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Repression of Zygotic Gene Expression in the Putative Germline Cells in Ascidian Embryos

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ABSTRACT—In germline cells of early *C. elegans* and *Drosophila* embryos, repression of zygotic gene expression appears to be essential to maintain the germ cell fate. In these animals, specific residues in the carboxy-terminal domain (CTD) of RNA polymerase II large subunit (RNAP II LS) are dephosphorylated in the germline cells, whereas they are phosphorylated in the somatic cells. We investigated, in early embryos of the ascidian *Halocynthia roretzi,* the expression patterns of three genes that are essentially expressed in the entire embryo after the 32-cell stage. We found that the expression of these genes was inactive in the putative germline cells during the cleavage stages. Once cells were separated from the germline lineage by cleavages, the expression of the genes was initiated in the cells. These results suggest that repression of transcription in germline cells may also be common in chordate embryos. We then examined the phosphorylation state of the CTD of RNAP II using a phosphoepitope-specific antibody. At cleavage stages after the 32-cell stage, CTD was phosphorylated in every blastomere, including the germline cells. Therefore, in the ascidian, the inactivation of zygotic transcription is not correlated with dephosphorylation of the CTD. These observations indicate that zygotic transcription is inactivated in ascidian germline cells, but the mechanism of the repression may differ from that in *C. elegans* and *Drosophila*.

Key words: ascidian embryo, germline, gene expression, RNA polymerase II, transcriptional repression

INTRODUCTION

The cells in multicellular embryos are primarily classified into germline cells and somatic cells. Several differences between these two cell types have been shown. In various species, the germline cells contain the germ plasm, which promotes the formation and the differentiation of the germline cells. In the early *C. elegans* and *Drosophila* germline, zygotic gene expression is generally repressed (Seydoux and Fire, 1994; Seydoux *et al*., 1996; Kobayashi *et al*., 1996; Van Doren *et al*., 1998). In *C. elegans*, 16 different early transcripts can be detected zygotically as early as the 4-cell stage in the somatic lineage, while transcriptional activity in the germline precursors begins around the 100-cell stage. In *Drosophila*, mRNA transcription begins in pole cells during gastrulation, approximately 2 hr after it begins in somatic cells. The repression of zygotic gene expression in the early embryonic stage appears to be essential to maintain the germ cell fate. Germline cells delay the initiation of zygotic transcription, otherwise it would promote somatic cell fates (reviewed by Seydoux and Strome, 1999; Matova and Cooley, 2001).

During the stage when transcriptional repression occurs in these animals, specific residues in RNA Polymerase II (RNAP II), serines at position 2 of the consensus repeat (YSPTSPS) of the carboxy-terminal domain (CTD), are dephosphorylated, while they are phosphorylated in the somatic cells (Seydoux and Dunn, 1997). Phosphorylation of the CTD is thought to occur during the transition from the initiation phase to the elongation phase of transcription (Dahmus, 1996). Dephosphorylation of CTD is correlated with transcriptional repression. RNAP II activity would be reduced in germline cells, although it is not known whether dephosphorylation of the CTD is a cause or a consequence of the lack of RNAP II activity.

In ascidian embryos, the origin of the germline cells has not been definitely identified. However, several lines of evidence suggest that cells in the posterior pole of the pre-gastrulation embryos are germline cells. First, after a series of unequal cleavages, the posteriormost (B7.6) blastomeres in the vegetal hemisphere of the 64-cell embryos cease cell division during embryogenesis, similarly to the germline cells of other organisms such as *Drosophila*, *C. elegans* and

