stronger staining in two dorsal stripes
		comma to pretzel	gut
	cey-2	1- to 32-cell	maintained in germ lineage, progressively lost in soma
(C)	Genes producing no maternal RNAs		
	pes-10	4- to 60-cell	newly arisen somatic blastomeres
	crf-2	12- to 200-cell	all cells, except P4 and unidentified posterior/middle cells
	lin-19	15- to 60-cell	somatic cells
	Histone H1	22- to 500-cell	all cells, except P4 and unidentified posterior/middle cells
	(cm14g5)	bean to comma	several groups of unidentified cells
		$1\frac{1}{2}$ fold	unidentified cells in tail
	hlh-1	60-cell to bean	body muscle precursors¶
	RP21C	550-cell	unidentified posterior cells
	(pPD33.24)	bean to pretzel	gut
	unc-54	bean to pretzel	body muscle cells

*When available, gene names are indicated in italics. Otherwise clone names are indicated in parentheses below the observed protein similarity.

†Ubiquitous distribution refers to staining which appeared to be present in all cells. In 100-cell and older embryos, however, we have not excluded the possibility that a minority of cells were actually negative for staining.

‡These maternal RNAs were absent from somatic cells at the 28-cell stage. Subsequent somatic accumulation is presumably due to embryonic transcription.

§*glp-1* RNA accumulation in Z2 and Z3 in bean and older embryos was detected only in a transgenic line carrying multiple copies of the *glp-1* gene and of a *glp-1::lacZ* transgene. In this line, we also detected RNA in cells outside of the pharynx, between the two bulbs, in the pretzel stage.

¶In one experiment, weak *hlh-1* RNA accumulation was detected in EMS in the 6-cell stage, and in 4 unidentified cells in the 24-cell stage.

Table 2. Distinguishing maternal from embryonically transcribed RNAs

Probe	Stage	Maternal genotype	
		+/+	+/*Df**
cey-1	1- to 4-cell	100% (50)	99% (73)
	200- to 400-cell	100% (59)	75% (81)
skn-1	1- to 4-cell	97% (40)	100% (44)
	6- to 28-cell†	95% (59)	100% (61)
dpy-30	1- to 4-cell	97% (35)	95% (167)
	6- to 28-cell	96% (52)	93% (206)
ama-1	1- to 4-cell	94% (70)	96% (85)
	6- to 28-cell	93% (82)	97% (109)

The percentage of embryos hybridizing with a particular probe is indicated for the progeny of wild-type mothers or mothers heterozygous for a chromosomal deficiency deleting the probe sequence used. Numbers of embryos scored are indicated in parentheses.

*Deficiencies used were *mnDf90* (*cey-1*), *nDf41* (*skn-1*), *ctDf1* (*dpy-30*) and *mDf4* (*ama-1*). Complete genotypes of parental strains are given in Materials and Methods.

†These embryos stained preferentially in the P lineage. All other embryos showed uniform staining. *skn-1*, *dpy-30* and *ama-1* RNAs are not detected reliably in embryos older than 28-cell; for this reason these probes were not scored in later stages.

reflected the distributions of maternal transcripts. This analysis was carried out for two class I genes (*dpy-30* and *ama-1*) and one class II gene (*skn-1)*. In each case, we found RNA distributions in early (1- to 28-cell) stages to be identical between wild-type embryos and deficiency homozygoytes lacking both genomic copies of the probed gene (Table 2).

Because our method of detection relies on the ability of an exogenous probe to hybridize to RNA in situ, it was important to verify that the apparent disappearance of class II RNAs from somatic cells was due to actual loss of RNA by degradation, rather than to the formation of double-stranded RNA or ribonucleoprotein complexes that were inaccessible to the probe. The quantitative masking of maternal mRNA by antisense RNA seems unlikely, given that we detected no antisense transcripts (nuclear or cytoplasmic) using appropriate sense probes for *cey-1*, *cey-2* and *skn-1*.

To address the possibility of masking by ribonucleoprotein complexes, we performed in situ hybridization experiments to *cey-2* and *CeIF* RNAs following digestion of embryos with proteinase K. Proteinase K treatment at a low concentration (1 μg/ml) did not change the hybridization patterns of the *cey-2* or *CeIF* probes at any stage (data not shown). Proteinase K treatment at a higher concentration (10 μg/ml) was sufficient to cause significant damage to the morphology of embryos, but still did not affect the distribution of *cey-2* and *CeIF* RNAs in 12-cell and older embryos (cell integrity in earlier embryos was too damaged for cell boundary identification; data not shown). Although we cannot rule out the possibility that some ribonucleoprotein complexes are still present under these conditions, these results are consistent with loss of RNA by degradation. This interpretation is further supported by experiments reported by Ahringer et al. (1992). Their northern analyses of RNA derived from populations of staged embryos indicate that the level of *glp-1* RNA, another class II maternal RNA, decreases dramatically during the first two hours of embryogenesis.

Embryonically transcribed RNAs

For 12 of the 21 genes in our survey, embryonically transcribed RNAs were easily recognized as new signals arising after a period when no RNA was detected. The timing of the onset of embryonic transcription varied greatly for different genes, from before gastrulation (which begins at the 26-cell stage) to after the onset of morphogenesis (550-cell stage), with no apparent preference for any particular developmental stage (Table 1). For a subset of genes in our survey (8/21), the onset of embryonic transcription could not be determined because newly transcribed RNAs could not be distinguished from perduring maternal RNAs.

