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Transcription in Leech: mRNA Synthesis Is Required for Early Cleavages in *Helobdella* Embryos

SHIRLEY T. BISSEN[1] AND DAVID A. WEISBLAT

Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

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Zygotic transcription was analyzed in embryos of the glossiphoniid leech *Helobdella triserialis* by autoradiographic detection of tritiated uridine incorporated in the presence or absence of low concentrations of α-amanitin. RNA synthesis was first detected after the second cleavage and α-amanitin-sensitive RNA synthesis was first detected during the divisions yielding the embryonic stem cells, or *teloblasts*. RNA synthesis increased as development progressed, and the bulk of α-amanitin-sensitive RNA synthesis was found in two classes of cells, the *blast cells*, which are the progeny of the teloblasts, and the micromere-derived cells. The time during which zygotic gene products are required was determined by observing the developmental consequences of α-amanitin exposure. Zygotes microinjected with α-amanitin underwent the first several cleavages with normal timing and symmetry, but underwent aberrant cleavages and produced supernumerary large blastomeres during the time that the control embryos generated teloblasts. Once the teloblasts were formed, the microinjection of α-amanitin did not affect the production of blast cells by the teloblasts, but it did block the divisions and movements of the blast cells and the micromere-derived cells. These data suggest that zygotic transcription is activated during the early cleavages of *Helobdella* embryos and that newly synthesized transcripts are required for the generation of teloblasts. Thus, there is an early, critical period of messenger RNA synthesis essential for teloblast production that is distinct from the later phase of messenger RNA synthesis required for cell divisions and cell movements during gastrulation. © 1991 Academic Press, Inc.

INTRODUCTION

Most early developmental processes are under the control of maternal gene products, but at some point during development there is a transition to control by zygotic gene products. It is important to distinguish between the onset of zygotic transcription and the onset of zygotic control. For example, in sea urchin embryos the onset of zygotic transcription occurs during the early cleavages (Wilt, 1963; Nemer and Infante, 1965), while in frog embryos the major onset of transcription occurs during the blastula stages (Bachvarova and Davidson, 1966; Newport and Kirschner, 1982a). Nonetheless, the transition to zygotic control occurs during later stages of development in both sea urchin and frog embryos because zygotic transcripts are not essential until gastrulation (reviewed by Davidson, 1976). As part of our ongoing investigations into the development of glossiphoniid leeches, we have recently analyzed the onset of zygotic transcription and the transition to zygotic control in their embryos and report on those studies here.

Leech embryos undergo stereotyped cleavages to yield individually identifiable cells, the most prominent of which are the blastomeres called *teloblasts*. Teloblasts are relatively large, yolk-filled stem cells; each cell divides repeatedly to generate a chain of much smaller segmental founder cells, or *blast cells*. The chains of blast cells converge in an ordered manner; the blast cells undergo stereotyped cell divisions and complex morphogenetic movements to generate the segmentally iterated structures of the juvenile leech (Whitman, 1887; Weisblat *et al.*, 1984; Zackson, 1984; Weisblat and Shankland, 1985; Torrence and Stuart, 1986; Shankland, 1987a,b; Bissen and Weisblat, 1987).

By analogy to other organisms, we predicted that the transition to zygotic control would be initiated in the blast cells in stage 7 leech embryos, i.e., mRNA synthesis would be required for their cell divisions and morphogenetic movements. To test this, we monitored the synthesis of RNA in intact embryos by the autoradiographic detection of incorporated tritiated uridine and analyzed the developmental effects of the transcriptional inhibitor α-amanitin. We found that the blast cells do synthesize RNA, as do most of the cells in stage 7 embryos, and that α-amanitin-sensitive RNA synthesis is required for the divisions and subsequent movements of the blast cells and the micromere-derived cells. These are not the first zygotically produced transcripts, however. Embryos synthesize RNA many hours earlier, and α-amanitin-sensitive RNA synthesis during the early cleavages is required for the formation of the teloblasts themselves. These data indicate that early leech em-

[1] To whom correspondence should be directed at Department of Biology, University of Missouri—St. Louis, 8001 Natural Bridge Road, St. Louis, MO 63121-4499.

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bryos are transcriptionally active and that there is an early requirement for mRNA synthesis during the teloblast forming cleavages.

MATERIALS AND METHODS

Embryos

Embryos of the glossiphoniid leech *Helobdella triserialis* were obtained from a laboratory breeding colony and grown at 23°C in a buffered embryo medium (Weisblat *et al.*, 1980; Blair and Weisblat, 1984). Embryos were staged and cells named according to the system of Fernandez (1980) as amended (Stent *et al.*, 1982; Weisblat and Blair, 1984; Bissen and Weisblat, 1989).

Lineage Tracer and α-Amanitin Injections

The procedures for pressure injecting identified blastomeres with lineage tracers or other compounds have been previously described (Weisblat *et al.*, 1984). Bandlets of blast cells were labeled by microinjecting the parental teloblast in early stage 7 embryos with 75 mg/ml rhodamine-dextran-amine (RDA) (Gimlich and Braun, 1985; Molecular Probes), 1% fast green FCF (Sigma), and 0.2 *M* KCl. Embryos of various stages were microinjected with 75 μg/ml α-amanitin (Sigma), 1% fast green, and 0.2 *M* KCl. The α-amanitin was microinjected into any cell and, because of its low molecular weight, it readily passed throughout the entire embryo having global effects. Since the injection volume was about 0.05% of the total volume of the embryo (S.T.B., unpublished observations), the final concentration of α-amanitin was approximately 40 ng/ml (ca. 40 n*M*). This concentration specifically inhibits RNA polymerase II purified from mouse myeloma cell lines (*in vitro* K_i ca. 4 n*M*; Vaisius and Faulstich, 1986). Control embryos were injected with 1% fast green and 0.2 *M* KCl.

Embryos not used in the tritiated uridine incorporation experiments (see below) were allowed to develop for varying periods of time and then were fixed in 3.7% formaldehyde and 100 m*M* Tris-HCl, pH 7.4, for 16 hr at 4°C. After being rinsed with 100 m*M* Tris-HCl, they were stained with 1 μg/ml bisbenzimide (Sigma) for 1 hr. They were either cleared in 70% glycerol or dehydrated in graded alcohols (30–100%) and cleared in methyl salicylate; embryos were viewed in wholemount using epifluorescence microscopy.

Autoradiography

RNA synthesis was monitored by the autoradiographic detection of incorporated tritiated uridine. An aliquot of tritiated uridine 5′-triphosphate ([^{3}H]UTP; 35 Ci/mmole, New England Nuclear) was dried under a stream of N_2 and resuspended at a concentration of 3 m*M* in a solution of 0.2 *M* KCl and 1% fast green. This solution was microinjected into macromeres of embryos, some of which contained RDA-labeled cells. Some embryos had also been microinjected with α-amanitin 30–60 min earlier. The [^{3}H]UTP injection volume was approximately 10 times that of the lineage tracer or α-amanitin injections; the phosphorylated nucleotide readily passed throughout the entire embryo and was thereby diluted several hundredfold. The embryos were incubated for periods ranging from 15 min to 4 hr and then fixed and rinsed as described above. Some embryos were treated with 20 μg/ml RNase A (Sigma) in 500 m*M* NaCl, 1 m*M* EDTA, and 10 m*M* Tris-HCl, pH 8, at 37°C for 1 hr and then rinsed with 100 m*M* Tris-HCl, pH 7.4.