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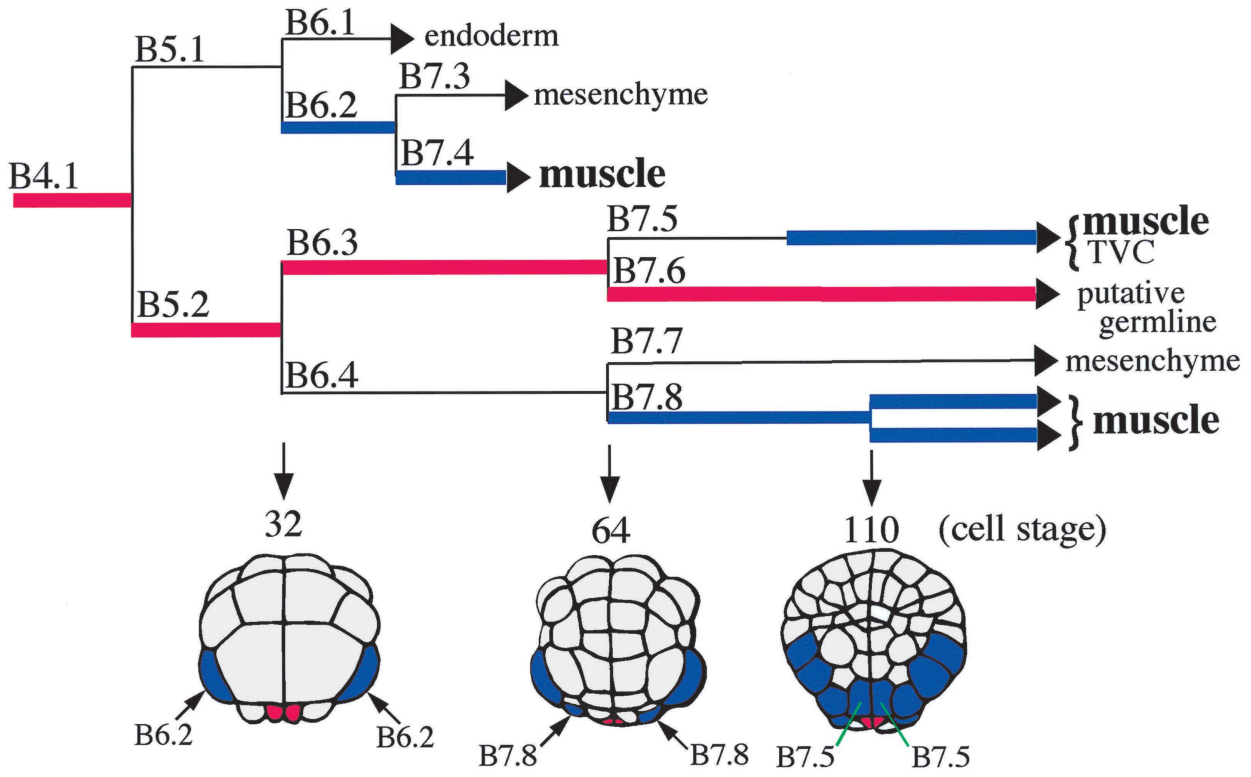


Fig. 1. Temporal and spatial expression patterns of muscle actin (*HrMA4*), myosin heavy-chain (*HrMHC1*), and Ca-transporter genes in the posterior-vegetal B-lineage. Red lines and blastomeres represent putative germline. Blue lines and blastomeres represent blastomeres that express muscle-specific genes. Blastomeres with arrows and numbers are the blastomeres that initiate the expression of muscle-specific genes. The muscle-specific gene expression appears to be initiated with an approximately one-cell cycle delay after the blastomeres are separated from the putative germline blastomeres. TVC, trunk ventral cell.

Xenopus (Matova and Cooley, 2001). Second, the cells of this lineage contain a structure designated the centrosome-attracting body (CAB), which plays a role in the unequal cleavages by attracting the centrosome and nucleus towards the posterior pole (Hibino *et al.*, 1998; Nishikata *et al.*, 1999). Ultrastructural studies have shown that the CAB contains a matrix with high electron density, that resembles the germ plasm reported in other animals (Iseto and Nishida, 1999). Third, *vasa* mRNA and protein are characteristic of the germline cells of various animals (reviewed by Ikenishi, 1998), and maternal transcripts of *Ci-DEAD1*, a *vasa* homolog in the ascidian, *Ciona intestinalis*, are concentrated in the posteriormost blastomeres (Fujimura and Takamura, 2000).

