Development 124, 2191-2201 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV6291

Transcriptionally repressed germ cells lack a subpopulation of phosphorylated RNA polymerase II in early embryos of *Caenorhabditis elegans* and *Drosophila melanogaster*

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SUMMARY

Early embryonic germ cells in *C. elegans* and *D. melanogaster* fail to express many messenger RNAs expressed in somatic cells. In contrast, we find that ribosomal RNAs are expressed in both cell types. We show that this deficiency in mRNA production correlates with the absence of a specific phosphoepitope on the carboxy-terminal domain of RNA polymerase II. In both *C. elegans* and *Drosophila* embryos, this phosphoepitope appears in somatic nuclei coincident with the onset of embryonic transcription, but remains absent from germ cells until these cells associate with the gut primordium during gastrulation. In contrast, a second distinct RNA polymerase II phosphoepitope is present continuously in both somatic and germ cells. The germ-line-specific factor PIE-1 is required to block mRNA production in the germ lineage of early *C. elegans* embryos (Seydoux, G., Mello, C. C., Pettitt, J., Wood, W. B., Priess, J. R. and Fire, A. (1996) *Nature* 382, 713-716). We show here that PIE-1 is also required for the germ-line-specific pattern of RNA polymerase II phosphorylation. These observations link inhibition of mRNA production in embryonic germ cells to a specific modification in the phosphorylation pattern of RNA polymerase II and suggest that repression of RNA polymerase II activity may be part of an evolutionarily conserved mechanism that distinguishes germ line from soma during early embryogenesis. In addition, these studies also suggest that different phosphorylated isoforms of RNA polymerase II perform distinct functions.

Key words: RNA polymerase II, CTD phosphorylation, mRNA, rRNA, germ line, embryogenesis, *Caenorhabditis elegans*, *Drosophila melanogaster*.

INTRODUCTION

The separation of germ line and soma is an essential process in the development of multicellular organisms, yet the molecular properties that initially differentiate between these two cell types during embryogenesis are still poorly understood. One characteristic that distinguishes germ cells from somatic cells in early *C. elegans* and *Drosophila* embryos is the ability to produce new messenger RNAs. In both organisms, several studies have suggested that somatic blastomeres activate mRNA production earlier than do germ-line blastomeres. This difference was first discovered in *Drosophila* embryos where somatic nuclei, but not germ-line nuclei, were shown to incorporate [^{3}H]UTP and to hybridize with [^{3}H]poly-U in the blastoderm stage (Zalokar, 1976; Lamb and Laird, 1976; Kobayashi et al., 1988). More recent experiments have demonstrated that the introduction of potent transcription factors in germ-line nuclei is not sufficient to activate mRNA production in these cells prior to the onset of gastrulation (A. Williamson, M. Van Doren and R. Lehmann, personal communication). These observations have led to the hypothesis that early germ cells may be refractory to transcriptional activation (Williamson and Lehmann, 1996).

A similar conclusion was recently reached in *C. elegans* (Seydoux et al., 1996). In this organism, several newly transcribed mRNAs have been detected in somatic blastomeres as early as the 4-cell stage, but none to date have been detected in germ-line blastomeres (Seydoux and Fire, 1994; Seydoux et al., 1996). This soma-germ line difference was shown to depend on the germ-line-specific factor PIE-1. In the absence of PIE-1, newly transcribed mRNAs are detected in both somatic and germ-line blastomeres (Seydoux et al., 1996) and descendants of germ-line blastomeres eventually differentiate inappropriately into somatic tissues (Mello et al., 1992). These data have suggested that germ cell fate in *C. elegans* depends on a PIE-1-mediated inhibitory mechanism that generally blocks mRNA production in the early embryonic germ lineage. We have hypothesized that this inhibitory mechanism may serve to protect germ-line blastomeres from the activity of transcription factors that would otherwise promote somatic development in these cells (Seydoux et al., 1996).

How is mRNA production globally inhibited in germ cells? One possibility is that newly transcribed mRNAs are rapidly

degraded in germ cells; another possibility is that mRNA transcription itself is blocked in germ cells. To begin to distinguish between these two possibilities, we have characterized the distribution of different isoforms of the large subunit of RNA polymerase II in *C. elegans* and *Drosophila* embryos. We find that germ cells lack a specific subpopulation of phosphorylated RNA polymerase II, consistent with the possibility that mRNA transcription is blocked in these cells. We also show that, unlike mRNA transcription, rRNA transcription is active in early germ cells.

MATERIALS AND METHODS

Strain maintenance

C. elegans and *D. melanogaster* stocks were maintained at 20°C and 25°C, respectively, using standard methods (Brenner, 1974; Ashburner, 1989). Wild-type stocks were *Caenorhabditis elegans* N2 and *Drosophila melanogaster* Oregon R. *C. elegans pie-1(–)* embryos were generated by self-fertilization of *pie-1(zu154) unc-25(e156)* hermaphrodites derived from the strain *pie-1(zu154) unc-25(e156)/qC1* (Mello et al., 1992). *D. melanogaster nos(–)* embryos were obtained from nos^{bn}/nos^{bn} females (Wang et al., 1994) crossed with Canton S males.

Antisense RNA injections

Antisense *ama-1* or *pes-10* (control) RNA was synthesized from plasmids pDB19 and pGS17.09, respectively, and injected into the gonad of wild-type adult hermaphrodites as described in Guo and Kemphues (1995) and Powell-Coffman et al. (1996). 18 to 24 hours after injections, injected hermaphrodites were squashed under coverslips to release embryos and processed for antibody staining.

Indirect immunofluorescence microscopy

C. elegans embryos obtained by Clorox treatment or squashing of gravid hermaphrodites were freeze-cracked in PBS between slide and coverslip, immersed in –20°C MeOH for 10 seconds to 1 minute, fixed in 1× PBS, 0.08 M Hepes (pH 6.9), 1.6 mM $MgSO_4$, 0.8 mM EGTA, 3.7% formaldehyde for 30 minutes, washed 3 times in PBT (1× PBS, 0.1% Triton, 0.1% BSA) and blocked for 30 minutes in PBT. 1° antibodies were applied overnight at 4°C and 2° antibodies for 2 hours at room temperature. In a control experiment presented in Fig. 3, fixed embryos were incubated with alkaline phosphatase (0.125 units/μl, Boehringer Mannheim) for 2 hours at 37°C, and washed several times in PBT, prior to incubation with the primary antibody.

Drosophila embryos collected on molasses or apple juice plates were dechorionated, fixed and permeabilized for 30 minutes in 50% heptane, 3.7% formaldehyde, 1× PBS, devitellinized in MeOH and blocked in PBT as described in Reuter et al. (1990). 1° and 2° antibodies were applied overnight at 4°C.

Primary antibodies used were: mAbs H5 and H14, two mouse IgM monoclonal antibodies that bind to distinct phosphorylated epitopes on the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II (Warren et al., 1992; Bregman et al., 1995; Kim et al., 1997; M. Patturajan and J. Corden, personal communication); P4G5, a mouse monoclonal IgG antibody raised against a PIE-1 peptide and received as a gift from C. Schubert, C. Mello and J. Priess; OIC1D4, a mouse monoclonal IgG antibody that recognizes P granules (Strome, 1986); a rabbit polyclonal serum raised against *C. elegans* RNAP II (Sanford et al., 1985); a goat polyclonal serum raised against *Drosophila* RNAP II (Weeks et al., 1982) and rabbit polyclonal serum raised against *Drosophila* VASA received as a gift from A. Williamson.

Secondary antibodies used were: FITC-conjugated goat anti-mouse IgM, rhodamine-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG, FITC-conjugated rabbit anti-goat IgG (Jackson Immuno Research) and Cy3-conjugated goat anti-rabbit IgG (Amersham Life Science). DAPI (1 μg/ml) was added to secondary antibody dilutions to visualize nuclei.