The embryos were stained with bisbenzimide, dehydrated in graded alcohols (30–95%), and embedded in glycol methacrylate (JB-4; Polysciences, Inc.). Serial sections (4–8 μm) were cut and mounted on gelatin-coated slides. The slides were coated with Kodak NTB2 emulsion (diluted 1:1 with distilled water), stored at −70°C (those with stage 7 embryos for 6–8 weeks and those with stage 2–stage 6 embryos for 3–4 months), and developed in 5% Kodak Dektol. The sections were examined using dark-field, differential interference contrast (DIC) and epifluorescence microscopy. Grains were counted using an oil immersion X63 objective (Zeiss).

RESULTS

Summary of Leech Development

After three rounds of cleavage [stages 1–4a; 0–9 hr after egg deposition (AED)] *Helobdella* embryos comprise four micromeres (a′–d′) and four macromeres (A′–D′), the largest of which, cell D′, inherits pools of yolk-free cytoplasm, or *teloplasm*, from the zygote. Subsequently, macromeres A′–C′ each produce two more micromeres, which contribute to nonsegmented structures. Macromere D′, in contrast, undergoes an extensive series of stereotyped cleavages (stages 4b–6c; 9–30 hr AED) to form 15 additional micromeres (Sandig and Dohle, 1988; Bissen and Weisblat, 1989), plus five bilateral pairs of stem cells, the M, N, O/P, O/P, and Q *teloblasts*, whose progeny give rise to all the segmented structures (Whitman, 1887; Weisblat *et al.*, 1984). Each teloblast divides repeatedly to generate *bandlets* of much smaller *primary blast cells* (stages 5–7; 14–78 hr AED). Blast cells are produced at a rate of about one per hour, are arranged in the bandlets according to order of birth, and are designated by the lower case letter corresponding to their parental teloblast. Each m, o, and p bandlet contains one class of blast cell, whereas each n and q bandlet contains two alternating classes of blast cells, i.e., nf and ns; qf and qs (Zackson, 1984; Weisblat and Shankland, 1985). The bandlets merge in a stereotyped manner to form right and left *germinal bands*,

which gradually coalesce along the ventral midline of the embryo (stage 8; 78–122 hr AED) into a structure called the *germinal plate.* Within the germinal bands and germinal plate, each class of blast cell undergoes a characteristic sequence of divisions (stages 7–10; 30–200 hr AED) to give rise to a distinct set of segmental progeny in the juvenile leech (Weisblat and Shankland, 1985).

Transcription in Stage 7 Embryos

In light of previous work in *Xenopus* and *Drosophila* embryos on the mechanisms of transcriptional activation (Newport and Kirschner, 1982a,b; Kimelman *et al.*, 1987; Edgar *et al.*, 1986; Edgar and Schubiger, 1986), we predicted that zygotic transcription would be initiated in the primary blast cells of leech embryos because of their high nuclear to cytoplasmic ratio and their long G2 phases (Bissen and Weisblat, 1989). To determine whether the blast cells synthesized RNA, we used autoradiography to analyze the incorporation of [^{3}H]UTP in intact embryos. Intact embryos were used so that the transcriptionally active cells could be readily identified and so we could be confident of observing normal development. Teloblasts in early stage 7 embryos were microinjected with RDA and allowed to develop for 24–32 hr. The embryos were then microinjected with [^{3}H]UTP, incubated for periods ranging from 15 min to 4 hr, and processed for autoradiography. A total of 12 embryos was examined; a representative section is shown in Figs. 1A and 1B. Silver grains were localized over the nuclei and yolk-free cytoplasm of many cells, indicating that [^{3}H]UTP had been incorporated. The density of grains was about 3–4 times higher over the yolk-free cytoplasm than over the yolk-filled cytoplasm of the cells. Although it was difficult to obtain accurate counts of the nuclear grains because of their high density, there were approximately 20–100 times more grains over the nuclei than over the yolk-filled cytoplasm of the cells.

We are confident that the [^{3}H]UTP was incorporated into RNA because the accumulation of silver grains relative to background was nearly eliminated in embryos that were treated with RNase A after nucleotide exposure (data not shown). There were a few grains over some of the nuclei in the RNase A-treated embryos. It is unlikely that these RNase-insensitive grains marked RNA primers for DNA synthesis or DNA that had incorporated ^{3}H-labeled deoxyribonucleotides synthesized from [^{3}H]UTP, because some cells known to be in S phase (Bissen and Weisblat, 1989) during the period of nucleotide incorporation lacked these grains. Presumably, these residual grains marked double-stranded DNA–RNA duplexes that were insensitive to RNase A. Additional evidence that [^{3}H]UTP was incorporated into RNA is provided by the observation that there was a differential distribution of silver grains depending upon the duration of nucleotide exposure (data not shown). Grains were observed over only the nuclei of cells following a brief (15 min) incubation period, whereas grains were found over both the nuclei and the yolk-free cytoplasm of cells after a longer (4 hr) incubation period, although the density over the nuclei was much greater. These findings suggest that, during the longer incubation period, the newly synthesized RNAs were processed and transported to the cytoplasm. For the experiments presented below, the embryos were incubated for 30–60 min and grains were present over both nuclei and cytoplasm, but were much more abundant over the nuclei (cf., Figs. 1–3). As judged by this incorporation assay, all cells (blast cells, micromere-derived cells, teloblasts, and macromeres) in stage 7 embryos were capable of synthesizing RNA.

Since the embryos contained bandlets of lineage tracer-labeled blast cells, specific classes of blast cells could be readily identified and followed. All classes of primary blast cells synthesized RNA, and they began to transcribe as soon as they were generated. Successive sections through an O/P teloblast and its bandlet of primary blast cells are presented in Figs. 1C and 1D. Each blast cell, including the most recently produced one that lies adjacent to the teloblast (arrowhead in Fig. 1C), had grains over its nucleus and cytoplasm. The density of grains gradually increased throughout the length of the bandlet; the older blast cells (i.e., those furthest from the teloblast) had about 3–4 times more cytoplasmic grains than the younger cells and at least 10 times more nuclear grains. All these blast cells were exposed to [^{3}H]UTP for the same period of time, which suggests that the older cells had increased rates of synthesis and processing and/or decreased rates of degradation. Furthermore, these data show that the blast cells were able to synthesize RNA during the S and G2 phases of their cell cycles; the youngest four to five cells were in S phase and the older cells were in G2 phase during the 30-min nucleotide exposure (Bissen and Weisblat, 1989).