The earliest embryonically transcribed RNA detected in our survey was derived from *pes-10*. *pes-10* is a gene of unknown function that we identified in a promoter-trap screen (carried out as in Hope, 1991) designed to identify transcripts expressed in early embryos (details of the screen will be published elsewhere). We found that *pes-10* RNA is present transiently in each somatic lineage starting in the 4-cell stage (Fig. 3). Because no *pes-10* RNA was detected at the 1-cell stage, and because the earliest *pes-10* RNA was detected in nuclei rather than cytoplasm, we believe this RNA to be dependent on embryonic transcription. To analyze the onset of *pes-10* transcription with a greater degree of sensitivity, we have taken advantage of the amplified signal derived from a chromosomally integrated, multicopy *pes10::lacZ* transgene. The *pes-10::lacZ* RNA is detected sequentially in each somatic lineage shortly after each lineage diverges from the germ lineage (Fig. 3). In each somatic blastomere, the *pes-10:: lacZ* RNA initially accumulates in two nuclear foci, which may correspond to the site of transcription on the two homologous chromosomes that carry the multi-copy transgene. These ‘double dots’ are absent from cells undergoing mitosis, suggesting that transcription may not be occurring during that time. In each somatic lineage, *pes-10* and *pes-10::lacZ* RNAs were detected for one to four cell cycles, before being rapidly degraded.

Transcription of *pes-10* and of the *pes-10::lacZ* transgene was never detected in the germ lineage (P_0-P_4) (Figs 3 and 4). Interestingly, embryonically transcribed RNAs from the other genes in our survey also appeared to be absent from the germ lineage in the early embryo. A particularly striking example comes from the *lin-19* RNA which is expressed in all somatic blastomeres from the 15-cell stage to the 60-cell stage, but was never detected in the germ lineage (Fig. 4).

When do embryonically transcribed RNAs first appear in the germ lineage? As discussed above, the presence of perduring maternal RNAs, particularly in the germ lineage, makes it difficult to determine whether embryonically transcribed RNAs are also present. For example, the *act-1* RNA is present in the germ lineage continuously from the 1-cell stage to the end of embryogenesis (Table 1). Because there are no available deficiencies that remove the *act-1* gene, we were not able to determine when this RNA becomes dependent on embryonic transcription. In one case, however, we detected a newly arisen, and therefore presumably embryonically transcribed, RNA in the germ lineage: this transcript was derived from an array containing multiple copies of the *glp-1* gene and of a *glp-1::lacZ* fusion, and was first detected in Z2 and Z3 in the bean stage, after the onset of morphogenesis (Table 1). This result

suggests that, at least by that stage, the germ cells Z2 and Z3 have become competent for transcription.

The *par-4* gene is required for the asymmetric distributions of maternal and embryonically transcribed RNAs

The generation of morphological asymmetry in early embryos has been shown to involve the products of the *par-1*, *par-2*, *par-3* and *par-4* genes (Kemphues et al., 1988). These genes were identified by maternal-effect mutations which lead to defects in early cleavage patterns and mislocalization of the germline-specific P granules. We used a mutation in the *par-4* gene to address the possibility that *par*-dependent mechanisms also function in the generation of asymmetry in maternal and embryonically transcribed RNA distributions. In *par-4* embryos, after a normal asymmetric first cleavage, both the AB and P_1 blastomeres divide symmetrically and synchronously (Kemphues et al., 1988; Morton et al., 1992). This and other characteristics led to the proposal that the P (germ) lineage is transformed to an AB-like lineage in *par-4* embryos (Morton et al., 1992). We found that the distributions of both the *cey-2* and *pes-10* RNAs in *par-4* embryos reflect this proposed P_1 to AB transformation. The *cey-2* RNA, a maternal transcript which in wild-type embryos is lost from the AB lineage but is maintained in the P lineage, was found to be lost rapidly from all blastomeres in *par-4* embryos (Fig. 5). Conversely, the *pes-10* RNA, a embryonically transcribed RNA which in wild-type is detected in the AB lineage but not in the P lineage, was detected in all blastomeres in *par-4* embryos (Fig. 5). These results indicate that the mechanism disrupted in *par-4* embryos is required for the asymmetric distributions of both maternal and embryonically transcribed RNAs.