In ascidian embryos, muscle formation has been particularly well studied. We noticed that expression of the muscle-specific genes such as muscle actin (*HrMA4*) and myosin heavy-chain (*HrMHC1*) genes appears to be inactive in the putative germline blastomeres (Fig. 1; Satou *et al.*, 1995). Both genes show exactly the same spatio-temporal expression pattern. The muscle-specific Ca^{2+}-transporter gene also shows the same expression pattern (Miya and Nishida, unpublished data). The expression starts in the two lateral cells (B6.2 cell pair) at the 32-cell stage, then gradually progresses in the posterior direction at the 64- and 110-cell stages. Looking at the cell lineage tree (Fig. 1), this expression pattern could be interpreted as indicating that expression of these genes is repressed in the posteriormost lineage cells (B5.2, B6.3 and B7.6; red lines in Fig. 1). When blastomeres are separated from the posteriormost lineage cells, which contain a CAB, the expression of these genes starts after a one-cell cycle delay (blue lines in Fig. 1). Because these genes are tissue-specific genes, the idea that zygotic expression is repressed in germline cells needs to be tested using non-tissue-specific genes that are ubiquitously expressed in embryos. In this study, we examined whether zygotic expression is generally inactivated in the putative germline cells in early ascidian embryos. We also used H5 antibody, which recognizes the phosphoepitope in the CTD, to examine whether RNAP II in the putative germline cells is specifically dephosphorylated.

MATERIALS AND METHODS

In situ hybridization

DIG-labeled RNA probes were produced and *in situ* hybridization was carried out according to the protocol described by Miya *et al.* (1994). DIG-labeled RNA probes were used at a concentration of 1 μg/ml in the hybridization buffer. The reaction time for coloring was 4 to 8 hr. After *in situ* hybridization the embryos were stained with DAPI at 0.2 μg/ml for 20 min for nuclear staining, then washed with PBS containing 0.1% Tween 20 and mounted for microscopic observation.

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Immunohistochemistry

Embryos were fixed with –20°C methanol for 30 min and then stored in ethanol at –20°C until use. The embryos were rehydrated by soaking in a graded series of ethanol and transferred to PBST (PBS containing 0.05% Triton-X 100). After the specimens were washed 4 times in PBST, they were treated with 3% H_2O_2 in PBST for 30 minutes at room temperature to inactivate endogenous peroxidase activity. After the specimens were washed in PBST, they were incubated in 0.5% blocking reagent (contents of TSA Kit; NEN Life Science Products, Boston) for 1 hr, and then incubated with H5 antibody, a mouse IgM monoclonal antibody against the phosphoserine at position 2 in the CTD heptapeptide of RNAP II LS (Babco, Richmond, CA) diluted 1:10000 in blocking solution overnight at 4°C. After the specimens were washed for 1 hr, they were blocked again for 1 hr. They were incubated with goat anti-mouse IgG (H+L) conjugated to horseradish peroxidase (Bio-Lad, Hercules, CA) diluted 1:500 in blocking solution overnight at 4°C. After washing for 20 min, they were immersed in TNT buffer (0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.05%Tween 20). The specimens were incubated with Biotinyl-Tyramide (TSA Kit, NEN Life Science Products, Boston) diluted 1:50 in amplification diluent for 20 min at room temperature, then washed once in TNT buffer. The specimens were washed with PBST for 15 min and then incubated with Alexa-streptavidin (Molecular Probes, Eugene, OR) diluted1:100 in PBST for 1 hr at room temperature. After the specimens were washed for 10 min, the nuclei were stained with DAPI (0.2 µg/ml) for 20 min. The embryos were mounted in 80% glycerol in PBS and observed under a confocal microscope. An equivalent amount of mouse IgM was used as a control for H5 primary antibody.

RESULTS AND DISCUSSION

Initiation of zygotic expression of the ubiquitously expressed genes

To examine whether the repression of zygotic transcription in the muscle-specific genes is also generally observed in ubiquitously expressed genes, we examined the timing of initiation of expression of three genes that are essentially expressed in the entire embryo. cDNA clones 10, 22 and 36 for these genes were isolated using differential screening between eggs and 110-cell embryos (Miya and Nishida, in preparation). The sequences of clones 10 and 36 show no homology to known proteins, while clone 22 encodes for a putative RNA-binding protein. These genes are expressed in almost every blastomere after the 32-cell stage.