As expected, blast cells did not synthesize RNA during M phase. Mitotic blast cells were identified by their rounded morphology and condensed chromosomes, which were visualized with bisbenzimide staining. An n bandlet in which an nf blast cell (open arrows) was in metaphase at the time of fixation is shown in Figs. 2A and 2B; this cell was nearly devoid of grains. Since the length of M phase of primary n blast cells is about 40 min (S.T.B., unpublished observations) this cell was in M phase for all or nearly all of the 30-min incubation period and incorporated little or no [^{3}H]UTP. Occasionally, cells in mitosis at the time of fixation had a much higher density of grains over their cell bodies than neighboring cells. Presumably, these cells had been in G2 phase during most of the incubation period and incor-

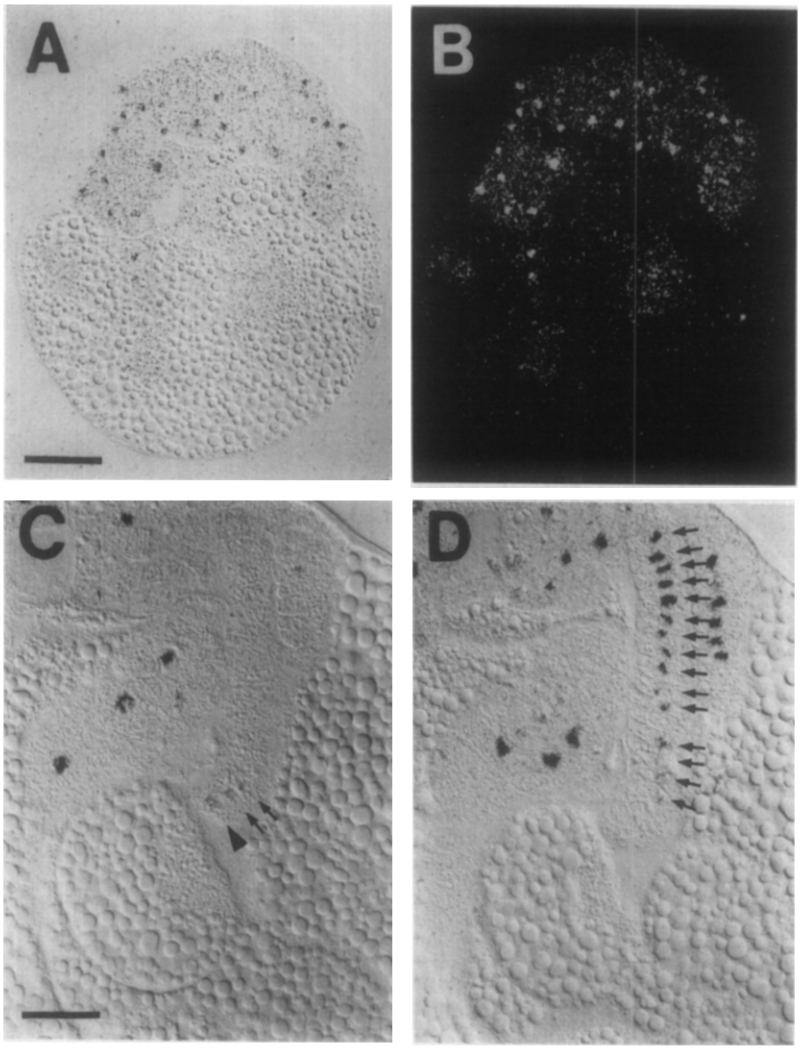

FIG. 1. Transcription in stage 7 embryos. Embryos were injected with [^{3}H]UTP and then fixed, sectioned, and processed for autoradiography after a brief incorporation period. Silver grains appear as black dots or dense clusters under DIC optics and as white dots or clusters under dark-field optics. In A and B the animal pole is up; in C and D anterior is up. (A, B) Corresponding DIC (A) and dark-field (B) photomicrographs of a horizontal 4-μm section showing bands of blast cells in the animal hemisphere; silver grains are localized over the nuclei of the blast cells and the yolk-free areas of all cells. The circular profiles in the lower portion in A are yolk platelets. (C, D) DIC photomicrographs of two successive 8-μm sections through an O/P teloblast and its bandlet of blast cells. There are grains over the nucleus and cell body of each o/p blast cell; three blast cells are visible in C and 15 more blast cells are visible in D (arrowhead indicates most recently produced cell and arrows indicate other cells). The nucleus of the eighth cell from the teloblast was in the next section, which is not shown, and it was covered with silver grains. Scale bar, 100 μm in A and B, 25 μm in C and D.

porated [^{3}H]UTP into newly synthesized RNAs which were then released into the cytoplasm upon dissolution of the nuclear membrane during early M phase.

The progeny of the primary blast cells, the *secondary blast cells*, also synthesized RNA as soon as they were generated and continued until they divided. For example, in the n bandlet shown in Fig. 2, the identities of the primary and secondary blast cells anterior to the mitotic nf blast cell were assigned on the basis of cell size and relative position (Zackson, 1984). Additionally, from our studies of cell cycle composition (Bissen and Weisblat, 1989), we know which phase of the cell cycle each of these identified cells was in during nucleotide exposure (see figure legend for details). There were silver grains over every interphase secondary blast cell, which indicates that the secondary blast cells synthesized RNA during the G1, S, and G2 phases, but not during the M phases of their cell cycles.

The teloblasts in stage 7 embryos also incorporated [^{3}H]UTP. In every teloblast, the density of grains was about three times higher over the yolk-free teloplasm than over the yolk-filled cytoplasm. There were no localized grains over the condensed chromosomes of mitotic teloblasts, but in 69% of the interphase teloblasts (136/198) there was an accumulation of grains over the nuclei (see arrow in Fig. 3A). The presence or absence of nu-

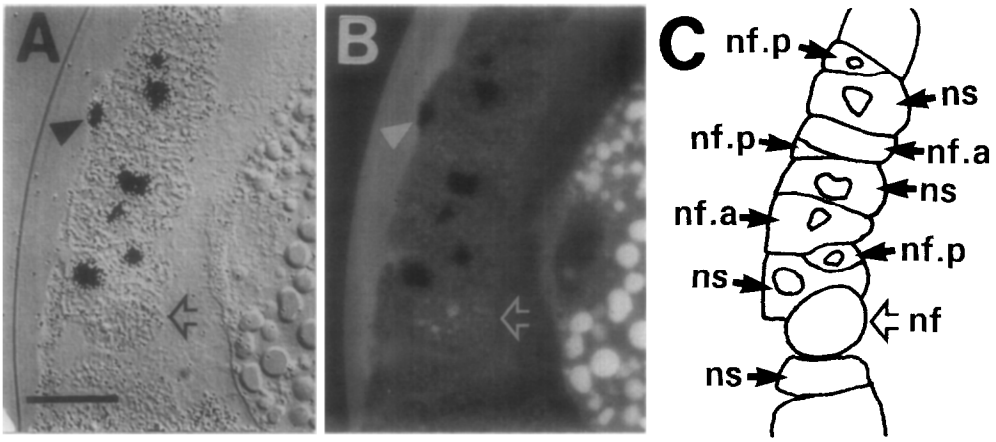

FIG. 2. Transcription in germinal band cells. (A, B) Photomicrographs of a 4-μm section through an n bandlet as viewed with DIC (A) and epifluorescence (B) optics. Anterior is up. Open arrows denote an nf blast cell that was in metaphase at the time of fixation (its condensed chromosomes are visible at a lower focal plane in B). (C) A tracing of the cells in this n bandlet, labeled to indicate their identities. The ns cells adjacent to the mitotic nf cell were in G2 phase, the posteriormost nf.p cell was in G1 phase, and the posteriormost nf.a cell was in S phase during the incorporation period (Bissen and Weisblat, 1989). Silver grains accumulated over the nuclei and cytoplasm of every interphase cell. The nuclei of the next anterior pair of nf.p and nf.a cells were in adjacent sections (not shown), and they had an accumulation of grains. Arrowheads denote the labeled nucleus of a micromere-derived cell in the epithelium that overlies the bands of blast cells. Scale bar, 25 μm.