In each experiment, a minimum of 10 embryos was analyzed for each developmental stage. Photographs were taken using Ektachrome 400ASA color slide film (Kodak) for immunofluorescence, or Ektachrome 160T color slide film (Kodak) for Nomarski. Images were digitized using a Vision 35 scanner (Agfa) and the digitized images were assembled using Adobe Photoshop 3.0 and Page Maker 6.0 programs (Adobe).

rRNA in situ hybridization

Drosophila ITS1 sequence (Tautz et al., 1988) was PCR amplified from *Drosophila* genomic DNA and cloned into pBluescript KS+ to generate digoxigenin-labeled, single-stranded DNA sense and antisense probes as described in Seydoux and Fire (1995). In situ hybridization to *Drosophila* embryos was carried out as described in Tautz and Pfeifle (1989), except that formaldehyde was used instead of paraformaldehyde and levamisole was omitted from the staining reaction.

C. elegans ITS1 and ITS2 sequences were PCR amplified from ribosomal DNA clone pCe7 (Emmons et al., 1979; Ellis et al., 1986) and cloned into pBluescript KS+ to generate digoxigenin-labeled, single-stranded DNA sense and antisense probes used for in situ hybridization as described in Seydoux and Fire (1995). Both ITS1 and ITS2 antisense probes hybridized to 1 or 2 foci per interphase nucleus, consistent with the fact that all rDNA genes have been mapped to a single region on LGI (Albertson, 1984). No staining was detected using ITS1 and ITS2 sense probes.

The 7 kb *Bam*HI rDNA insert from pCe7 (Emmons et al., 1979) was cloned into pBluescript KS+ to generate a rDNA repeat where the 18 S gene is interrupted by full-length Bluescript sequence. This construct (100 μg/ml) was injected with pRF4 (10 μg/ml; Mello et al., 1991) to generate an heritable extrachromosomal array. Roller males carrying the array were mated with *tra-2(q122)* females and their progeny processed for in situ hybridization using a pBluescript-specific probe. We observed variable expression of the transgene in embryos, with a fraction of embryos showing expression in all blastomeres, including the germ-line blastomere. Because the transgene was on an extrachromosomal array with pRF4 DNA (encoding a RNAP II transcript), it may have had difficulty localizing to the nucleolus for efficient RNAP I-dependent expression.

RESULTS

RNA polymerase II is present in both somatic and germ-line nuclei in pregastrulation *C. elegans* and *Drosophila* embryos

In *C. elegans* and *Drosophila*, germ cells are formed during early embryogenesis (Fig. 1). To visualize the distribution of RNA polymerase II (RNAP II) in early *C. elegans* and *Drosophila* embryos, we used two polyclonal antibodies raised against RNAP II purified from *C. elegans* and *Drosophila* extracts, respectively (Sanford et al., 1985; Weeks et al., 1982). These antibodies recognize several subunits of RNAP II, and do not distinguish between the phosphorylated and non-phosphorylated forms of the large subunit (Sanford et al., 1985; Weeks et al., 1982). We found that these antibodies label the nucleoplasm of both somatic and germ-line interphase nuclei in *C. elegans* and *Drosophila* embryos (Fig. 2). In many nuclei, these antibodies also highlighted two subnuclear foci, which were often barely visible above the strong nucleoplasmic staining (data not shown). These results indicate that RNAP II is present in both somatic and germ-line nuclei in early embryos of *C. elegans* and *Drosophila*.

A subpopulation of phosphorylated RNA polymerase II is absent from germ-line nuclei in pregastrulation *C. elegans* and *Drosophila* embryos

mAbs H5 and H14, two monoclonal antibodies that recognize phosphorylated RNA polymerase II

Phosphorylation of the carboxy-terminal domain (CTD) of the large subunit of RNAP II (RNAP II LS) has been linked with the process of transcriptional elongation (Dahmus, 1996 for review). To visualize phosphorylated RNAP II LS, we have used two monoclonal IgM antibodies, mAbs H5 and H14. By western analysis, these antibodies have been shown to bind to phosphorylated RNAP II LS in a wide range of eukaryotes, including *C. elegans* and *Drosophila* (Warren et al., 1992; Bregman et al., 1995; M. A. D. and G. S., unpublished data). Recently, these antibodies were shown to recognize distinct phosphorylated epitopes on the CTD of RNAP II LS (Kim et al., 1997; M. Patturajan and J. Corden, personal communication; also see Discussion).

To verify the specificity of mAbs H5 and H14 binding to RNAP II LS in fixed tissues, we have compared mAbs H5 and H14 in situ staining patterns in wild-type embryos and embryos lacking RNAP II LS. *C. elegans* embryos lacking RNAP II LS [*ama-1(as)* embryos] were generated by injecting the gonad of adult hermaphrodites with antisense *ama-1* RNA, which encodes RNAP II LS (Bird and Riddle, 1989). This treatment causes hermaphrodites to produce embryos lacking RNAP II LS, which fail to activate mRNA transcription and arrest around the 100-cell stage (Powell-Coffman et al., 1996). Whereas high levels of mAbs H5 and H14 staining were detected in embryos derived from hermaphrodites injected with a control antisense RNA (Fig. 3A,C), no mAb H5 or mAb H14 staining was detected in interphase nuclei of *ama-1(as)* embryos (Fig. 3B,D). These results confirm that mAbs H5 and H14 can recognize RNAP II LS specifically in fixed interphase nuclei of *C. elegans*. Occasionally, some mAbs H5 and H14 immunoreactivity was detected on condensed mitotic chromosomes of *ama-1(as)* embryos (Fig. 3B), raising the possibility that this aspect of mAbs H5 and H14 staining may not be specific to RNAP II LS. For this reason, mitotic nuclei were not considered further in our analysis of mAbs H5 and H14 staining.

To verify that mAbs H5 and H14 recognize phosphorylated epitopes, we treated fixed *C. elegans* embryos with alkaline phosphatase prior to immunostaining (see Materials and methods). We found that this treatment completely abolishes staining with mAbs H5 and H14 (Fig. 3F,G), but does not affect staining with a polyclonal antibody that recognizes both phosphorylated and non-phosphorylated forms of RNAP II (Fig. 3E). We conclude that mAbs H5 and H14 bind specifically to phosphorylated RNAP II LS in fixed *C. elegans* embryos.

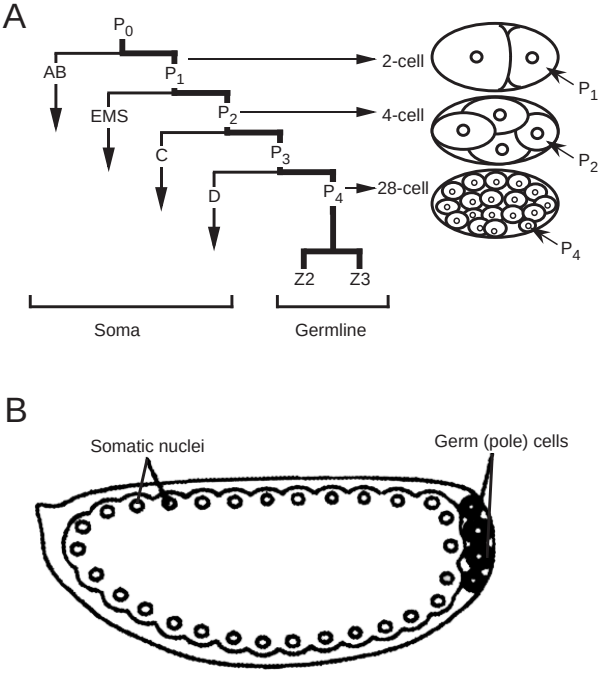

Fig. 1. Origin of the germ line in *C. elegans* and *Drosophila*. (A) In *C. elegans*, the zygote (P_0) undergoes a series of asymmetric cleavages (horizontal bars) to generate 4 somatic blastomeres (AB, EMS, C and D) and successive germ-line blastomeres (P_1, P_2, P_3 and P_4). In the 100-cell stage, P_4 divides symmetrically to give rise to the two primordial germ cells Z2 and Z3 (Sulston et al., 1983). (B) In *Drosophila*, the zygotic nucleus undergoes a series of rapid nuclear divisions to give rise to a multinucleate syncytium. The first nuclei to migrate to the posterior pole are cellularized precociously and form the germ cells (also called pole cells) (after Williamson and Lehmann, 1996).