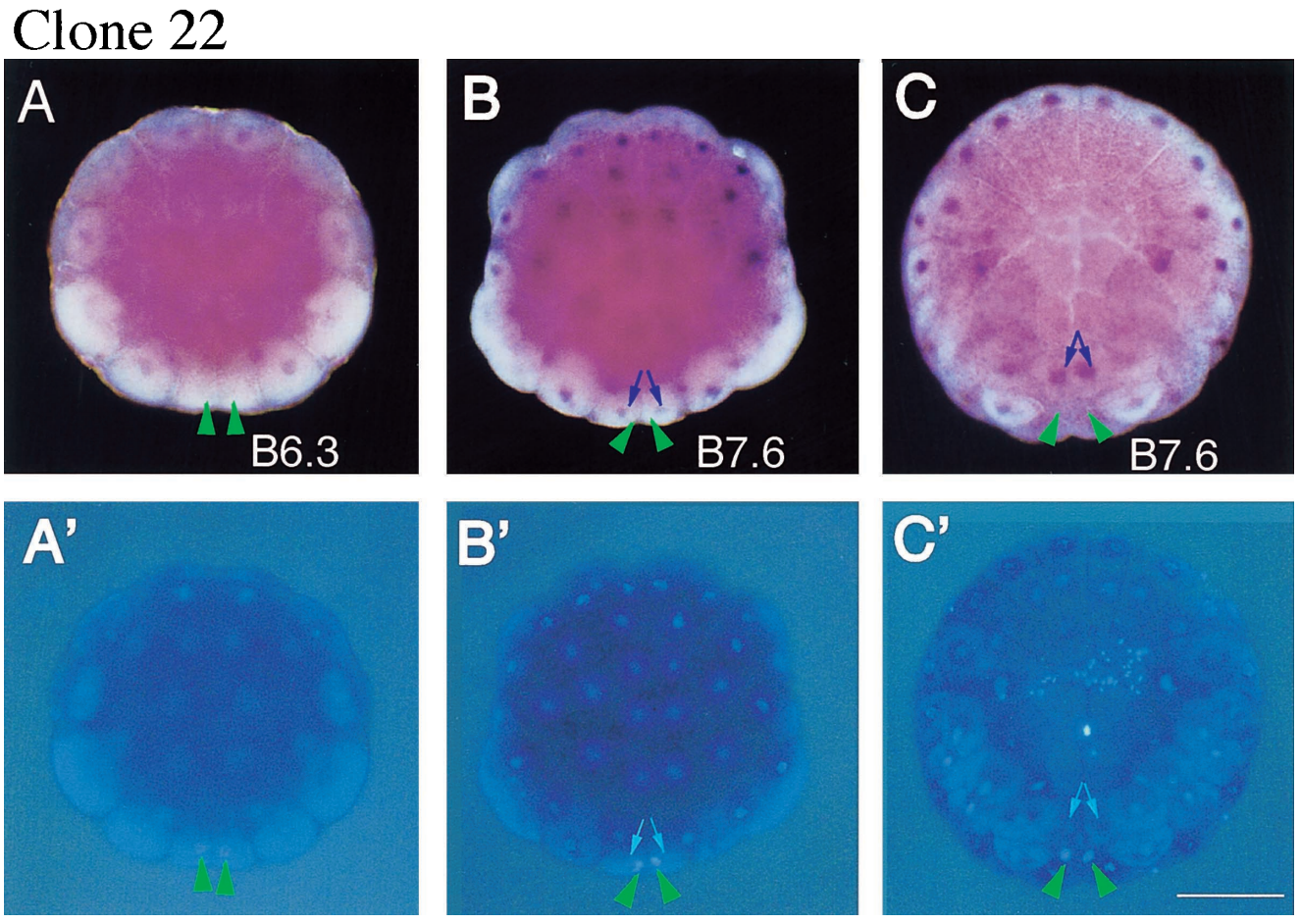


Fig. 2. Expression profile of clone 22. (A) 32-cell stage. (B) 64-cell stage. (C) 118-cell stage. (A, B, C) *In situ* hybridization with clone 22 antisense probe. (A', B', C') The same embryos were stained with nuclear fluorescent dye. Hybridization signals indicating expression of the clone 22 gene were detected in every blastomere except for the putative germline blastomeres (arrowhead). Blue arrows indicate the B7.5 cells, which are sister cells of the B7.6 cells. (A, B) Animal views. (C) Vegetal views. In some blastomeres, the expression was already terminated at the 118-cell stage. Scale bar, 100 µm.