clear grains in the interphase teloblasts was not lineage-specific, but it did correlate with cell cycle composition. The approximate 30-min interphase period consists of a 10-min S phase and a 20-min G2 phase (Bissen and Weisblat, 1989). Since the proportion of interphase teloblasts that incorporated [^{3}H]UTP was about the same as the proportion of teloblasts expected to be in G2 phase, these findings suggest that teloblasts synthesized RNA during G2 phase, but not during S and M phases.

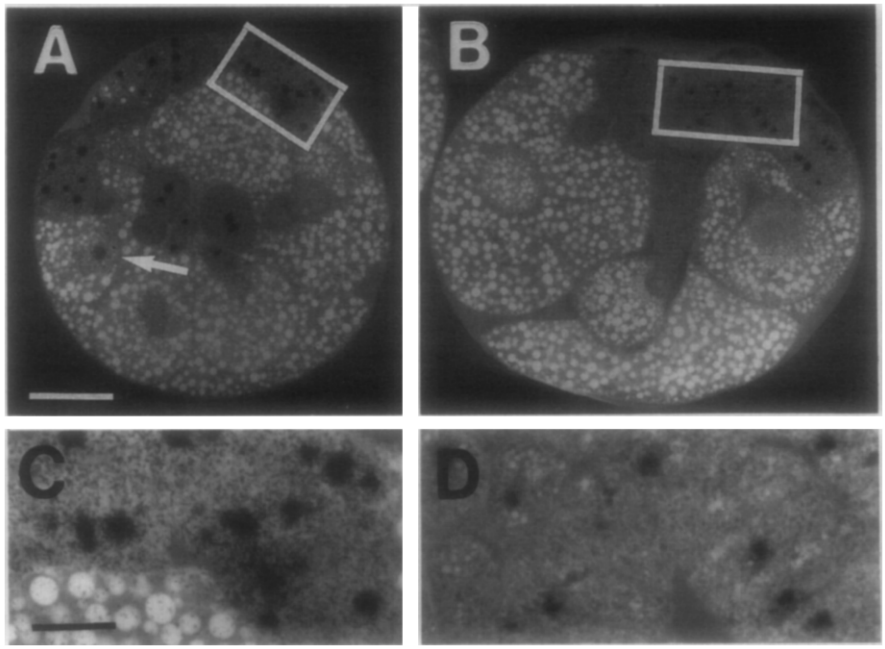

FIG. 3. Transcription is partially inhibited by α-amanitin. Horizontal 4-μm sections, viewed with epifluorescent optics, through stage 7 embryos that were sham injected (A, C) or injected with α-amanitin (B, D) prior to [^{3}H]UTP injection. After fixation, embryos were embedded side by side so that subsequent processing for autoradiography would be carried out under identical conditions. (A, B) Animal pole is up; small bright spots are yolk platelets. The arrow in A points to the labeled nucleus of an O/P teloblast. (C, D) Higher power views of the areas designated in A and B, respectively. There are grains over the cytoplasm and nuclei of many cells under both conditions, but the density of grains is lower in the α-amanitin-injected embryo. Scale bar, 100 μm in A and B; 25 μm in C and D.

The micromere-derived cells and the macromeres were also transcriptionally active in stage 7 embryos. A micromere-derived cell in the provisional epithelium overlying the blast cells is shown in Figs. 2A and 2B (arrowheads). This and other micromere-derived cells had silver grains over their nuclei and cytoplasm, at levels comparable to those observed in the blast cells. Since the lengths and compositions of the cell cycles of the micromeres are not known, we cannot determine whether the transcriptional activity of micromeres resembles that of blast cells (i.e., transcribe during G1, S, and G2) or teloblasts (i.e., transcribe during G2). Most of the macromeres had low, uniform levels of grains over their nuclei, perinuclear cytoplasm, and yolk-filled cytoplasm. In 11% (10/87), however, there were grains concentrated over the nuclei, indicating that they too had synthesized RNA. This transcription occurred during G2 because all the macromeres are in G2 phase throughout stage 7 (Bissen and Weisblat, 1989).

Some of the RNA Synthesized during Stage 7 Is mRNA

Incorporation of [^{3}H]UTP is a general assay that measures the synthesis of all species of RNA. To assess the contribution of mRNA and its precursors, low concentrations of α-amanitin were used to selectively inhibit RNA polymerase II. Stage 7 embryos, some of which contained RDA-labeled blast cells, were injected with α-amanitin and incubated for 30–60 min. They were then injected with [^{3}H]UTP and processed for autoradiography after an incorporation interval of 30–60 min. A total of 18 α-amanitin-treated embryos and 17 control embryos prepared in parallel were examined.

In the blast cells and the micromere-derived cells of the α-amanitin-injected embryos, the density of grains over the yolk-free cytoplasm was reduced 1.5- to 2-fold relative to that in control embryos (Fig. 3). The density of grains over the nuclei was also reduced in the α-amanitin-injected embryos, but the high density of grains prevented an accurate quantification. In contrast, the density of grains over the nuclei and teloplasm of the teloblasts or over the nuclei of the macromeres was not altered by α-amanitin. These findings suggest that mRNA precursors were synthesized by blast cells and micromere-derived cells, but not by teloblasts and macromeres, in amounts detectable by this method.

mRNA Synthesis Is Required for the Divisions of Blast Cells and Micromeres, but Not for the Divisions of Teloblasts

Although the experiments presented above suggested that only the blast cells and micromere-derived cells synthesized mRNAs, it is possible that the teloblasts and macromeres did synthesize some developmentally important but rare transcripts that were not detected by autoradiography. A more sensitive assay for such transcripts would be to analyze the developmental consequences of α-amanitin exposure. Accordingly, N teloblasts in early stage 7 embryos were microinjected with either RDA or a mixture of RDA and α-amanitin. The embryos were fixed after 32 hr of development and the effects of α-amanitin on the divisions of the teloblasts and blast cells were analyzed.