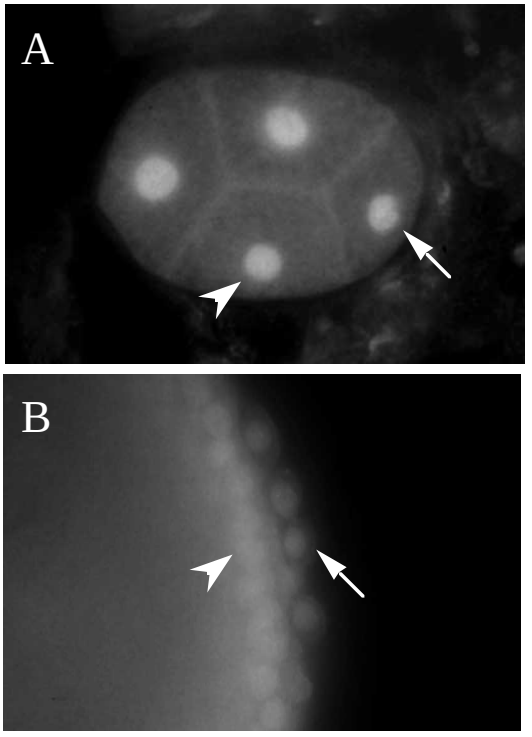

Fig. 2. RNAP II is present in both somatic and germ-line nuclei in early *C. elegans* and *Drosophila* embryos. (A) *C. elegans* 4-cell embryo stained with a polyclonal antibody raised against *C. elegans* RNAP II (Sanford et al., 1985). This antibody also stains the cytoplasm of mitotic cells (not shown). (B) Posterior pole of a *Drosophila* blastoderm embryo stained with a polyclonal antibody raised against *Drosophila* RNAP II (Weeks et al., 1985). Representative germ-line nuclei are marked by an arrow and somatic nuclei by an arrowhead. In this and all subsequent figures, embryos are oriented with anterior to the left and posterior to the right. *C. elegans* and *Drosophila* embryos are approximately 45 and 450 μm in length, respectively.

mAb H5 immunostaining pattern

To determine the pattern of mAb H5 immunoreactivity in *C. elegans* embryos, we co-immunostained embryos with mAb H5, and with an antibody specific for the germ-line factor PIE-1 (Fig. 4). As reported previously (Mello et al., 1996), PIE-1 was detected in germ-line blastomeres from the 1-cell stage to the 100-cell stage (Fig. 4, second column). In contrast, mAb H5 staining was restricted to somatic blastomeres (Fig. 4, third column). We first detected mAb H5 staining in the interphase nucleus of the somatic blastomere EMS in the 4-cell stage (Fig. 4I, arrowhead). In this stage, somatic blastomeres are known to initiate mRNA transcription (Seydoux and Fire, 1994, Seydoux et al., 1996; the somatic blastomeres ABa and ABp are transcriptionally active for a very short time in the early part of the 4-cell stage before they resume mitosis, which may explain our inability thus far to detect mAb H5 staining in these cells). From the 8-cell stage to the 100-cell stage, mAb H5 immunoreactivity was detected in the nuclei of all somatic blastomeres in interphase; in contrast, no staining was detected in germ-line blastomeres (Fig. 4L,O). Occasionally, two faint foci of staining were detected in the germ-line blastomere P_4 in 50-cell and older embryos, but this aspect of staining was not always reproducible (data not shown). In 100-cell and older embryos, mAb H5 staining began to be detected in the nucleoplasm of the 2 daughters of P_4, the primordial germ cells Z2 and Z3 (Fig. 4R). By this stage, PIE-1 staining was no longer detected in the germ lineage (Fig. 4Q; the primordial germ cells were identified here using another germ cell marker, the P granule antibody OIC1D4).

To determine the pattern of mAb H5 immunoreactivity in *Drosophila* embryos, we co-immunostained embryos with mAb H5, and with an antibody against the germ cell protein VASA (Lasko and Ashburner, 1990; Hay et al., 1990; A. Williamson and R. Lehmann, personal communication) (Fig. 5A-C). mAb H5 staining was first detected weakly in somatic nuclei of stage 3 embryos and with increased intensity from stage 4 onward (Fig. 5C). During this time, somatic nuclei acquire general competence for transcription (Edgar and Schubiger, 1986). In contrast, no staining was detected in germ cell (pole cell) nuclei until stage 7, when these cells began to show faint mAb H5 staining (data not shown).

We conclude that the phosphoepitope on RNAP II that is recognized by mAb H5 is present in somatic nuclei, but not in germ-line nuclei, in pregastrulation *C. elegans* and *Drosophila* embryos.

mAb H14 immunostaining pattern

We determined the pattern of mAb H14 staining using the same approach used for mAb H5; embryos were co-stained with mAb H14 and with antibodies specific for germ cells (anti-PIE-1 or anti-P granule antibodies for *C. elegans* and anti-VASA antibody for *Drosophila*) (Figs 5, 6 and data not shown). In both *C. elegans* and *Drosophila*, mAb H14 immunostaining pattern differed in two respects from that of mAb H5. First, unlike mAb H5, which only stained nuclei, mAb H14 also stained the cytoplasm of dividing cells throughout embryogenesis, even prior to the onset of mRNA transcription (Fig. 6B). Second,

