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Early transcription in *Caenorhabditis elegans* embryos

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SUMMARY

We have analysed early transcription in devitellinized, cultured embryos of the nematode *Caenorhabditis elegans* by two methods: measurement of [^{32}P]UTP uptake into TCA-precipitable material and autoradiographic detection of [^{3}H]UTP labelling both in the presence and absence of α-amanitin. RNA synthesis was first detected at the 8- to 12-cell stage, and α-amanitin sensitivity also appeared at this time, during the cleavages establishing the major founder cell lineages. The requirements for maternally supplied versus embryonically produced gene products in early embryogenesis were examined in the same culture system by observing the effects of α-amanitin on cell division and the early stereotyped lineage patterns. In the presence of high levels of α-amanitin added at varying times from two cells onward, cell division continued until approximately the 100-cell stage and then stopped during a single round of cell division. The characteristic unequal early cleavages, orientation of cleavage planes and lineage-specific timing of early divisions were unaffected by α-amanitin in embryos up to 87 cells. These results indicate that embryonic transcription starts well before gastrulation in *C. elegans* embryos, but that although embryonic transcripts may have important early functions, maternal products can support at least the mechanics of the first 6 to 7 cell cycles.

Key words: autoradiography, α-amanitin, maternal effects, transcription, *Caenorhabditis*

INTRODUCTION

The stage at which embryonic transcription begins varies widely among embryos of different organisms, ranging from the 2-cell embryo in the mouse to several thousand cells in *Xenopus* (reviewed in Davidson, 1986). The relative importance of maternally supplied and embryonically produced gene products in the early events of establishing embryonic axes, patterning of cleavages and determining of cell fates likewise seems to vary between different embryos. In *Drosophila*, for example, initial asymmetries and regional specification in the embryo are established under maternal instruction, but genes important in determining cell fate and refining the pattern for subsequent morphogenesis are among the earliest to be embryonically expressed, transcribed in localized patterns one to two cell cycles before high levels of general transcription are attained following cellularization of the blastoderm (Edgar and Schubiger, 1986; Ingham et al., 1985; Karr et al., 1985; Knipple et al., 1985; Weir and Kornberg, 1985). In embryos of another invertebrate, the leech *Helobdella triserialis*, embryonic transcription is detected considerably earlier, at 5 cells for RNA polymerases I and III, and at about 25 cells for α-amanitin-sensitive transcription by RNA polymerase II. The early process of teloblast formation at stage 5 is affected by drug treatment, but subsequent blast cell divisions occur, suggesting that some early steps in differentiation require embryonically produced gene products even as other processes involve only maternally supplied components until a more general transition at stage 7, corresponding to a mid-blastula transition (Bissen and Weisblat, 1991). We report here an analysis of the onset of embryonic transcription and some aspects of the transition from sufficiency of maternally supplied gene products to dependency on embryonic transcription in early embryos of the nematode *Caenorhabditis elegans*.

Early embryogenesis in *C. elegans* follows an invariant pattern in which several unequal and asynchronous cleavages produce individually recognizable founder cells for the major somatic lineages (Deppe et al., 1978; Sulston et al., 1983). By the 30-cell stage, about 90 minutes after fertilization at 20°C, all five somatic lineages and the germ-line lineage have been established, and gastrulation begins. An ensuing period of about 4 hours includes gastrulation, continuing cell division and the early stages of organogenesis and morphogenesis. Cell division is essentially completed during this period and, in the final 7.5 hours before hatching, the embryo elongates, secretes a cuticle and completes morphogenesis (for review see Wood, 1988).

The early determination of several lineages appears to depend on segregated maternal products (Laufer et al., 1980; Strome and Wood, 1983; Cowan and McIntosh, 1985; Kemphues et al., 1988; Bowerman et al., 1993). Genetic evidence, in the form of an abundance of maternal-effect embryonic lethal mutations, indicates that much of early embryogenesis is controlled by maternal information (Kemphues et al., 1988; Kemphues, 1989). A recent study of the defective phenotypes of embryos homozygous for chromosomal deficiencies totalling about half the genome also suggests that the patterning of early cleavages is dictated almost exclusively by maternal gene products (Storfer-Glazer and Wood, 1994).

However, this deficiency analysis also indicates that successful morphogenesis requires the activity of many genes transcribed in the embryo. At least some genes associated with differentiation functions are expressed very early during embryogenesis. For example, the MyoD homolog *hlh-1* is expressed in four muscle precursor cells at the 28-cell stage (Krause et al., 1990), and the appearance of gut granules, an early lineage-specific marker related to tryptophan degradation, can be blocked by α-amanitin treatment of 16-cell embryos, only one cell cycle after birth of the E cell, which is the clonal progenitor of the gut (Edgar and McGhee, 1986; 1988).