Clusters of poly(A)$^+$ RNAs co-localize with P granules

So far, our analysis of maternal and embryonically transcribed RNAs has been limited to the 21 genes included in our survey. To complement this analysis, we have examined the overall distribution of poly(A)$^+$ RNAs using a digoxigenin-labeled oligo(dT) probe. We found that this probe hybridized uniformly to the cytoplasm of all somatic cells. In the P blastomeres, we detected additional clusters of strong staining above a uniform background (Fig. 6). In P_0, P_1, and P_2, these clusters were preferentially associated with the posterior half of the cell cortex prior to each division. In P_3 and P_4, the clusters coalesced and

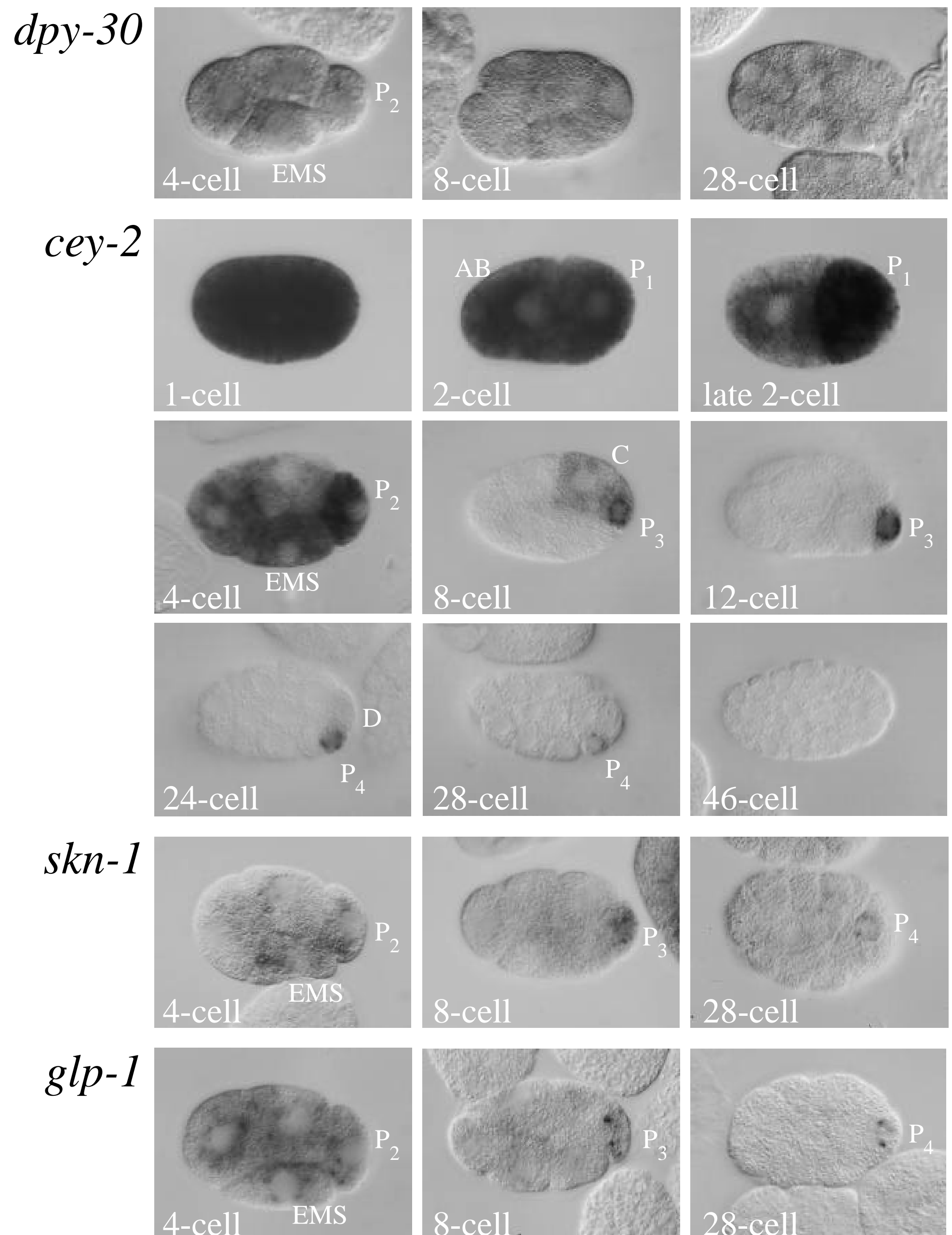

Fig. 2. Localization of maternal RNAs in early embryos. Embryos were hybridized to *dpy-30, cey-2*, *skn-1* or *glp-1* digoxigenin-labeled antisense probes. Probes were visualized using an anti-digoxigenin antibody coupled to alkaline phosphatase, which was reacted with chromogenic substrates to give a dark color. These maternal RNAs appear predominantly cytoplasmic, although we have not excluded the possibility that they are also present in nuclei. In this and all other figures, anterior is to the left and dorsal is up. Embryos measure approximately 50 μm along their anterior-posterior axis.