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In ascidian embryos, initiation of zygotic expression can be clearly detected based on the fact that newly synthesized transcripts are first accumulated in the nuclei (e.g., Yasuo and Satoh, 1994). *In situ* hybridization signals indicating expression of the clone 22 gene and nuclear staining with a fluorescent dye, DAPI, after *in situ* hybridization are shown in Fig. 2A-C, 2A'-C', respectively. The signals were first detected in the nuclei of every blastomere except for the posteriormost B6.3 cells at the 32-cell stage (Fig. 2A). At the 64-cell stage (Fig. 2B), B6.3 cells divide into larger anterior B7.5 cells (blue arrows) and smaller posterior B7.6 cells (green arrowheads). The B7.6 cells, which are located at the tip of the green arrowheads, are invisible in Fig. 2B, but are recognizable in nuclear staining shown in Fig. 2B'. The expression of the clone 22 gene was initiated in B7.5 cells but not in B7.6 cells (Fig. 2B). The inactivation in the B7.6 cells was still observed at the 118-cell stage (Fig. 2C).

In Fig. 2A'-C', nuclei were stained with a fluorescent dye after *in situ* hybridization. When the nuclei were stained purple by *in situ* hybridization, the DAPI fluorescence was quenched somewhat and the nuclei looked less bright. When there was no hybridization signal, the nuclei looked brighter. In these figures, one can notice that the nuclei of B6.3 and B7.6 cells are brighter than those of the other cells. Thus, the clone 22 gene was not expressed only in the posteriormost blastomeres at each stage, which are putative germline cells, even when transcription of this gene was initiated in almost the entire embryo.The expression profiles of clone 10 (Fig. 3A–C, A'–C') and clone 36 (Fig. 3D–F, D'–F') were similar to each other, but were slightly different from that of clone 22. At the 32-cell stage, the hybridization signals were first detected in the nuclei of every blastomere except for the posteriormost B6.3 cells and their sister B6.4 cells at the 32-cell stage (Fig. 3A, D). At the 64-cell stage, the expression was initiated in the B6.4 daughter cells, but not in the B6.3 daughters, B7.5 and B7.6 (Fig. 3B, E). At the 118-cell stage, the expression started in B7.5 cells but still not in B7.6 cells (Fig. 3C, F). Thus, the expression of the clone 10 and 36 genes was inactive in putative germline cells and their latest sister cells at the 32- and 64-cell