In these experiments, the RDA-labeled N teloblasts divided, even in the α-amanitin-injected embryos, producing bandlets of tracer-labeled primary blast cells. Each RDA-labeled N teloblast in the 12 control embryos produced about one primary blast cell per hour, i.e., 32 blast cells (Fig. 4A). Each RDA-labeled N teloblast in the 12 α-amanitin-injected embryos produced 25 $\pm$ 2 (mean $\pm$ SD) blast cells (Fig. 4B). Thus, although the rate of blast cell production was slightly reduced in the α-amanitin-injected embryos, the teloblasts were able to divide and produce blast cells in the presence of α-amanitin at a concentration that blocked blast cell divisions (see below). This result did not reflect a quantitative difference in the sensitivity of teloblasts and blast cells to α-amanitin because similar results were observed by R. K. Ho and D.A.W. after the injection of 200-fold higher concentrations of α-amanitin (personal communication). Thus, it seems that newly synthesized mRNAs are not required for the blast cell producing divisions of the teloblasts.

As expected, the blast cells in the α-amanitin-injected embryos did not divide. Blast cells normally undergo very stereotyped, reproducible divisions. The nf blast cells, for example, divide asymmetrically about 22 hr, and the ns blast cells divide symmetrically about 28 hr, after they are generated (Zackson, 1984). In the control embryos, the n blast cells underwent their normal divisions, as shown in Fig. 4A. The arrow denotes the division of cell nf, at a distance of about 22 cells from the teloblast (or 22 hr after birth), and the arrowhead denotes the division of cell ns. In the α-amanitin-injected embryos, however, none of the RDA-labeled n blast cells divided, even though the oldest cells could have been at least 32 hr old (Fig. 4B). The divisions of the blast cells, therefore, were blocked by α-amanitin.

Since the blast cells generated in the presence of α-amanitin did not enter the germinal bands (see below), it seemed possible that they were unable to divide because they were not in the correct position in the embryo, even though they were of the proper age. To determine whether blast cells in the germinal bands could divide in the presence of α-amanitin, N or O/P teloblasts in early stage 7 embryos were microinjected with RDA and allowed to develop for 24 hr so that the RDA-labeled (n, o, and p) blast cells were beginning to undergo their first divisions in the germinal bands. At that time, 18 embryos were microinjected with α-amanitin and 11

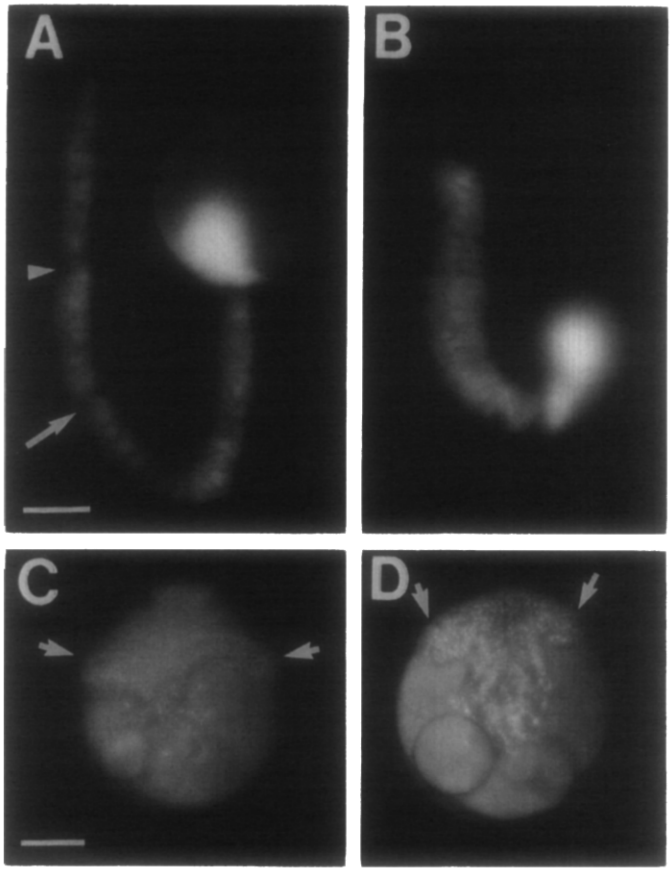

FIG. 4. Blast cells are produced but do not divide in the presence of α-amanitin. (A, B) Fluorescence micrographs of RDA-labeled N teloblasts and their bandlets of n blast cells; N teloblasts were injected with RDA (A) or a mixture of RDA and α-amanitin (B) and the embryos were fixed 32 hr later. In A, the arrow indicates the first division of cell nf and the arrowhead indicates the first division of cell ns. No blast cells in B have divided; all the nuclei are the same size. (C, D) Bisbenzimide staining of whole mounted embryos that were allowed to develop 32 hr after sham (C) or α-amanitin (D) injections; animal pole is up. In the embryo shown in C, the bands of blast cells (arrows) have moved apart and lie near the equator (the anterior ends of the bands are beginning to coalesce on the other side of the embryo), the micromere-derived cells at the animal pole have expanded, and there are *few* blast cells in the bandlets between the teloblasts and the germinal bands. In the embryo featured in D, however, the germinal bands (arrows) have not moved apart (they lie at the top of the embryo), the micromere-derived cells have not expanded, and there are *many* blast cells in the bandlets between the teloblasts and the germinal bands. Scale bar, 50 μm in A and B; 150 μm in C and D.

were sham injected; the embryos were fixed after 0, 4, 8, or 24 hr of incubation.

During the first 4 hr, the RDA-labeled blast cells in the experimental embryos continued to divide, presumably because enough of the transcripts needed for cell division had already been synthesized in those cells at the time of the α-amanitin injection. With longer periods of incubation, however, there were no further blast cell divisions in the germinal bands of the α-amanitin-injected embryos (data not shown). Thus, α-amanitin blocked the divisions of the blast cells regardless of their position in the embryo. Using bisbenzimide staining to reveal chromatin morphology, we found a virtually complete disappearance of mitotic figures in the blast cells and micromere derivatives of embryos exposed to α-amanitin for longer than 4 hr, again supporting the notion that, in these cells, mRNA synthesis was necessary for their subsequent divisions. But, as described previously, the teloblasts in the α-amanitin-injected embryos continued to produce blast cells, and long chains of undivided blast cells accumulated between the teloblasts and the germinal bands.

α-Amanitin affected the morphogenetic movements as well as the divisions of the blast cells and micromere derivatives. When stage 7 embryos were injected with α-amanitin, the germinal bands did not lengthen and move over the surface of the embryos, and the micromere-derived cells lying between the germinal bands did not expand (see arrows in Figs. 4C and 4D). Additionally, the bandlets of blast cells in the experimental embryos did not coalesce into or enter the existing germinal bands. Normally, as more blast cells are produced, the bandlets continue to merge into the germinal bands so that only about 6 to 8 blast cells lie between the parental teloblasts and the germinal bands (Fig. 4C). In the α-amanitin-injected embryos, however, there were about 18 to 22 blast cells in the unmerged bandlets between the teloblasts and the germinal bands (Fig. 4D). Thus, even though the teloblasts continued to produce blast cells in the presence of α-amanitin, the bandlets of blast cells did not merge and become organized into parallel arrays on the surface of the embryo.