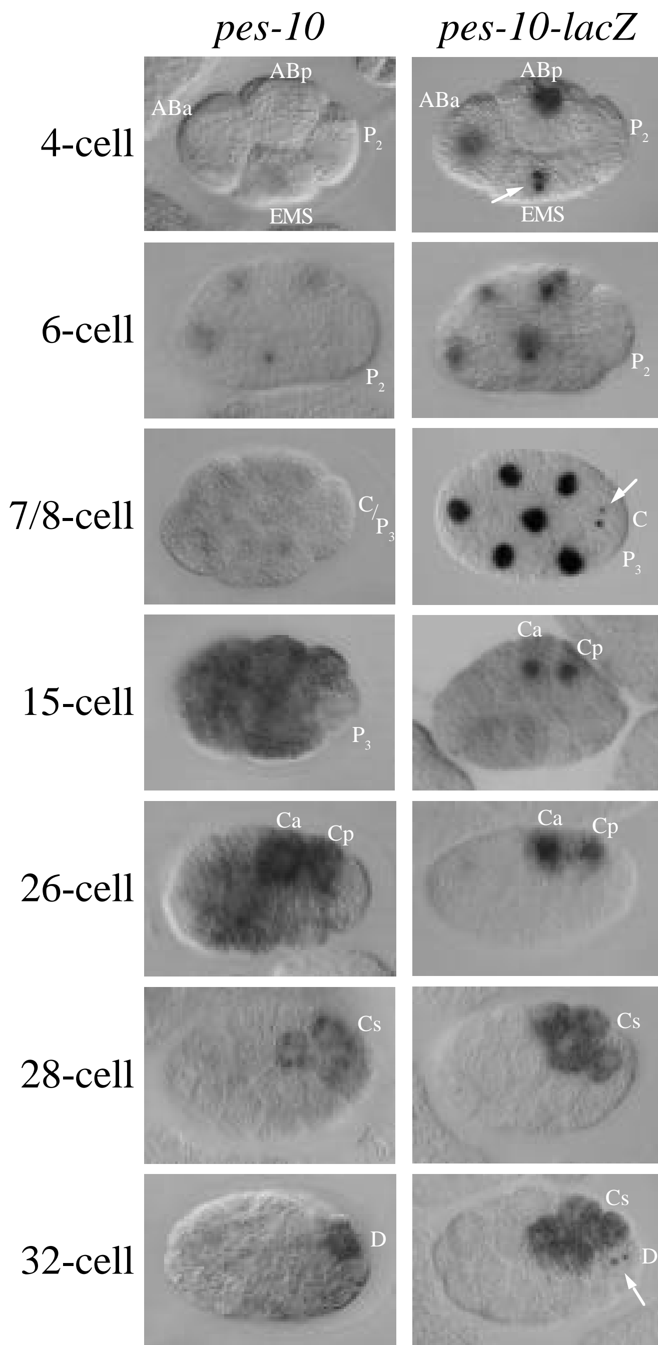

Fig. 3. Localization of *pes-10* and *pes-10::lacZ* embryonically transcribed RNAs in early embryos. Wild-type embryos (first column) or embryos transformed with a *pes-10::lacZ* transgene (second column) were hybridized, respectively, to *pes-10* or *lacZ* anti-sense probes. Probes were visualized as described in the legend of Fig. 2. Arrows point to nuclei of recently arisen somatic founder blastomeres where the *pes-10::lacZ* message has just started to accumulate in two foci. In contrast to the endogenous *pes-10* message, which quickly becomes cytoplasmic (see 7/8-cell stage), the *pes-10::lacZ* RNAs remains predominantly nuclear until the 26-cell stage. This apparent block in nuclear export (or cytoplasmic instability) has also been observed for other *lacZ* fusion RNAs (data not shown), and could be due to the long intron-less coding region of *lacZ*. Cs refer to the 4 C-derived blastomeres: Caa, Cap, Cpa, Cpp.

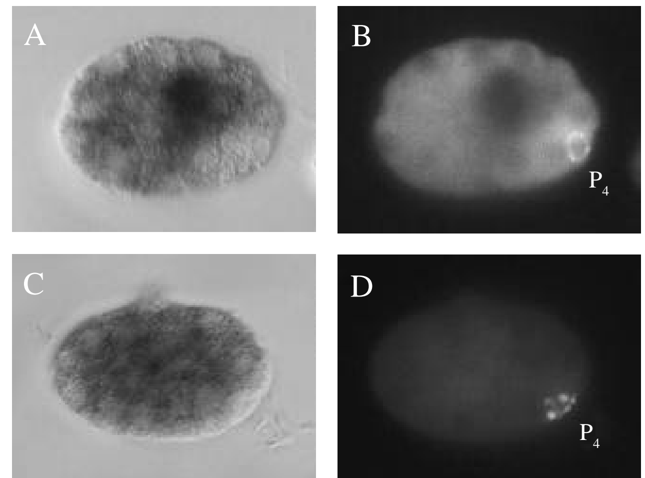

Fig. 4. *pes-10* and *lin-19* embryonically transcribed RNAs are absent from the germ lineage. Wild-type embryos were hybridized to *pes-10* or *lin-19* antisense probes and stained with anti-P granule antibody, which identifies blastomeres from the germ lineage. (A) 24-cell embryo hybridized to *pes-10* antisense probe. (C) 28-cell embryo hybridized to *lin-19* antisense probe. (B,D) Same embryos as in A or C, respectively, showing anti-P granule staining in P_4.

became peri-nuclear. This dynamic pattern of localization is identical to the distribution of P granules (Strome and Wood, 1982). To determine whether the oligo(dT)-hybridizing clusters were coincident with P granules, we performed double label in situ experiments with P granules labeled with an FSE-conjugated anti-P granule antibody (OIC1D4; Strome, 1986). In these experiments, we found that the majority of the oligo(dT)-hybridizing clusters co-localized with P granules (compare Figs 6C and D). These results suggest that poly$(A)^+$ RNA is present in P granules.

As a control for the specificity of the oligo(dT) staining pattern, we have used an oligo-dA probe, which gave no staining (Table 3). In addition, pre-treatment of embryos with RNAse A or RNAse I eliminated staining with oligo(dT) (Table 3).