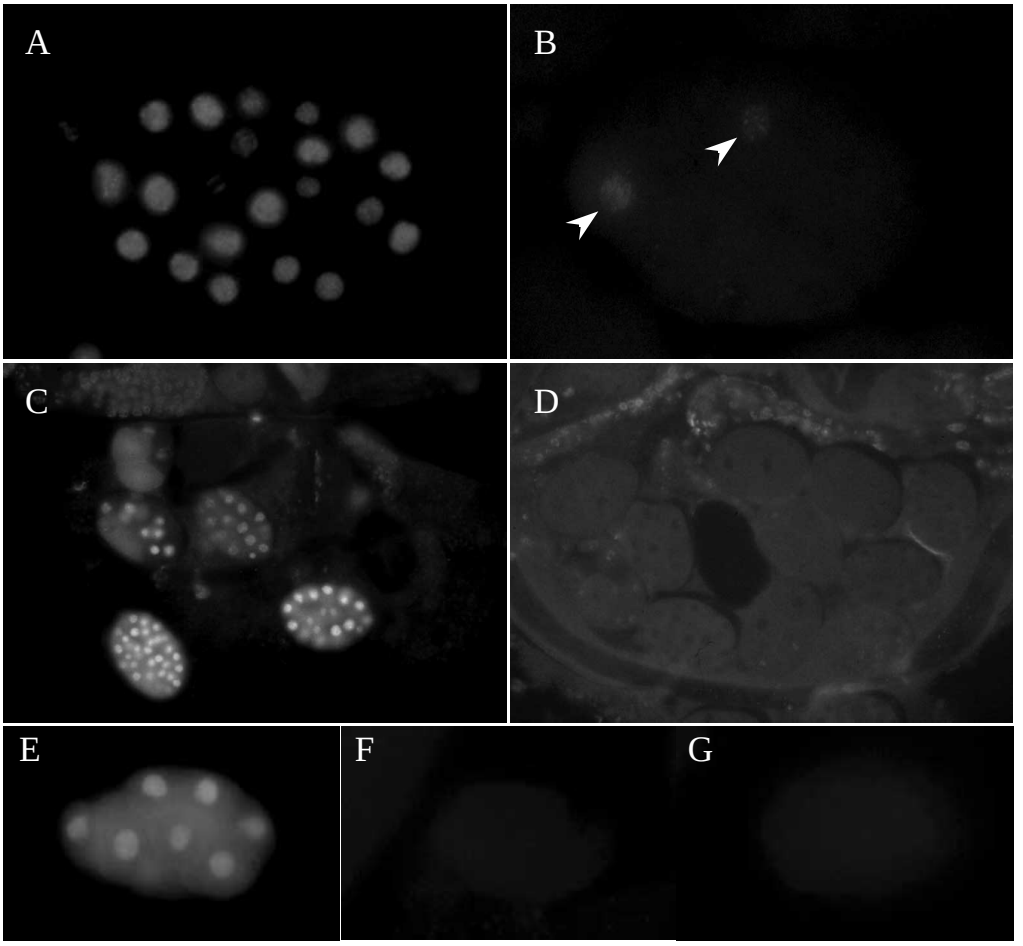

Fig. 3. The monoclonal antibodies H5 and H14 bind to phosphorylated forms of the large subunit of RNAP II in *C. elegans* embryos. (A-D) *C. elegans* embryos derived from hermaphrodites injected with *pes-10* (control, A,C) or *ama-1* (B,D) antisense RNAs and stained with mAb H5 (A,B) or mAb H14 (C,D). Low level mAbs H5 and H14 staining is detected on mitotic chromosomes (prophase, arrow heads) in *ama-1(as)* embryos. We have also observed mAb H5 staining on metaphase chromosomes (not shown). A and B each show one 28-cell embryo, whereas C and D show several embryos of various stages. (E-G) 15-cell *C. elegans* embryos treated with alkaline phosphatase (see Materials and Methods) and stained with (E) a polyclonal antibody that recognizes both phosphorylated and unphosphorylated forms of *C. elegans* RNAP II (Sanford et al., 1985), (F) mAb H5 or (G) mAb H14.

mAb H14 stained both somatic and germ cell nuclei from early embryogenesis (4-cell stage in *C. elegans*, stage 3 in *Drosophila*) onward. In both cell types, mAb H14 labeled the nucleoplasm and two prominent foci per nucleus (Figs 5F, 6D,F,H; similar foci were also visible in early somatic nuclei with mAb H5, data not shown). These foci became more difficult to detect in somatic nuclei of later stage embryos, perhaps due to increased nucleoplasmic staining. In contrast, in germ cell nuclei, nucleoplasmic staining intensity remained low and the two foci of staining could be detected throughout early embryogenesis (4- to 100-cell stage in *C. elegans*, stage 4 to stage 6 in *Drosophila*). After the onset of gastrulation, (100-cell stage in *C. elegans*, stage 7 in *Drosophila*), staining in germ cells began to resemble that observed in somatic cells (diffuse nucleoplasmic staining; data not shown).

We conclude that the phosphoepitope on RNAP II that is recognized by mAb H14 is present in two subnuclear domains in both somatic and germ-line nuclei in early embryos. This phosphoepitope is also present in the nucleoplasm, but is detected there at a higher level in somatic nuclei compared to germ-line nuclei in pregastrulation embryos.

pie-1 activity is required for the germ-line-specific patterns of mAbs H5 and H14 immunoreactivity in *C. elegans*

In *C. elegans* embryos, the PIE-1 protein is detected in germ-line blastomeres from the 1-cell stage to the 100-cell stage (Mello et al., 1996). As described above, this period corresponds to the interval during which germ cells do not stain with mAb H5 (Fig. 4) and stain with lowered intensity with mAb H14 (Fig. 6). We have previously shown that *pie-1* activity is required to keep mRNA production off in germ cells of early embryos (Seydoux et al., 1996). To test whether *pie-1* is also required for the germ-line-specific patterns of mAbs H5 and H14

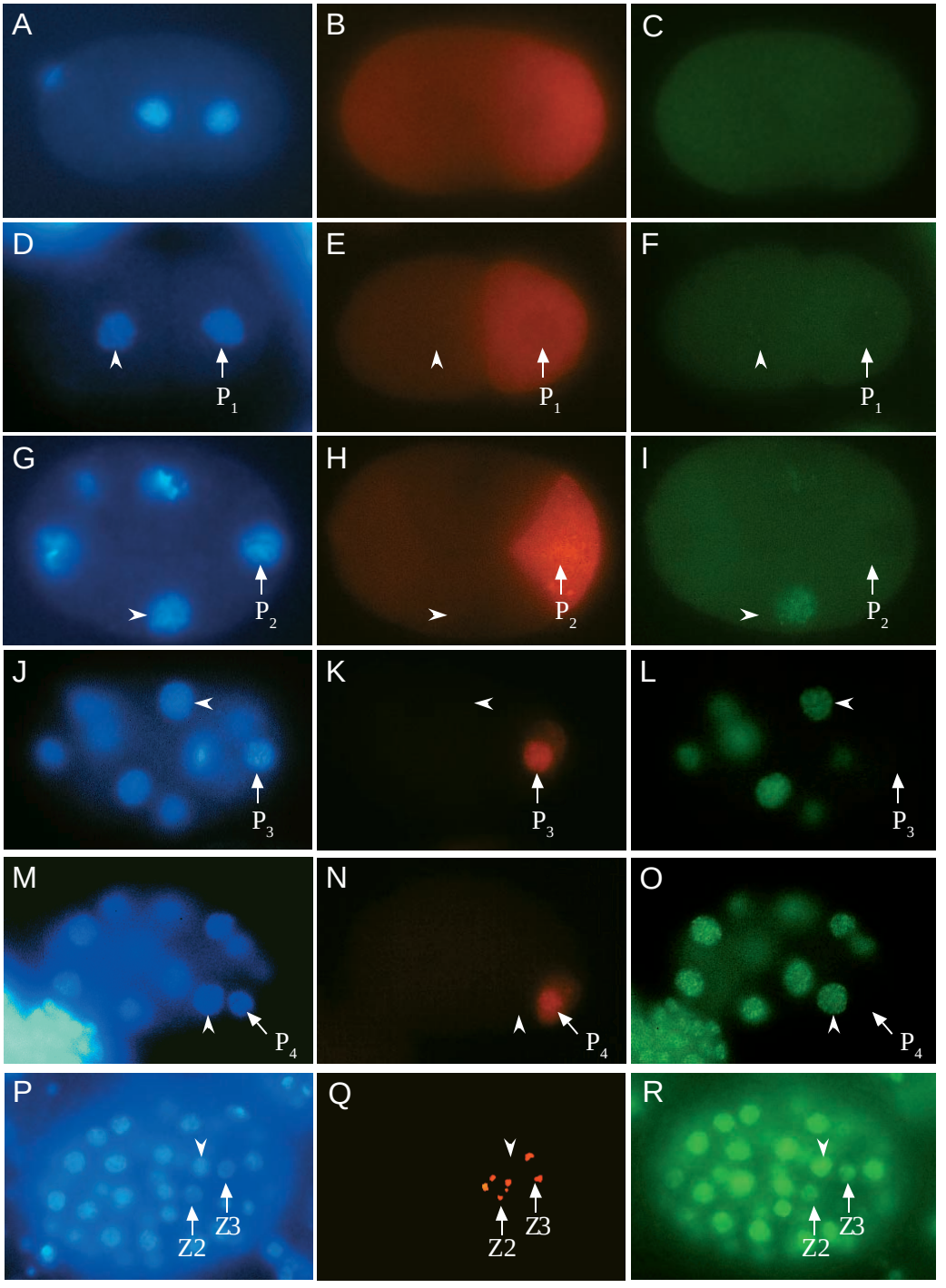

Fig. 4. Immunolocalization of RNAP II-H5 in *C. elegans* embryos. Wild-type *C. elegans* embryos triply stained with the DNA dye DAPI (1st column), an antibody specific for the germ-line factor PIE-1(2nd column) and mAb H5 (3rd column). In each panel, an arrow points to the germ-line nucleus, and an arrowhead points to a representative somatic nucleus in a comparable stage of the cell cycle (interphase). (A-C) 1-cell embryo, (D-F) 2-cell embryo, (G-I) 4-cell embryo, (J-L) 15-cell embryo, (M-O) 28-cell embryo, (P-R) ca. 100-cell embryo; in addition to DAPI (blue), anti-PIE-1 antibody (red) and mAb H5 (green), this latter embryo was also stained with OIC1D4 (red), an antibody specific for P granules (germ-line organelles), used here to identify the primordial germ cells Z2 and Z3. These cells stain with OIC1D4 in a perinuclear pattern typical for P granules at this stage, but no longer show any PIE-1 staining.