Synthesis of mRNA Is Required during Teloblast Formation

If the α-amanitin-sensitive RNAs produced by the blast cells and micromeres in stage 7 embryos were the first zygotically produced mRNAs, then α-amanitin should have no effect on the development of embryos at earlier stages. We found, however, that α-amanitin perturbed the formation of teloblasts. When zygotes were microinjected with α-amanitin, the first several divisions proceeded with normal timing and symmetry (Fig. 5A), which suggests that maternal mRNAs sufficed for the first several cell cycles. During early stage 5, however, the D′-derived cells (NOPQ proteloblasts and M teloblasts) in the α-amanitin-injected embryos underwent abnormal cleavages and generated extra blastomeres (Fig. 5B). These progeny cells continued to cleave abnormally, yielding supernumerary yolk-filled blastomeres during the time the control embryos generated the normal complement of teloblasts and micromeres (Fig. 5C). Thus, zygotically produced transcripts are required during the teloblast forming cleavages.

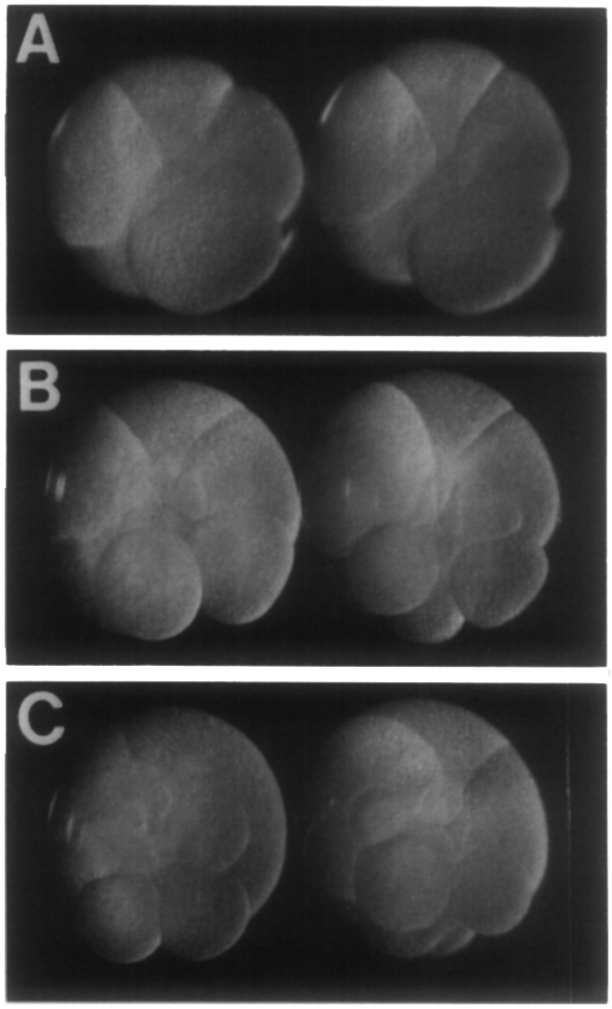

FIG. 5. α-Amanitin perturbs cleavages during teloblast formation. Animal views of live embryos; control embryos are at the left and α-amanitin-injected embryos are at the right in each panel. (A) Stage 4b; cell DNOPQ is positioned near 5 o'clock and cell DM lies more vegetally and is positioned near 7 o'clock. The α-amanitin-injected embryo is indistinguishable from the control. (B) The control embryo is at early stage 5; cell DNOPQ has yielded $NOPQ_l$ and $NOPQ_r$ and cell DM has cleaved into two M teloblasts, which are not visible. The D′-derived cells in the α-amanitin-treated embryo have undergone abnormal cleavages. (C) The control embryo is at stage 6a; the NOPQ cells have generated N teloblasts and OPQ proteloblasts, which are the larger two cells. The D′-derived cells in the α-amanitin-injected embryo have undergone additional, aberrant cleavages. The embryos are approximately 500 μm in diameter.

These same cleavage alterations were observed when α-amanitin was injected at any time prior to stage 5. Although the D′-derived cells of most of the experimental embryos began to undergo abnormal cleavages when their control siblings were at early stage 5, the onset varied somewhat among batches of embryos: 13% (13/102) deviated visibly from normal development during the 2 hr just prior to the beginning of stage 5 in sibling controls, 77% (79/102) deviated within the first 2 hr of sibling stage 5, and 10% (10/102) during the period 2–4 hr after the onset of sibling stage 5. When early stage 5 embryos were injected with α-amanitin, the D′-derived cells underwent aberrant cleavages within 1–2 hr. The supernumerary cells were randomly positioned throughout the embryo and, upon cessation of the abnormal cleavages, these embryos appeared to develop no further, i.e., no visible germinal bands were formed, and they died after 5–6 days. These findings suggest that zygotic transcripts were synthesized during and required for the teloblast forming cleavages of the D′-derived cells of *Helobdella* embryos.

Further Analysis of Transcription in Cleavage Stage Embryos

Since the α-amanitin studies suggested that mRNA synthesis was initiated much earlier than stage 7, we sought to determine which cells of cleavage stage embryos incorporated [^{3}H]UTP and whether the incorporation was sensitive to low concentrations of α-amanitin. Embryos from stage 2 to stage 6 (46 sham-injected and 45 α-amanitin-injected) were microinjected with [^{3}H]UTP, incubated for 30–60 min, and processed for autoradiography.

The youngest embryos in which grains were selectively localized over the nuclei and yolk-free cytoplasm were stage 3 (4-cell) embryos (data not shown). In every cell, there were about 3–4 times more grains over the yolk-free cytoplasm than over the yolk-filled cytoplasm and about 10–20 times more grains over the nuclei than over the yolk-filled cytoplasm. Neither the localization nor the density of grains was affected by α-amanitin. These data suggest that zygotic transcription was initiated by at least the 4-cell stage and that, since it was not reduced by low concentrations of α-amanitin, it was carried out by pol I and/or pol III.

A similar pattern of RNA synthesis was observed in all stage 3–stage 6 embryos. Although not every cell in a given embryo had localized grains, all large A-, B-, C- and D-derived cells, as well as the micromeres and their derivatives, were capable of synthesizing RNA. There were about three to four times more grains over the teloplasm than over the yolk-filled cytoplasm of the large D-derived cells. In the 41 control embryos (stage 3–stage 6) there were 158 large D-derived cells; 46 were in M phase and 112 were in interphase. None of the M phase cells had grains localized over the mitotic apparatus, but about 60% (66/112) of those in interphase had an accumulation of grains over their nuclei, which suggests that these cells transcribed in a cell cycle-dependent manner. Since G2 phase comprises about 70% of the interphase period of these cells (Bissen and Weisblat, 1989), it appears that the large D-derived cells synthesized RNA only during the G2 phases. Some of the large A-, B-, and C-derived cells had about three to four