To characterize further the P granule-associated RNAs, we used a probe complementary to the 22 nucleotides of SL1, a short sequence which is trans-spliced upstream of many RNAs (Krause and Hirsh, 1987; Bektesh et al., 1988). Like the oligo(dT) probe, this anti-SL1 oligonucleotide probe hybridized to the cytoplasm of all cells and to cytoplasmic granules in the P cells (presumably P granules; data not shown). In contrast, an oligonucleotide complementary to the 3′ region of the unspliced SL1 precursor RNA did not show any staining (data not shown). We have also used a monoclonal antibody (K121; Krainer, 1988) that recognizes the trimethyl-guanosine cap found at the 5′ end of many small RNAs, including SL1 (Liou and Blumenthal, 1990; Van Doren and Hirsh, 1990). Like the oligo(dT) and anti-SL1 probes, this antibody recognizes putative P granules (data not shown). Together, these results suggest that SL1-containing RNAs are present in P granules.

What is the identity of the RNAs associated with P granules? Because these RNAs are detected as early as the 1-cell stage, we presume that they are maternal RNAs. Although all the

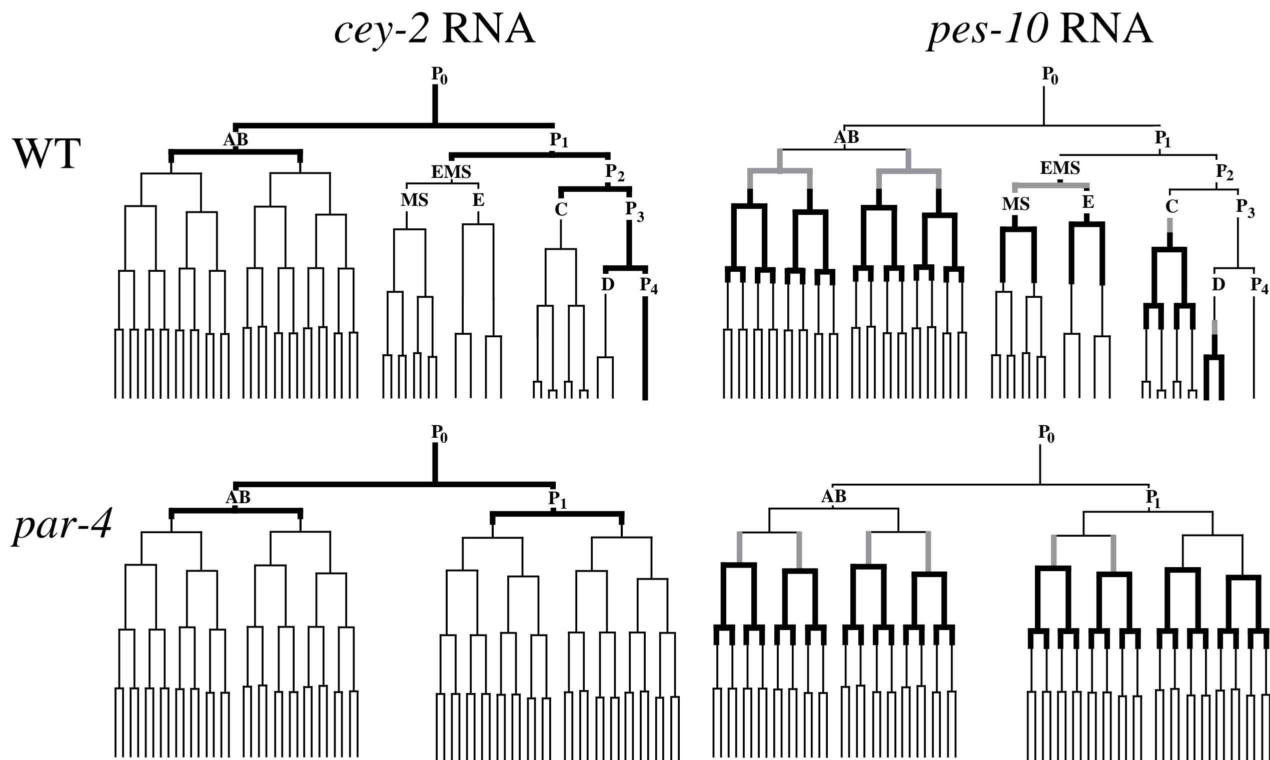

Fig. 5. Distribution of *cey-2* and *pes-10* RNAs in wild-type and *par-4* embryos. Early embryonic lineages are depicted for both wild-type and *par-4* embryos. Thick black lines indicate cells where *cey-2* (maternal) or *pes-10* (embryonically transcribed) cytoplasmic RNAs were detected. Thick gray lines represent cells where nuclear *pes-10* RNA was detected.

specific maternal RNAs included in our survey were present in the P lineage, none appeared to be preferentially associated with P granules in the 1- to 4-cell stage (eg. compare Fig. 6B,G). Later, when the P granules start to coalesce around the nucleus in P_3 and P_4, class II maternal RNAs were often found to also adopt this peri-nuclear localization (eg. Figs 2, 6H), but the significance of this late association remains unclear. These results suggest that RNAs associated with P granules in early embryos belong to a class of maternal RNAs not represented in the 21 genes chosen for our survey. We refer to these RNAs as class III maternal RNAs.