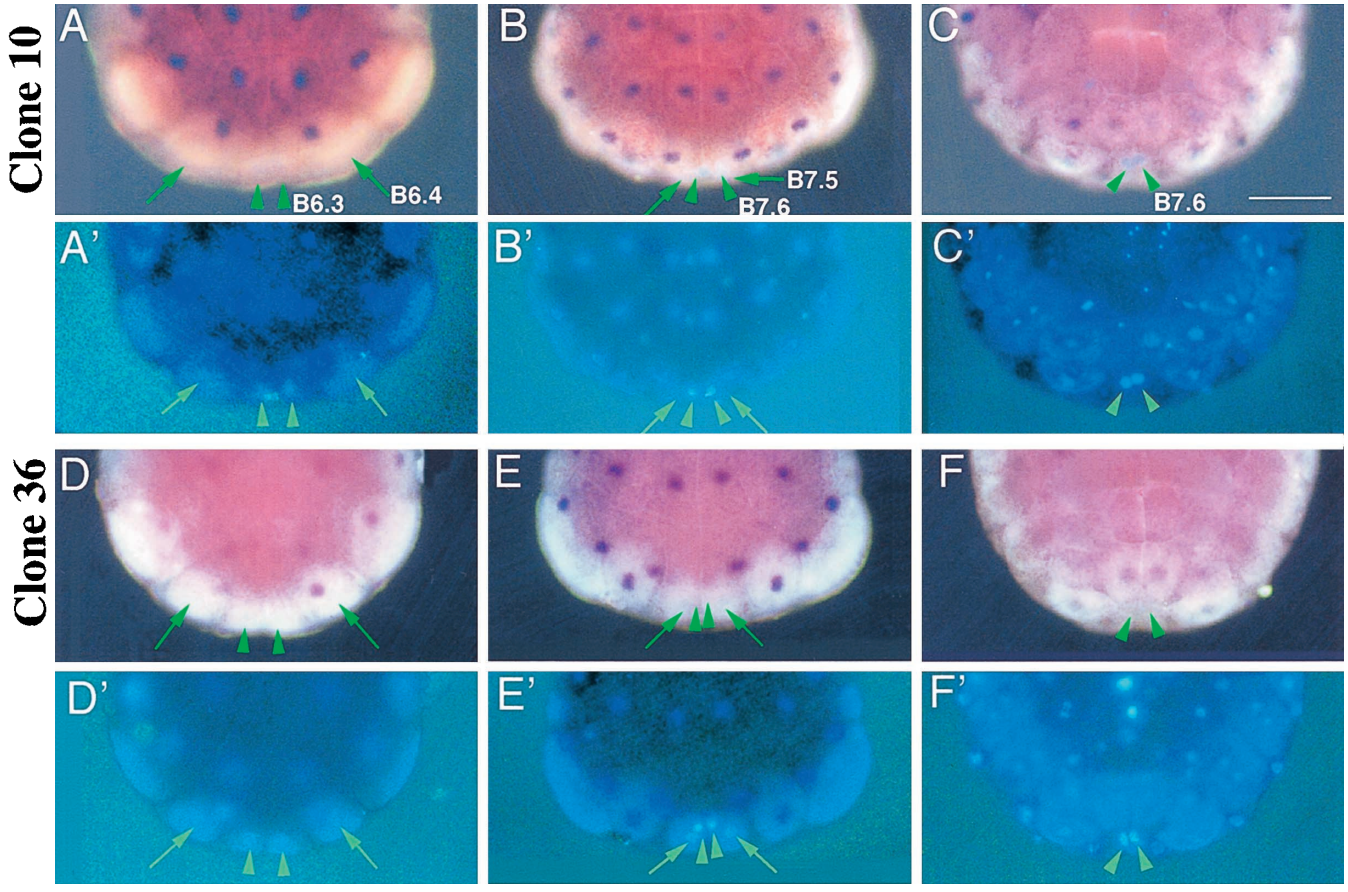


Fig. 3. Expression of clone 10 (A–C) and clone 36 (D–F) in the posterior half of embryos. (A, D) 32-cell stage. (B, E) 64-cell stage. (C, F) 118-cell stage. (A', B', C', D', E', F') The same embryos were stained with nuclear fluorescent dye. The hybridization signals were detected in every blastomere except for the putative germline blastomeres (arrowhead), and their latest sister cells (arrows) at the 32- and 64-cell stages. The stained nuclei overlapping on B6.4 cells in the 32-cell embryos are the nuclei of animal blastomeres, but not the nuclei of B6.4 cells. The nuclei in the B6.2 cells (the cells next to the B6.4 cells in A and D) show faint expression and are out of focus, and can not be seen in these photographs. In B', the nuclei of B7.5 cells are also out of focus. Eventually at the 118-cell stage, B7.6 cells are the only cells that do not express these genes. (A, B, D, E) Animal views. (C, F) Vegetal views. Scale bar, 100 μm.