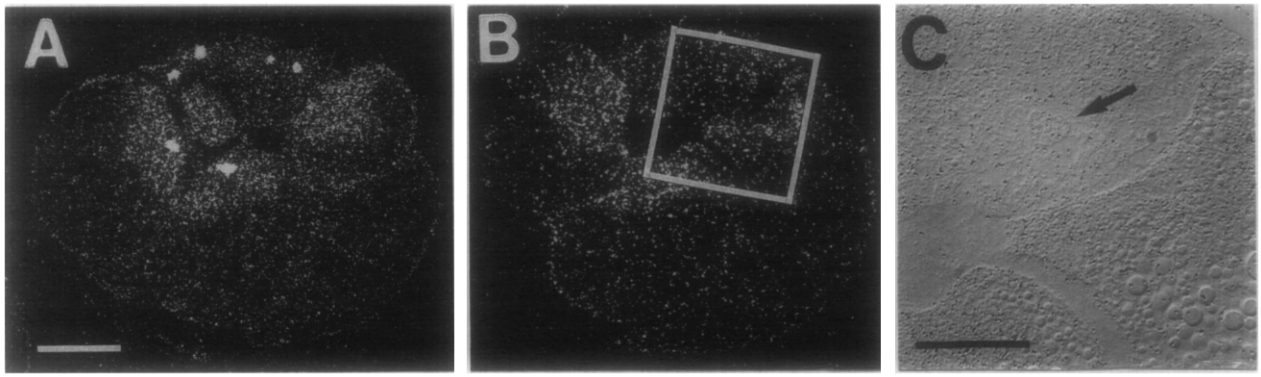

FIG. 6. α-Amanitin-sensitive transcription in stage 5 embryos. (A, B) Dark-field photomicrographs of 4-μm sections through embryos that had been injected with KCl (A) or α-amanitin (B), and then injected with [^{3}H]UTP and processed for autoradiography. Animal pole is up. In A, grains are localized over the nuclei of cells NOPQ$_1$ and M$_r$, as well as several micromeres near the animal pole. None of the cells in the embryo presented in B has an accumulation of grains over the nucleus. (C) Higher power view of the area designated in B; the nucleus of a micromere is visible with DIC optics (arrow) and there are no localized grains. Scale bar, 100 μm in A and B; 50 μm in C.

times more grains over the yolk-free perinuclear cytoplasm than over the yolk-filled areas, and this cytoplasmic labeling appeared to be stage-dependent: about 80% of these cells were labeled in stage 3–stage 4 embryos but only about 20% of these cells were labeled in stage 5–stage 6 embryos. (As noted above, none of these cells in stage 7 embryos had grains concentrated over the perinuclear cytoplasm.) About 35% (40/116) of the large A-, B-, and C-derived cells had abundant nuclear grains. Since these cells cease dividing and enter prolonged G2 phases during stage 5, we are unable to determine in this population of embryos whether these cells transcribed only during G2. (As noted above, only 11% of these cells in stage 7 embryos had localized nuclear grains.) The micromeres and their progeny also had a preponderance of grains over their nuclei and about four times more grains over their cell bodies than over the yolk-filled areas of the embryos.

Sensitivity to α-amanitin was first detected in stage 5 embryos. The density of grains over the yolk-free cytoplasm in the α-amanitin-injected embryos was not noticeably reduced, but the density of grains over the nuclei of the large D′-derived cells and the micromeres was drastically reduced (Figs. 6A and 6B). There was no accumulation of grains above background levels over the nuclei of any cell in the α-amanitin-injected embryos (Figs. 6B and 6C). These findings complement and extend those presented earlier and suggest that pre-mRNA synthesis was initiated not only in the proteloblasts and teloblasts but also in the micromeres of stage 5 embryos.

The proteloblasts and teloblasts appear to partake in α-amanitin-sensitive RNA synthesis for a limited period of time, i.e., only during stage 5 and early stage 6. RNA synthesis in the proteloblasts, teloblasts, and micromeres of early stage 6 embryos was partially inhibited by α-amanitin, whereas only RNA synthesis in the micromeres (and blast cells) of late stage 6 embryos was affected by α-amanitin. The teloblasts of late stage 6 embryos, therefore, resemble those of stage 7 embryos in their insensitivity to α-amanitin. These results are in accord with our earlier observations of the developmental consequences of α-amanitin injection, i.e., teloblast formation (stages 5–6) was perturbed by α-amanitin, but once the teloblasts were formed (stage 7) they were able to generate blast cells in the presence of α-amanitin.

DISCUSSION

A Comparative Analysis of the Onset of Zygotic Transcription

Through the autoradiographic detection of [^{3}H]UTP incorporated in the presence or absence of α-amanitin we have determined that general RNA synthesis is initiated by the 4-cell stage and α-amanitin sensitive RNA synthesis is initiated by stage 5 (~25 cells) in *Helobdella* embryos. Further support for this conclusion can be drawn from the observation that the transcription of a specific gene (htr-wnt-1) has been demonstrated in stage 4c *Helobdella* embryos (R. G. Kostriken and D.A.W., in preparation). Zygotic transcription is activated during the first several cell cycles in numerous other embryos. For example, mRNA precursors are synthesized at high rates during the early cleavages of sea urchin, clam, snail, ascidian, mouse, and nematode embryos (Wilt, 1963; Nemer and Infante, 1965; Kidder, 1976; Meedel and Whittaker, 1978; Knowland and Graham, 1972; Clegg and Piko, 1983; Cleavinger *et al.*, 1989; Schauer and Wood, 1990).

Although RNAs are synthesized in *Helobdella* embryos during the early cleavages, the synthesis of total RNA and pre-mRNA (as judged its α-amanitin sensitivity) increases markedly in the blast cells and micromere

derivatives during stage 7 (~100–1000 cells). This increase in transcription is analogous to the global activation of transcription after the 12th cleavage division (~4000 cells) in *Xenopus* embryos (Bachvarova and Davidson, 1966; Newport and Kirschner, 1982a) and after the 13th mitotic cycle (~8000 cells) in *Drosophila* embryos (Zalokar, 1976; Anderson and Lengyel, 1979; Edgar *et al.*, 1986). But, in *Xenopus* and *Drosophila*, as reported here in *Helobdella*, transcription is initiated, at low levels, earlier in development. Low levels of transcription have been detected by at least the 6th or 7th division in *Xenopus* embryos (Brown and Littna, 1964; Nakakura *et al.*, 1987; Kimelman *et al.*, 1987) and the 9th or 10th mitotic cycle in *Drosophila* embryos (McKight and Miller, 1976; Anderson and Lengyel, 1980; Weir and Kornberg, 1985; Karr *et al.*, 1989). It appears, therefore, that *Helobdella* embryos are similar to *Xenopus* and *Drosophila* embryos in that transcription is activated at low levels during early development and the levels of transcription increase markedly at a later stage in development.

Helobdella Embryos Have an Early Onset of Zygotic Control

We have identified an early, critical period of mRNA synthesis that is required for the formation of the leech embryonic stem cells, or teloblasts. Following the inhibition of mRNA synthesis by low concentrations of α-amanitin, the timing and/or symmetry of the divisions of the D′-derived cells were altered during teloblast formation (stages 4c–6c). Normally during this time, the large D′-derived cells, DNOPQ and DM, generate five bilateral pairs of teloblasts and 15 micromeres, but in embryos derived from zygotes injected with α-amanitin, the cleavages of these cells were perturbed and supernumerary large yolk-filled cells were generated. Since similar effects were obtained when α-amanitin was injected as late as stage 5, we conclude that the essential transcripts were produced during the teloblast forming cleavages of the D′ derivatives, in accord with our observation that α-amanitin-sensitive [^{3}H]UTP incorporation was detected in these cells at these stages.