DISCUSSION

We have developed an in situ hybridization protocol suitable for the detection of mRNA in whole-mount *C. elegans* embryos. We have used this protocol to analyze the expression pattern of 21 genes (summarized in Fig. 7) and the overall distribution of poly(A)$^+$ RNAs. We detect asymmetry in the distribution of maternal and embryonically transcribed RNAs as early as the 2- and 4-cell stages.

The fate of maternal RNAs in early embryos

Among the 21 genes included in our survey, we observed two distinct classes of maternal RNAs: maternal RNAs that are maintained equally in all cells during early cleavages (class I RNAs), and maternal RNAs that are maintained preferentially in germ cells and are rapidly lost in somatic cells (class II RNAs).

Why are some maternal RNAs rapidly degraded in somatic cells while others are not? Among RNAs that are degraded, we find genes that function in processes ranging from mRNA translation (*CeIF*, Roussell and Bennett, 1992) to blastomere fate determination (*glp-1,* Priess et al., 1987; *skn-1*, Bowerman et al., 1992). Proteins encoded by this class of RNAs include some that are present in all cells in the early embryo (CEY-1, Jantsch-Plunger, 1993), and others that are localized to specific blastomeres (SKN-1, Bowerman, 1993; GLP-1, Evans et al., 1994). From these observations, it is difficult to find a common functional property that distinguishes these class II maternal RNAs from class I maternal

Table 3. Specificity of oligo(dT) hybridization

Probe	Treatment	Number of embryos with P granule-like staining/total: AP staining	Rhodamine staining
oligo(dT)	BSA	38/41	27/31
	RNAse A	0/48	1/56
	RNAse I	ND	0/ >50
oligo(dA)	–	0/45	0/ >50

Embryos were treated with BSA (Fraction V; 200 µg/ml in 10 mM Tris pH 7.5, 10 mM KCl, 1 mM $MgCl_2$), DNAse-free RNAse A (200 µg/ml in 10 mM Tris pH 7.5, 10 mM KCl, 1 mM $MgCl_2$) or DNAse-free *E. coli* RNAse I (Promega, 0.6 units/µl in 20 mM Tris pH 7.5) for 4 hours at 37°C, prior to hybridization with DIG-labeled oligo(dT) or oligo(dA), and incubation with AP or rhodamine labeled anti-DIG antibody. RNAse A cleaves phosphodiester bonds adjacent to pyrimidines while RNAse I cleaves phosphodiester bonds between all bases. ND, not determined.

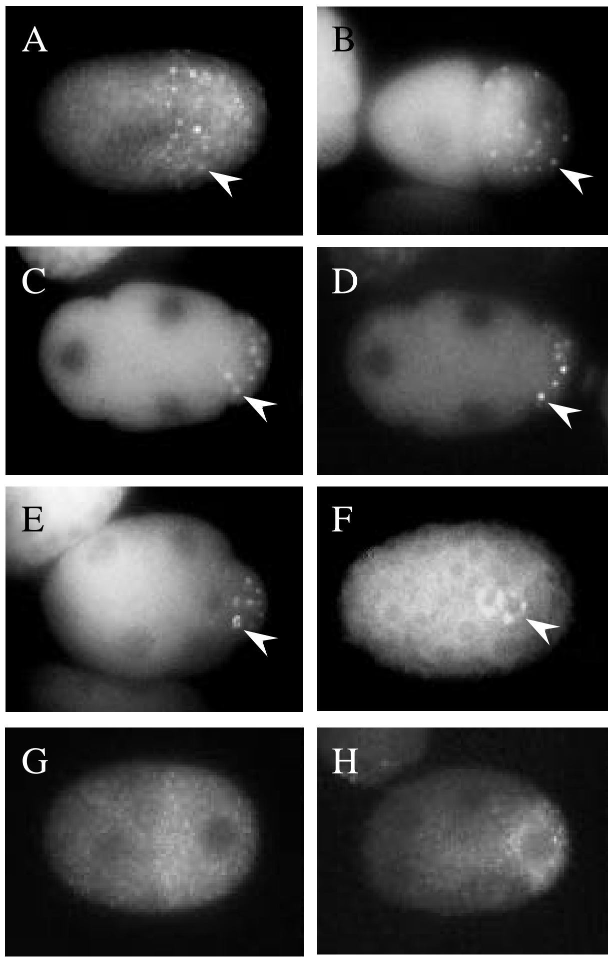

Fig. 6. Distribution of poly(A)$^+$ RNAs. Arrows point to representative P granule-like staining. (A-C,E,F) Wild-type embryos hybridized to an oligo(dT) probe visualized using an anti-digoxigenin antibody coupled to rhodamine. (D) Same embryo as in C labeled with a FSE-conjugated anti-P granule antibody. Embryos stages are as follows: (A) 1-cell, (B) 2-cell, (C,D) 4-cell, (E) 8-cell, (F) 100-cell. (G,H) Wild-type 2- and 6-cell embryos hybridized to an antisense *cey-2* probe visualized using an unlabeled anti-digoxigenin antibody and a 2° antibody coupled to fluorescein.