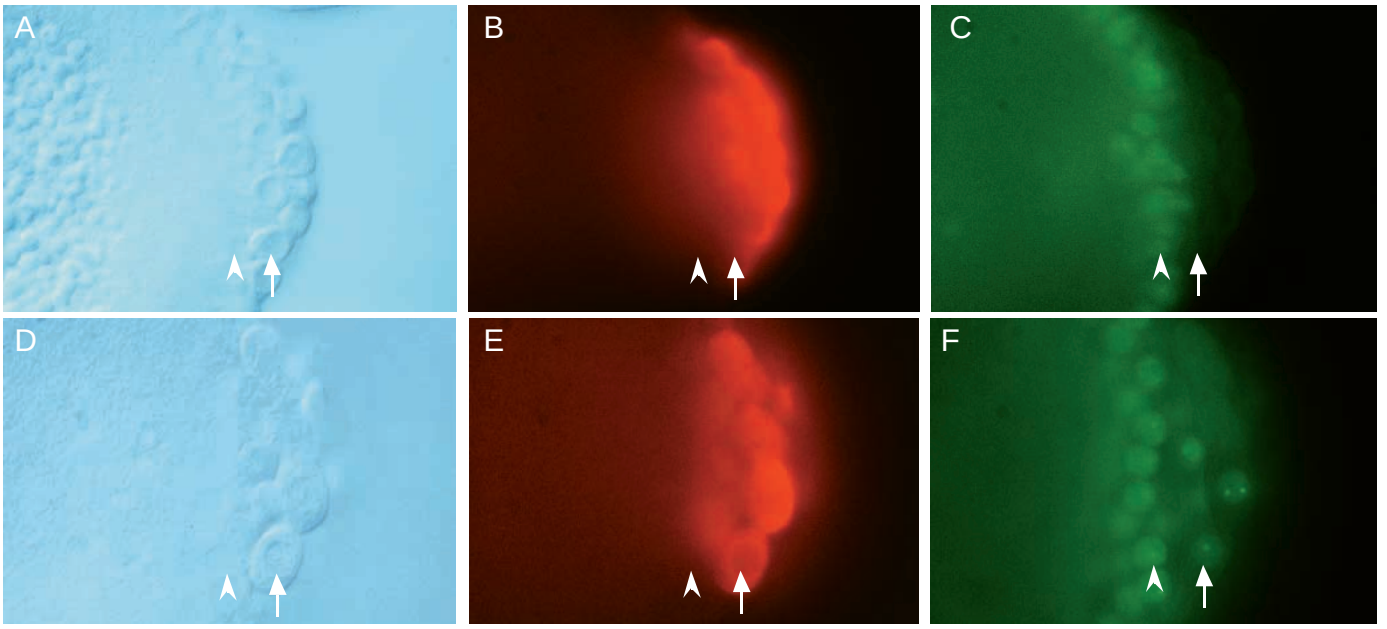

Fig. 5. Immunolocalization of RNAP II-H5 and RNAP II-H14 in *Drosophila* embryos. (A-C) Posterior pole of a wild-type stage 5 *Drosophila* embryo doubly stained with anti-VASA antibody to identify germ cells (B), and mAb H5 (C). (D-F) Posterior pole of a wild-type stage 4 *Drosophila* embryo doubly stained with anti-VASA antibody (E), and mAb H14 (F). In each panel, an arrow points to germ cell nuclei and an arrow head points to somatic nuclei.

staining, we stained embryos derived from *pie-1(–)* mothers with mAbs H5 and H14. We find that, in the absence of *pie-1* activity, germ cells stain with mAbs H5 and H14 in a pattern identical to that observed in somatic cells (Fig. 7). These results indicate that *pie-1* activity is required for the soma-germ line differences observed in mAbs H5 and H14 staining.

The *nanos* (*nos*) gene is required for the correct temporal expression pattern of several germ cell-specific transcripts in *Drosophila* (Kobayashi et al., 1996). In the absence of *nos* activity, expression of these transcripts, which normally initiates when the germ cells have reached the gonads (stage 14), begins prematurely during germ cell migration (stage 7-8) (Kobayashi et al., 1996). To test whether *nos* activity is also required for the proper onset of mAb H5 immunoreactivity in germ cells (stage 7), we stained embryos derived from *nos(–)* mothers with mAb H5. We found that the temporal pattern of mAb H5 staining was unaffected in *nos(–)* embryos (mAb H5 staining was detected in germ cells of *nos(–)* embryos no earlier than stage 7, as is observed in wild type; data not shown). These results indicate that *nos* activity is not required for the proper onset of mAb H5 immunoreactivity in germ cells and suggests that *nos* regulates the expression of specific germ-line transcripts after transcription has been generally activated in the germ line.

RNA polymerase I transcripts are transcribed in both somatic and germ-line cells in early *C. elegans* and *Drosophila* embryos

Our analysis of gene expression in embryonic germ cells has focused so far on RNA polymerase II transcription. To begin to test whether other polymerases are similarly regulated, we have analyzed the transcriptional pattern of ribosomal RNAs in early *C. elegans* and *Drosophila* embryos. To distinguish newly transcribed ribosomal RNAs from maternally inherited mature rRNAs, we used probes complementary to internal spacer sequences (ITS1 and ITS2 in *C. elegans* and ITS1 in *Drosophila*). These sequences are present in the nascent rRNA transcript, but are eliminated during rRNA processing in the nucleolus, and thus are not expected to be present in maternally inherited, mature rRNAs.

In *Drosophila*, nascent rRNA transcripts were first detected in stage 4 embryos, consistent with the observations of Edgar and Schubiger (1986), which showed that rRNA transcription begins during this stage. Initially, nascent rRNA transcripts were detected in all somatic nuclei, and in a subset of germ-line nuclei (Fig. 8A,B). By stage 5, nascent rRNAs were detected consistently in all somatic and germ-line nuclei (Fig. 8C,D).

In *C. elegans*, nascent rRNA transcripts were first detected in the decondensed maternal and paternal pronuclei of 1-cell stage embryos (Fig. 8F). Subsequently, these transcripts were detected in all nuclei (both somatic and germ line; Fig. 8G,I), with the exception of nuclei undergoing mitosis (Fig. 8H). Nascent rRNA transcripts were observed continuously throughout interphase in all blastomeres, except in the germ-line blastomere P_4, where they were transiently absent from the 28-cell stage to the 50-cell stage (data not shown). During this time, the chromosomes of P_4 adopt a unique morphology, which is retained in P_4 and its descendants throughout embryogenesis (G. S., unpublished observations); the significance of this morphology and of the apparent transient lack of rRNA transcription in P_4 is not known.

To rule out the possibility that the unprocessed transcripts that we observed were actually maternally derived, we used an additional strategy to visualize patterns of rRNA transcription in *C. elegans* embryos. We inserted pBluescript sequences in

the 18S gene of a ribosomal DNA repeat (see Materials and Methods) and transformed this tagged rDNA construct into worms to establish a heritable line. Males carrying the tagged rDNA construct were mated to untransformed females and the resulting cross-progeny were analyzed by in situ hybridization for expression of the tagged 18S rRNA. We detected tagged 18S rRNA in all nuclei as early as the 2-cell stage (with the exception of P_4 in 28-cell to 50-cell embryos as described above) (Fig. 8J). Because the tagged transgene was not present in the maternal germ line, expression of the tagged rRNA must have derived from the paternal copy of the transgene and therefore is most likely to be due to embryonic transcription.