Even though mRNA synthesis is required for the formation of the teloblasts in *Helobdella* embryos, the transition to zygotic control is most likely not complete at that time. Presumably, both maternal and zygotic messages are translated during that early period and the shift to complete zygotic control occurs later in development. Our data, however, clearly indicate that zygotically produced transcripts are essential for the early teloblast forming cleavages.

This early requirement for zygotically produced mRNAs differs markedly from what has been observed in most other organisms. Studies of the onset of zygotic genome utilization indicate that, for most embryos, zygotically produced transcripts are not essential until the time of gastrulation. The early developmental processes of cleavage and blastulation appear to rely mainly on maternally supplied gene products (reviewed by Davidson, 1976). For example, the inhibition of transcription by α-amanitin blocks gastrulation in *Xenopus* embryos (Newport and Kirschner, 1982a) and blastoderm cell formation in *Drosophila* embryos (Arking and Parente, 1980; Gutzeit, 1980; Edgar *et al.*, 1986). Mouse embryos differ, however, in that the transition to zygotic control occurs during the first several cell cycles; zygotic mRNAs are required for the early cleavages and morulation in mouse embryos (reviewed by Johnson, 1981).

What do leech and mouse have in common? One thing is that both leech and mouse embryos develop more slowly than other commonly studied embryos, such as, sea urchin, frog, and fruit fly. Thus, it is possible that absolute time as well as developmental time plays an important role in determining when zygotic gene products are required. At the time when leech and mouse embryos require zygotic mRNAs for their early cleavages (15 hr and ~48 hr from the onset of development, respectively), most other embryos have already advanced to the gastrula stage or beyond. In another slowly developing embryo, the nematode *Ascaris*, mRNA synthesis is detected in 4- to 8-cell embryos, at which time the embryos are about 3–4 days old (Cleavinger *et al.*, 1989), but it is unknown at what stage during development these zygotic transcripts are needed.

Transcriptional Activation and Cell Cycle Composition

The mechanism of the large increase in zygotic transcription prior to gastrulation has been investigated in *Xenopus* and *Drosophila*. A key observation was that this transcriptional activation is tied to the increasing nucleo-cytoplasmic ratio in *Xenopus* (Newport and Kirschner, 1982b) and in *Drosophila* (Edgar *et al.*, 1986). Subsequently, it was shown that the nucleo-cytoplasmic ratio acts indirectly by increasing the duration of the cell cycle, an effect that can be mimicked by cycloheximide treatment in both *Drosophila* (Edgar and Schubiger, 1986) and *Xenopus* (Kimelman *et al.*, 1987). Thus, the lengthening of the cell cycle appears to be a more immediate event regulating the onset of transcription in *Xenopus* and *Drosophila* embryos; the machinery of the very rapid early cell cycles may preclude transcription (Kimelman *et al.*, 1987).

The length of the cell cycle per se cannot be the sole factor in activating transcription, however. The early cell cycles of sea urchin, clam, snail, and nematode embryos are relatively rapid and yet RNA is synthesized at high rates during these early cell cycles (Wilt, 1963; Nemer and Infante, 1965; Kidder, 1976; Schauer and

Wood, 1990). One major difference between embryos in which transcription is initiated at high rates during the early cleavages and those in which the major onset of transcription occurs later in development is the presence of G2 phases in the early cell cycles. The early cell cycles of sea urchin, mouse, and snail embryos comprise M, S, and G2 phases (Hinegardner *et al.*, 1964; Dalq and Pasteels, 1955; van den Biggelaar, 1971), whereas the early cycles of frog and fruit fly embryos comprise only S and M phases (Graham and Morgan, 1966; Foe and Alberts, 1983). Thus, it appears that not only length but also composition of the cell cycle is important for transcription of the zygotic genome. In accord with this view, the elongation of the cell cycles associated with the major onset of transcription in frog and fruit fly embryos is achieved largely by the acquisition of G2 phases (Graham and Morgan, 1966; Foe and Alberts, 1983; Edgar *et al.*, 1986). It has been suggested that transcription occurs only during G phases during embryogenesis; the rationale being that chromosome condensation during M phase is incompatible with transcription and that DNA replication during S phase of the early cell cycles may prevent the formation of stable transcription units. The observation that there are two bursts of transcription during the second cell cycle of mouse embryos, i.e., one preceding and the other following S phase (Bolton *et al.*, 1984), has been taken as support for this notion.

Overall, our results with leech embryos are in general agreement with those summarized above. But our examination of transcription at various stages of development, in identified cells for which we had already described the cell cycle composition (Bissen and Weisblat, 1989), revealed two systematic exceptions to the generalization that being in G phase is a necessary and sufficient condition for transcription to occur in a normal embryo.

First, even though all cell cycles in *Helobdella* comprise prominent G2 phases (Bissen and Weisblat, 1989), zygotic transcription was not detected until the 4-cell stage. The failure of these early blastomeres to transcribe DNA cannot be ascribed to the brevity of the first two cell cycles, since they are in fact longer than later cell cycles in the D′ cell line in which transcription (including α-amanitin-sensitive transcription) is observed. Thus, there seems to be an early block to transcription in *Helobdella* that operates independently of cell cycle length and composition.

Second, although it appears that only G2 phase blastomeres in stage 3–stage 7 embryos can transcribe, blast cells in stage 7–stage 8 embryos clearly *do* synthesize RNA during S phase as well as during both G phases. The appearance of transcription during S phase is correlated with increases in the length of S phase; S phase increases from an average of 15 ± 4 min in the early blastomeres and teloblasts, which do not transcribe during S phase, to an average of 3.3 ± 1.4 hr in the primary blast cells and their progeny, which do transcribe. Thus, the S phase block to transcription during development is not absolute. Our results are consistent with the notion that rapid replication during early cell cycles is incompatible with transcription. Presumably under such conditions all possible transcription sites are occupied by replication machinery.

What Are the Early Transcripts That Are Needed for Teloblast Production?

The requirement for mRNA synthesis during the early cleavages is transient and appears to be more for teloblast *production* than for teloblast *function*. Once the teloblasts have been generated (stage 7), they are able to produce bandlets of blast cells in the presence of α-amanitin, which suggests that they undergo their typical unequal divisions without mRNA synthesis. The essential transcripts that are produced during teloblast formation could be involved in specifying the identity of the individual teloblasts; for example, zygotically produced determinants could be selectively partitioned into the mesoteloblasts or the ectoteloblasts. Alternatively, the requisite transcripts could be involved in shifting the mode of cleavage from the predominately equal cleavages characteristic of the early embryo to the highly unequal stem cell divisions characteristic of the teloblasts. This latter possibility is supported by the observation of supernumerary large yolk-filled cells after the injection of α-amanitin. The supernumerary cells could result from changes in the orientation of cleavage planes during the divisions of the D′-derived cells so that they divide equally instead of producing micromeres. Additionally, α-amanitin could alter the length of the cell cycles such that the large D′-derived cells cleave more frequently.

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