RNAs. One possibility is that maternal RNAs that are required only in early development (e.g. *skn-1, glp-1;* Priess et al., 1987; Bowerman et al., 1992) or for which embryonically transcribed RNAs arise early (*cey-1*, *CeIF*) are degraded rapidly, while maternal RNAs whose products are required throughout development (e.g. *ama-1*; Bird and Riddle, 1989) are maintained in all cells to ensure continuous expression.

Why are maternal RNAs protected from rapid degradation in the germline? This phenomenon has also been observed in *Drosophila* for the maternal transcript of the *Hsp83* gene. This transcript is initially uniformly distributed throughout the embryo. One hour after fertilization, it is degraded rapidly everywhere except in the polar plasm at the posterior pole of the syncytial embryo (Ding et al, 1993). Components of the pole plasm, in particular the RNA-containing polar granules, function in the determination of pole cells, the *Drosophila* primordial germ cells (Illmensee and Mahowald, 1974). One possibility is that protection of RNA from degradation is an essential property of the polar plasm to maintain RNAs that are essential for the development of germ cells. A similar phenomenon may be occurring in *C. elegans*. As discussed below, the germline cells of *C. elegans* may also contain specialized RNAs. Mechanisms that evolved to maintain these RNAs may protect all RNAs present in germline cells from rapid degradation.

Clusters of poly(A)$^+$ RNAs are associated with P granules

All class I and class II RNAs detected in our survey using gene-specific probes were uniformly distributed in the 1-cell stage embryo. Using an oligo(dT) probe, however, we found evidence for a third class of maternal RNAs. We observed clusters of poly(A)$^+$ RNAs that co-localized with the germline-specific P granules. Because these RNAs are present at the 1-cell stage, they are likely to be maternal in origin. In addition, because none of the class I and class II maternal RNAs detected in our survey appeared to be associated with P granules, the poly(A)$^+$ P granule-associated RNAs likely belong to a third class of maternal RNAs not included in our survey. Whether these class III RNAs are exclusively associated with P granules or are also present in the cytoplasm of somatic cells remains to be determined.

What is the function of P granules and their associated class III maternal RNAs? The *C. elegans* P granules are similar to the polar granules, dense bodies or nuage that have been observed in the germline of many organisms (Eddy, 1975). Much like the *C. elegans* P granules, the *Drosophila* polar granules contain RNA and are localized to the posterior pole of the early embryo prior to their segregation in germ cells (Mahowald, 1962, 1971). As mentioned above, the *Drosophila* polar granules and their associated polar plasm and RNAs are known to be required for the development of posterior structures including germ cells. No such function, however, has yet been demonstrated for the P granules of *C. elegans*. Nevertheless, it remains a reasonable hypothesis that P granules and class III RNAs function in some aspect of germ line and/or posterior development. Asymmetrically localized cytoplasmic factors have been postulated to function in the determination of the anterior-posterior axis in the 1-cell embryo (Schierenberg and Strome, 1992). Isolation of class III RNAs and subsequent genetic analysis will help determine what role if any these RNAs have in these processes.

Embryonic transcription can be detected in somatic cells as early as the 4-cell stage

Using a probe specific for the *pes-10* RNA, we detected embryonically transcribed RNAs in the nuclei of somatic cells as early as the 4-cell stage. Similarly, Edgar et al. (1994) were able to detect [^{3}H]UTP incorporation in permeabilized embryos as early as the 8-cell stage (background levels precluded detection in earlier stages). These observations indicate that, in *C. elegans*, embryonic transcription is not delayed by the rapid first mitotic cycles (also see Edgar et al., 1994). In this regard, *C. elegans* differs from other organisms with rapid initial mitoses such as *Drosophila* and *Xenopus*, where the bulk of embryonic transcription starts only after cell cycles slow down (Newport and Kirschner, 1982; Edgar and