Taken together, these results indicate that rRNAs, unlike mRNAs, are transcribed in both somatic and germ-line cells in early *C. elegans* and *Drosophila* embryos.

DISCUSSION

In this study, we describe the distribution of different RNA polymerase II isoforms in *C. elegans* and *Drosophila* embryos. These observations have led to three major conclusions. First, germ cells lack a specific RNAP II phosphoepitope (RNAP II-H5), which is present in transcriptionally active somatic cells. Second, a distinct RNAP II phosphoepitope (RNAP II-H14) is present in both somatic and germ cells. Third, in *C. elegans*, the germ-line-specific pattern of RNA polymerase II phosphorylation is dependent on *pie-1* activity. We also describe the distribution of newly transcribed rRNAs in early *C. elegans* and *Drosophila* embryos and show that, unlike mRNA transcription, rRNA transcription is active in both somatic and germ-line blastomeres. We discuss the implications of each of these findings below.

Evidence for a block in RNA polymerase II activity in the early germ lineage of *C. elegans* and *Drosophila*

To test whether the lack of newly synthesized mRNAs in embryonic germ cells is due to a block in RNAP II activity, we have characterized the distribution of phosphorylated RNAP II in early *C. elegans* and *Drosophila* embryos. Towards this purpose, we used two monoclonal antibodies, mAbs H5 and H14, which recognize distinct phosphorylated epitopes on the carboxy-terminal domain (CTD) of the large subunit of RNAP II (Bregman et al., 1995; Kim et al., 1997; M. Patturajan and J. Corden, pers. comm.). Phosphorylation of the CTD has been linked temporally with transcriptional elongation (Dahmus, 1996 for review); indeed, we find that mAbs H5 and H14 begin to label somatic nuclei at stages coincident with the onset of embryonic transcription (4-cell stage in *C. elegans*, stage 3/4 in

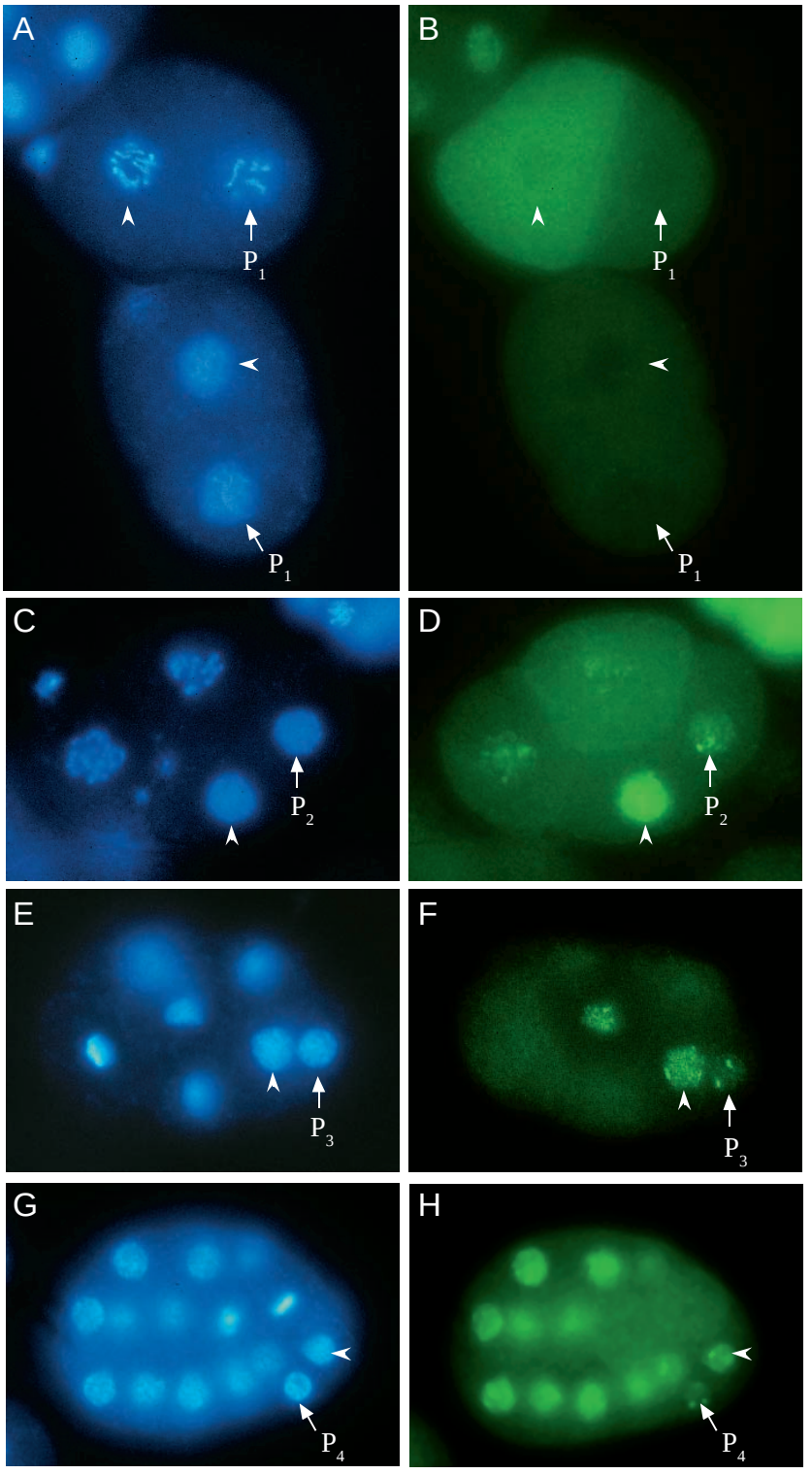

Fig. 6. Immunolocalization of RNAP II-H14 in *C. elegans* embryos. Wild-type *C. elegans* embryos doubly stained with the DNA dye DAPI (1st column), and mAb H14 (2nd column). (A,B) Two 2-cell embryos, (C,D) 4-cell embryo, (E,F) 8-cell embryo, (G,H) 28-cell embryo. In each panel, an arrow points to the germ-line nucleus and an arrowhead points to a representative somatic nucleus.

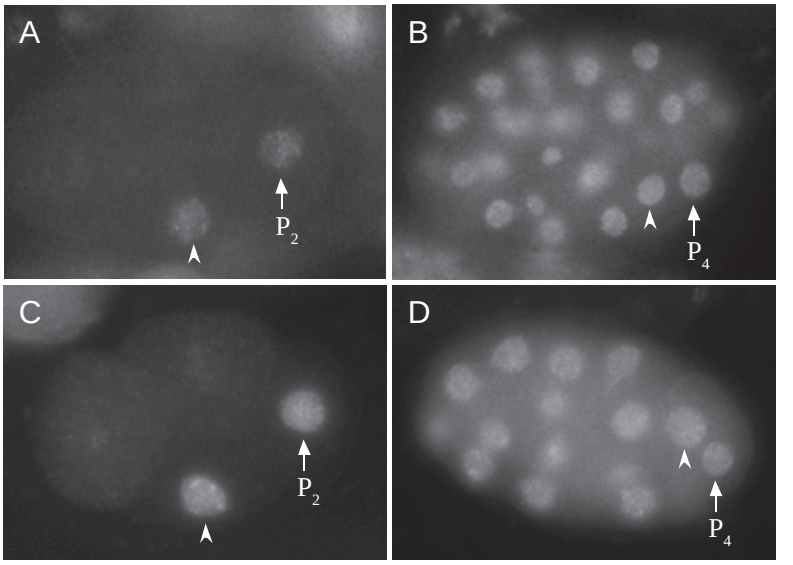

Fig. 7. RNAP II-H5 and RNAP II-H14 immunolocalization in *pie-1(–)* embryos. *pie-1(–)* 4-cell (A,C), 44-cell (B) and 28-cell (D) *C. elegans* embryos stained with mAb H5 (A,B) or mAb H14 (C,D). Germ-line nuclei are marked by an arrow and representative somatic nuclei are marked by an arrowhead. [In 4-cell embryos, the germ-line blastomere P_2 was identified based on its position; in older embryos, the germ-line blastomere P_4 was identified using mAb OIC1D4 to label the germ-line-specific P granules (not shown)].