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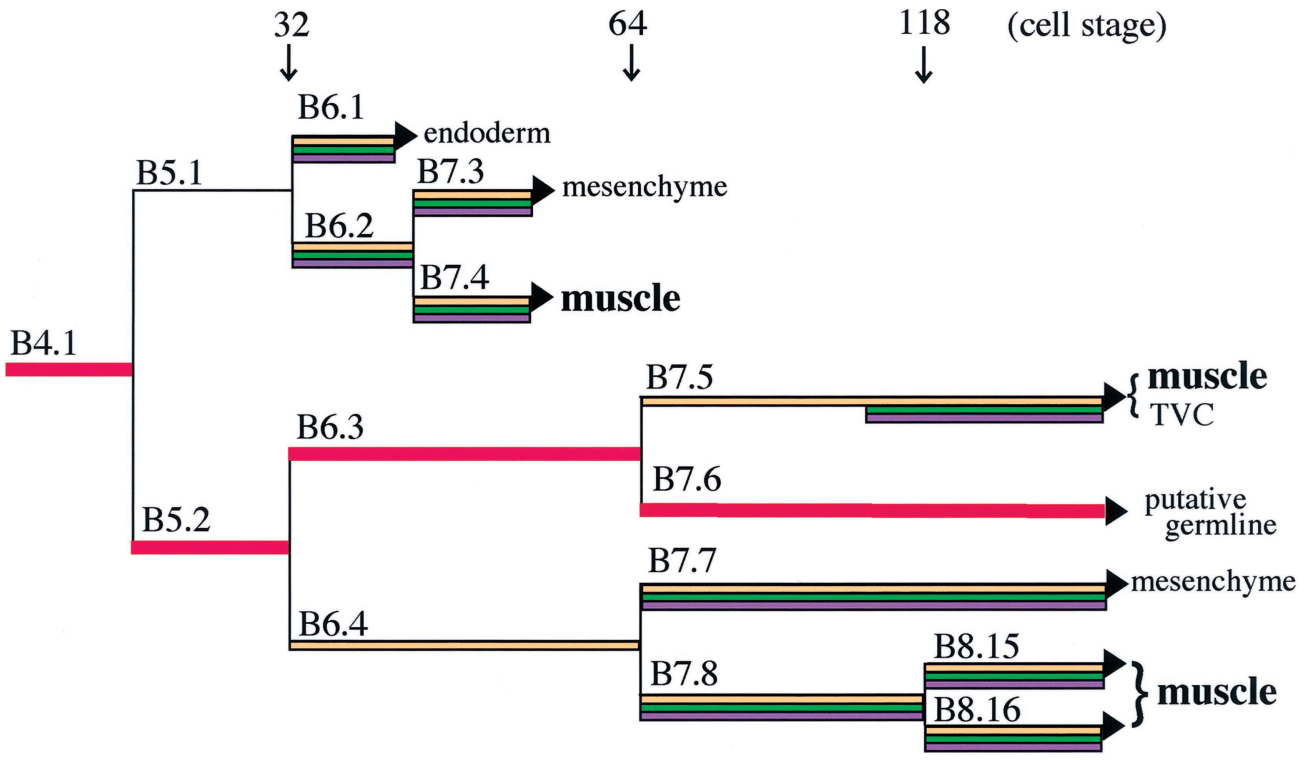


Fig. 4. Temporal expression pattern of three clones: 10, 22, and 36. After the 32-cell stage, expression of clone 22 (orange line) is initiated immediately after the blastomeres are separated from the putative germline (red lines). Initiation of the expression of clones 10 and 36 (green and violet lines, respectively) shows some delay after separation from the putative germline. B7.6 cell is the only cell that never express these genes. TVC, trunk ventral cell.

stages. The posteriormost germ line cells (B7.6) cease to divide during embryogenesis. Eventually at the 118-cell stage, B7.6 cells were the only cells that had never expressed these genes. This expression pattern is similar to that of the muscle-specific genes.

Fig. 4 summarizes temporal sequence of the expression of the clones 22, 10, and 36. The results support the idea that zygotic expression is generally repressed in germline cells (red lines). After the 32-cell stage, the expression of clone 22 was initiated immediately after separation from the germline. Clones 10 and 36 showed an approximately one-cell-cycle delay in initiation of transcription after separation from the germline. This expression pattern is similar to that observed for muscle-specific genes (Fig. 1) except that clones 10 and 36 are also expressed in non-muscle lineages. Therefore, inactivation of transcription occurs in both