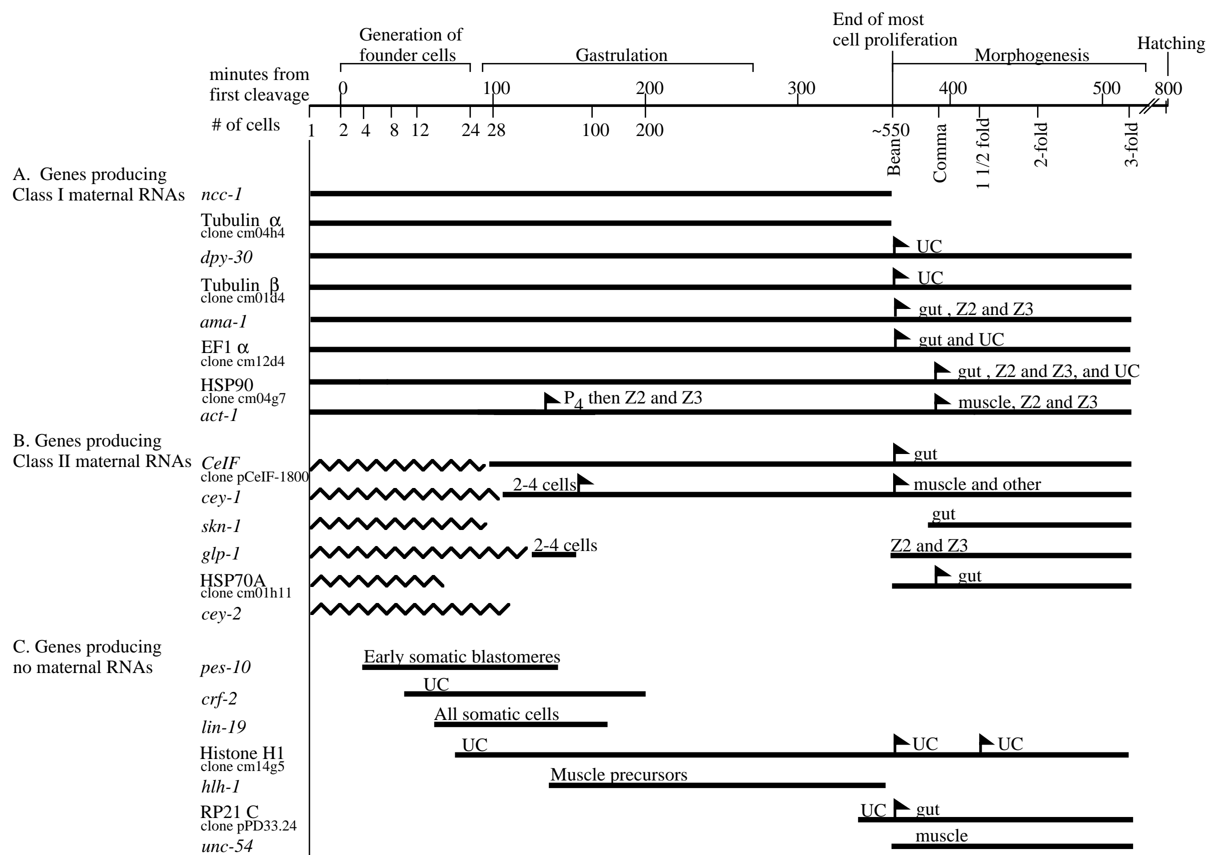

Fig. 7. Embryonic expression of 21 genes as determined by in situ hybridization. Horizontal lines delineate the period during which a specific RNA was detected as listed in Table 1. Flags indicate changes in tissue specificity. Tissue specificity is indicated above each line (only major tissues are listed, UC refers to expression restricted to unidentified cells); otherwise expression is ubiquitous. Genes are grouped according to the type of maternal transcripts detected. (A) Genes producing class I maternal RNAs: maternal transcripts that appear to be maintained equally in all cells at least up to the 28-cell stage. The onset of embryonic transcription (if any) could not be determined for these genes because of the perdurance of maternal transcripts. (B) Genes producing class II maternal RNAs: maternal transcripts that appear to be lost from somatic cells but are maintained in germline cells. These maternal RNAs are represented with jagged lines to distinguish them from the later arising embryonically transcribed RNAs (straight lines). (C) Genes producing no maternal RNAs. Only transcripts arising after the 1-cell stage (presumably embryonically transcribed) were detected for these genes.

Schubiger, 1986). One possibility is that the relatively small size of *C. elegans* genes permits them to be transcribed during rapid mitotic cycles.

Asymmetry in the distribution of newly transcribed RNAs was apparent from the onset of embryonic transcription. Newly transcribed RNAs were detected in all somatic blastomeres from the 4-cell stage onward, but were not detected in the germ lineage during early cleavages. A lack of embryonic transcription in the germ lineage of the early embryo has been observed in *Drosophila*, where germ cell nuclei failed to incorporate [^{3}H]UTP (Zalokar, 1976) and to hybridize with [^{3}H]poly(U) (Lamb and Laird, 1976) in the blastoderm stage. The mechanisms that inhibit transcription in early germ cells are not known. Although we cannot rule out the production of specific transcripts in the *C. elegans* embryonic germ lineage, our observations support a model where factor(s) required for the repression (or activation) of transcription are asymmetrically segregated at each unequal germline cell division. An intriguing possibility is that such factors might also function to maintain (or degrade) maternal RNAs.

Mechanisms required for unequal divisions in the early embryo affect the distributions of maternal and embryonically transcribed RNAs

As described in the Introduction, previous studies have shown that somatic and germline cells differ in size, cleavage pattern, and P granule content. This study now shows that somatic and germline cells also differ in three aspects of RNA metabolism. First, germline cells protect maternal RNAs from the rapid degradation observed in somatic cells. Second, germline cells contain clusters of poly(A)$^+$ RNAs not seen in somatic cells. Third, as early as the 4-cell stage, somatic cells transcribe certain RNAs, which are off in germ cells. Using a mutation in the *par-4* gene, we have shown that a failure to maintain unequal cell division in the early embryo affects at least two of these processes (maternal RNA degradation and embryonic transcription). [*par-4* embryos also missegregate P granules (Morton et al., 1992) and would therefore also be expected to mislocalize poly(A)$^+$ RNA clusters, although this was not tested.] These results indicate that mechanisms required for the establishment of asymmetry in the early embryo have

immediate effects on the distribution of RNAs. Whether these effects in turn result in the determination or execution of distinct germline and somatic fates will be the subject of further studies.

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