Drosophila). In early somatic nuclei, mAbs H5 and H14 stained the nucleoplasm and two discrete subnuclear domains. This pattern is reminiscent of the staining pattern of mAbs H5 and H14 in mammalian cells, where these antibodies were shown to label the nucleoplasm and several discrete subnuclear sites closely linked to 'speckle' domains (Bregman et al., 1995). These observations have suggested that phosphorylated RNAP II may exist in two forms in vivo: a storage form sequestered in discrete subnuclear domains and an active form engaged in transcription in the nucleoplasm (Bregman et al., 1995; Kim et al., 1997).

Remarkably, we detected different mAbs H5 and H14 staining patterns in early embryonic germ cells. mAb H5 failed to stain germ cells entirely in both *C. elegans* and *Drosophila* pregastrulation embryos. With mAb H14, some staining could be detected in early *C. elegans* and *Drosophila* germ cells, but this staining was concentrated in two subnuclear foci and was often barely detectable in the nucleoplasm. These observations suggest that early embryonic germ cells lack the phosphoepitope recognized by mAb H5 and have reduced nucleoplasmic levels of the phosphoepitope recognized by mAb H14. This germ-line-specific staining pattern persisted until early gastrulation (100-cell stage in *C. elegans*, stage 7 in *Drosophila*); at that time, the germ cells associate with the gut primordium inside the embryo and begin to stain with mAbs H5 and H14 in patterns similar to those observed in somatic nuclei. This stage is likely to correspond to the onset of mRNA transcription in germ cells; indeed, embryonically transcribed *vasa* transcripts have been detected in *Drosophila* germ cells as early as stage 9-10 (Williamson and Lehmann, 1996).

Our data suggest that CTD phosphorylation is transiently blocked (or reversed by dephosphorylation) in early *C. elegans* and *Drosophila* germ cells. Since CTD phosphorylation has been linked to the transcriptional cycle, these results support the hypothesis that RNAP II activity is transiently inhibited in *C. elegans* and *Drosophila* embryonic germ cells. Interestingly, this inhibition appears to eliminate completely mAb H5-specific CTD phosphorylation, but only reduces H14-specific CTD phosphorylation in the nucleoplasm. mAbs H5 and H14 recognize different phosphorylated serines on the CTD (serine at position 2 of each CTD repeat for mAb H5, and serine at position 5 of each CTD repeat for mAb H14; M. Patturajan and J. Corden, pers. comm.). Suppression analysis in yeast have shown that these two serines are genetically distinct, raising the possibility that phosphorylation at these two sites may have different functional consequences (Yuryev and Corden, 1996). Indeed, whereas mAb H5-specific CTD phosphorylation was detected only in transcriptionally active nuclei, mAb H14-specific CTD phosphorylation was also detected in the cytoplasm of mitotic cells, indicating that this latter pattern of phosphorylation is not necessarily linked to transcription. We do not know, however, whether residual RNAP II-H14 present in germ cell nuclei is inactive and represents only a storage form of the polymerase, or is active and is transcribing those few (so far undescribed) germ-line mRNAs that may escape transcriptional repression. In addition, our observations do not address whether reduction in CTD phosphorylation is a cause or a consequence of the lack of RNAP II activity in germ cells.

In the discussion above, we have assumed that the absence of mAb H5 immunoreactivity in germ cells reflects a lack of H5-specific CTD phosphorylation in these cells. An alternative interpretation of our results, however, is that the mAb H5 epitope is present in germ cells, but is not accessible to the antibody (i.e. the mAb H5 epitope is 'masked'). In this hypothesis, H5-specific CTD phosphorylation would still occur in germ cells, but in a context no longer recognizable by mAb H5. Although we know of no precedent for masking of the CTD, our observations so far have not ruled out this possibility formally. However, because this hypothesis implies that RNAP II-H5 exists in a unique context in early germ cells, this alternative interpretation is still consistent with our main conclusion, namely that phosphorylated RNAP II exists in a different form in germ cells compared to somatic cells.

Unlike mRNAs, ribosomal RNAs are transcribed in the early germ lineage of *C. elegans* and *Drosophila*

The observation that mRNA production is repressed in germ cells led us to investigate whether rRNA production is similarly regulated. Surprisingly, we found that rRNA transcription occurs in both somatic and germ-line cells in early *C. elegans*

and *Drosophila* embryos. In *Drosophila*, nascent rRNAs were detected in both somatic and germ-line nuclei starting in stage 4, although consistent expression in all germ-line nuclei was not detected until stage 5. In *C. elegans*, rRNA transcription was detected as early as the 1-cell stage, and continued in all somatic and germ-line interphase nuclei throughout early embryogenesis. The only exception was the germ-line blastomere P_4 where nascent rRNA transcripts were transiently absent from the 28-cell stage to the 50-cell stage. These observations indicate that, unlike mRNA transcription, rRNA transcription is not subject to continuous repression in the early germ lineage. We do not know whether differences between mRNA and rRNA transcription are due to the nucleolar localization of rRNA synthesis, or to the fact that rRNAs and mRNAs are transcribed by different polymerases, or both. In any case, these observations indicate that early germ cells in *C. elegans* and *Drosophila* are not completely transcriptionally repressed, and that at least a portion of their genome is accessible to transcription factors.

The germ-line-specific factor PIE-1 is required to repress RNAP II phosphorylation and mRNA production in early germ cells of *C. elegans*

We have previously shown that the germ-line factor PIE-1 is required to repress mRNA production in the early embryonic germ lineage of *C. elegans*; in *pie-1(–)* embryos, germ-line blastomeres activate mRNA production (Seydoux et al., 1996). In this study, we show that *pie-1* is also required for the germ-line-specific pattern of RNAP II phosphorylation; in *pie-1(–)* embryos, germ-line blastomeres stain with mAbs H5 and H14 in patterns identical to those observed in somatic blastomeres. This result is particularly significant since *pie-1* activity is *not* required for other aspects of early germ-line blastomere identity, such as maintenance of maternal RNAs or segregation of P granule-associated RNAs (Seydoux et al., 1996). The fact that *pie-1* activity is required specifically for both