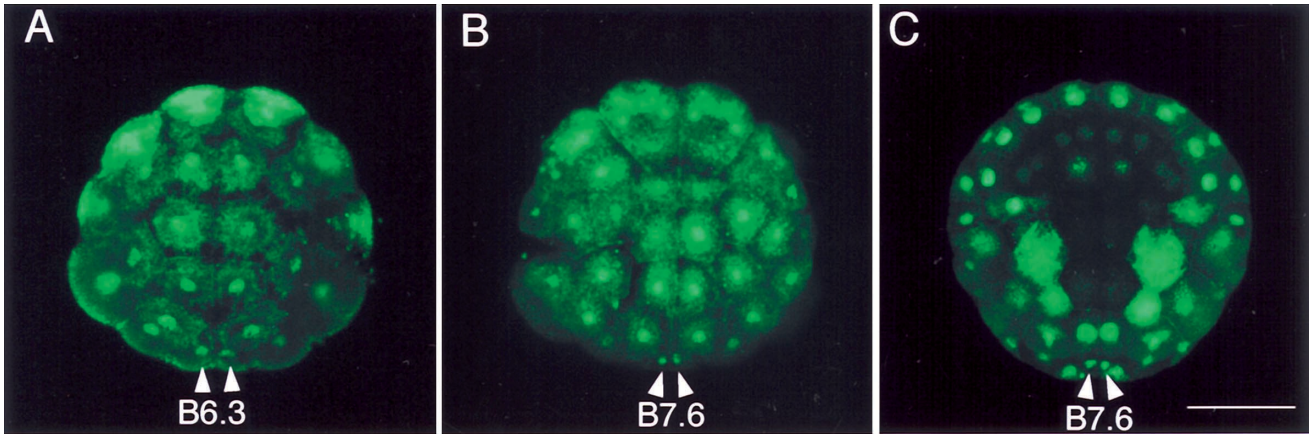


Fig. 5. Immunostaining of phosphoepitope in the CTD of RNAP II with monoclonal antibody H5. (A) 32-cell stage. (B) 64-cell stage. (C) 118-cell stage. Each figure was generated by stacking confocal images of various focal planes to show many nuclei. Staining was detected in every nucleus, including those in the putative germline blastomeres (arrowhead). (A, B) Animal views. (C) Vegetal view. Scale bar, 100 μm.

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of tissue-specific genes and ubiquitously expressed genes. Our results suggest that repression of zygotic gene expression in germline precursors would be a common phenomenon in various kinds of animals, including chordates.

Phosphoepitope of RNAP II is present in all blastomeres including germline blastomeres

The CTD of RNAP II contains tandem repeats of the consensus sequence YSPTSPS, which is conserved among eukaryotes. *Halocynthia* RNAP II also contains the CTD (K. Sawada, personal communication). During transcription, the CTD is phosphorylated on serines at positions 2 and 5, and this phosphorylation is linked with transcriptional initiation and subsequent elongation (Dahmus, 1996). H5 monoclonal antibody recognizes the CTD in which the serine at position 2 is phosphorylated in RNAP II from a wide range of eukaryotes (Warren *et al*., 1992; Bregman *et al*., 1995; Kim *et al*., 1997; Patturajan *et al*., 1998). This antibody has also been used to show the phosphorylation state of RNAP II in the *Drosophila* and *C. elegans* germlines (Seydoux and Dunn, 1997).

To examine whether the serine at position 2 in the CTD is dephosphorylated in the putative germline cells in the ascidian embryos, we immunostained the embryos with H5 antibody. Fig. 5A–C are stacks of confocal images, to show as many nuclei as possible. Staining was detected in the nuclei of all blastomeres including the putative germline blastomeres, the B6.3 cells at the 32-cell stage, and the B7.6 cells at the 64- and 118-cell stages. Equivalent amount of mouse IgM was used as control for H5 antibody, and no staining was observed. Therefore, the inactivation of zygotic gene expression observed in this study in the putative germline cells is not correlated with dephosphorylation of the CTD. This result indicates that the mechanism of repression of zygotic gene expression in ascidian embryos differs from that in *C.elegans* and *Drosophila* embryos. There may be yet unknown genes zygotically expressed in the putative germline cells, but at least the expression of the genes we examined in this study is not controlled by phosphorylation of the CTD of RNAP II. Recently, a similar observation was reported in zebrafish embryos, i.e., that H5 antibody staining simultaneously appears in both the somatic and germline lineages at the 256-cell stage (Knaut *et al*., 2000). This is further evidence that phosphorylation of the CTD occurs irrespective of whether cells are germline or not in chordate embryos.

In *C. elegans*, the maternal pie-1 product is required for both of dephosphorylation of the CTD and repression of zygotic transcription (Mello *et al*., 1996; Seydoux and Dunn, 1997; Tenenhaus *et al*., 1998; Batchelder *et al*., 1999). To analyze the mechanism of repression of zygotic transcription in ascidians, it will be important to examine the functions of molecules that are segregated into the posteriormost germline cells in *Halocynthia* embryos. A number of such localized maternal mRNAs have been identified in a recent large scale screening of maternal mRNAs called the MAGEST project (Makabe *et al*., 2001).

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