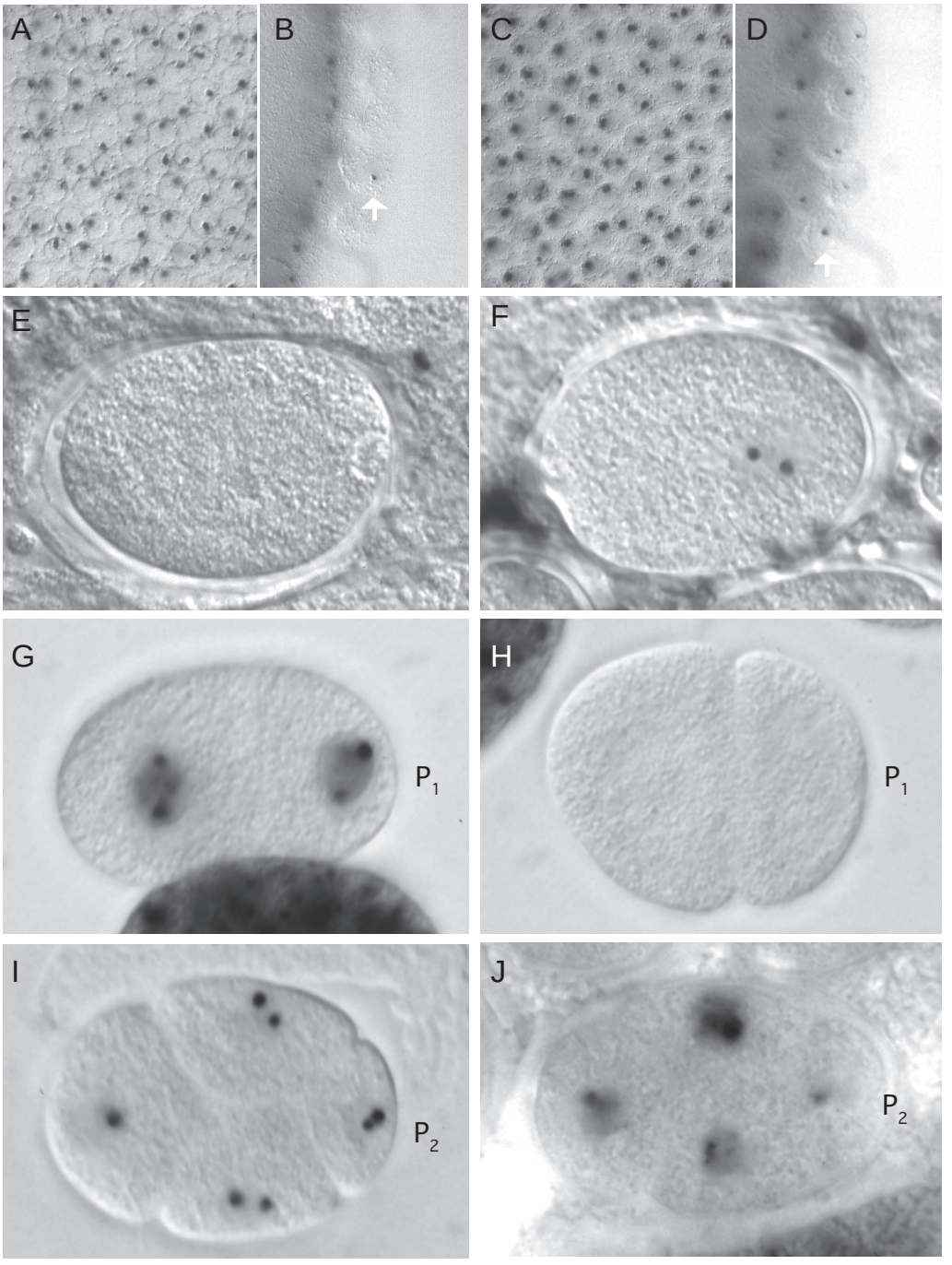

Fig. 8. Newly transcribed rRNAs are detected in both somatic and germ-line nuclei in early *C. elegans* and *Drosophila* embryos. (A-D) Wild-type *Drosophila* embryos hybridized to an ITS1 antisense probe. (A) Stage 4 somatic nuclei, (B) stage 4 germ cells, (C) stage 5 somatic nuclei, (D) stage 5 germ cells. (E-I) Wild-type *C. elegans* embryos hybridized to an ITS1 antisense probe. (E) Newly fertilized egg; (F) 1-cell embryo at pronuclear meeting; (G) 2-cell embryo with both nuclei in interphase; (H) 2-cell embryo with both nuclei in mitosis; (I) 4-cell embryo. (J) 4-cell *C. elegans* embryo derived from a cross between wild-type hermaphrodites and males transformed with a rDNA-pBluescript transgene, and hybridized to a pBluescript antisense probe. rDNA-pBluescript transcripts are detected in both somatic and germ-line (P_2) blastomeres. No signal was detected with a pBluescript sense probe (not shown).

repression of mRNA production and repression of RNAP II phosphorylation suggests that these two phenomena are linked, and supports the hypothesis that the lack of newly transcribed mRNAs in germ cells is due to a block in RNAP II-dependent transcription.

How might PIE-1 function to repress RNAP II activity in the germ lineage? The PIE-1 protein is translated from maternally derived RNA starting in the 1-cell stage, and accumulates in germ-line nuclei from the 2-cell stage to the 100-cell stage (Mello et al., 1996). Strikingly, PIE-1's disappearance from the germ lineage in the 100-cell stage coincides with the appearance of mAb H5 immunoreactivity in that lineage. Thus, both the spatial and temporal distributions of PIE-1 are consistent with the possibility that PIE-1 itself functions directly to block RNAP II activity in the germ lineage. One possibility is that PIE-1 interacts with the transcriptional machinery to prevent transcriptional activation and RNAP II phosphorylation. Equally plausible, however, are more complex models where PIE-1 effects on transcription and RNAP II phosphorylation are indirect. For example, PIE-1 could function to modify chromatin structure in germ cells in such a way as to prevent access of the transcriptional machinery to DNA. In this model, absence of RNAP II phosphorylation in germ cells would be a secondary consequence of a lack of template available for transcription. Such indirect models, however, must account for the fact that, unlike mRNA transcription, rRNA transcription is not repressed in early germ cells, indicating that rRNA genes are accessible to RNA polymerase I.

So far, the structure of the PIE-1 protein has not helped distinguish among different models for PIE-1 action. PIE-1 contains 2 zinc-finger-like motifs of the CCCH class (Mello at al., 1996). These motifs have been found in a number of proteins including the mammalian growth-factor response protein TIS11/Nup475 (Dubois et al., 1990, Varnum et al., 1991), the splicing factor U2AF^{35} (Zhang et al., 1992), and the *Drosophila* proteins Suppressor of Sable (Voelker et al., 1991), Unkempt (Mohler et al., 1992) and Clipper (Bai and Tolias, 1996). Recently, a region in Clipper that contains 5 CCCH fingers was shown to have RNA endonuclease activity in vitro (Bai and Tolias, 1996). It remains to be determined whether the PIE-1 fingers, which have diverged significantly from those of Clipper, will have the same function.

Repression of mRNA transcription: an evolutionarily conserved mechanism that distinguishes germ line from soma during early embryogenesis?

Perhaps the most significant finding in this study is the observation that embryonic germ cells of both *C. elegans* and *Drosophila* show an identical lack of a specific phosphoepitope of RNAP II. Since *C. elegans*, a nematode, and *Drosophila*, an insect, are separated by more than 1,000 million years of evolution (Vanfleteren et al., 1994), it is tempting to speculate that this similarity reflects the existence of a very ancient mechanism that inhibits RNAP II function in germ cells and thus insulates germ line from soma during early embryogenesis. We have shown that, in *C. elegans*, this mechanism depends on PIE-1, a germ-line factor essential for maintenance of the germ-line fate. Future studies will determine whether a PIE-1 homologue performs a similar function in *Drosophila*, and whether similar mechanisms are operating in other metazoans.

We are especially grateful to Jeff Corden and Andy Fire for many stimulating discussions and insightful suggestions during the course of this work. We thank Deborah Andrew and the Andrew laboratory for generously sharing their *Drosophila* expertise and protocols; Steve Warren; Miriam Golomb; Arno Greenleaf; Charlotte Schubert, Craig Mello and Jim Priess; Meera Patturajan and Jeff Corden; and Anne Williamson and Ruth Lehmann for antibodies; Jo-Anne Powell-Coffman and Bill Wood for the DB19 plasmid and antisense injection protocol; Barbara Sollner-Webb for advice on rDNA tagging; Liz Gavis for *nos^{bn}* flies; Randy Reed for the use of his scanner; Meera Patturajan and Jeff Corden, Anne Williamson and Ruth Lehmann for sharing data prior to publication; and Andy Fire, Bill Kelly, Jeff Corden, Dan Nathans and Deborah Andrew for critical reading of the manuscript. This work was supported by the March of Dimes and the American Cancer Society. G. S. is a Packard fellow.

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(Accepted 24 March 1997)