The time of onset for embryonic transcription in *C. elegans* has previously been assessed indirectly by two different methods, with somewhat differing results. In situ hybridization with labelled oligo(dT) probes to detect poly(A)$^+$ mRNAs showed the first detectable increase in nuclear labelling at approximately 80-100 cells (Hecht et al., 1981). Subsequently, a more sensitive assay using run-on transcription assays in vitro showed that extracts from staged early embryos of less than 30 cells produced a high level of incorporation into mRNAs, an estimated 20 to 30 of which are expressed at high levels only during this period (Schauer and Wood, 1990). However, these experiments provided only an approximation of the time of transcription onset.

We have carried out in vivo assays of early transcription to define more precisely when it begins. Utilizing methods for digesting the chitinous eggshell of *C. elegans*, mechanically stripping off the inner vitelline membrane and culturing the resulting permeabilized embryos, we have followed early transcription directly as incorporation of radioactively labeled nucleotides into RNA, detected by acid precipitation and scintillation counting or by autoradiography. The same culture system has been used to determine how far embryos will develop when α-amanitin is used to inhibit RNA polymerase II, and to ask whether pregastrulation cleavages are affected by inhibition of RNA polymerase II-mediated transcription. While these methods necessarily limit experiments to small numbers of embryos, statistical analysis of our results clearly shows that embryonic transcription begins before the 16-cell stage. The results of inhibiting RNA polymerase II transcription with α-amanitin indicate that, up to about 100-cell stage, maternally supplied gene products are sufficient to support cell division, with normal patterning and timing of cleavages. However, this result does not preclude the likely possibility that early embryonic transcripts are important for cell fate specification (see Discussion).

MATERIALS AND METHODS

Embryo permeabilization, culture and α-amanitin treatment

Embryos were made permeable to nucleotides and α-amanitin by chitinase digestion of the eggshell, followed by mechanical stripping of the vitelline membrane with a narrow pipette (Edgar and McGhee, 1988). Up to 200 early embryos could be staged and permeabilized in each preparation. Permeabilized embryos were cultured at 24 °C in embryonic growth medium (EGM), modified slightly from Edgar and Wood (1993) by the addition of trace minerals ($ZnSO_4{\cdot}7H_2O$, 5×10^{-6} M $FeSO_4{\cdot}7H_2O$, 1×10^{-6} M $MnSO_4{\cdot}7H_2O$, 5×10^{-6} M Na_2SeO_3, 1×10^{-6} M) and 0.1 volume LP3R-1 [low protein serum replacement, (Sigma L5263)]. Cell division in untreated permeabilized embryos proceeded to approximately the normal number of 550 cells, with a rate of about 75% that of nonpermeabilized embryos (data not shown). For α-amanitin treatment, 100-200 μl/ml of the drug was added to the medium. In this enriched medium, 100 μl/ml of α-amanitin produced the same physiological level of blocking on gut granules as 30 μl/ml using the earlier EGM recipe (Edgar and McGhee, 1988); presumably the higher level is required due to increased non-specific adsorption of the drug by media components (see Greenleaf et al., 1979). Embryos were incubated in drops of medium covered with silicon oil (Dow Corning) or in covered slide chambers for longer experiments.

[^{32}P]UTP labelling, TCA precipitation and counting

[^{32}P]UTP medium was prepared by mixing 5 volumes 2× EGM (made without uridine), four volumes of aqueous of [^{32}P]UTP (NEN, in 0.01 M tricine, pH 7.6, 3000 Ci/mmol, 10 mCi/ml, final concentration approximately 1.3 μM), and one volume of either H_2O or α-amanitin stock solution (1.5 mg/ml in H_2O, Sigma). Groups of embryos were staged at 2, 4 or 8 cells, incubated to the desired age and permeabilized. For labelling, embryos of a given age were incubated at 24 °C in 8 μl drops of [^{32}P]UTP EGM with or without α-amanitin at 150 μg/ml, under silicon oil for 20 minutes. Embryos were then washed twice in nonradioactive EGM, pipetted into 10 μl of GITC lysis buffer (4 M guanidium isothiocyanate; 0.5% Sarkosyl; 5 mM NaCl; 100 mM β-mercaptoethanol), containing 42.5 μg/ml yeast tRNA (Boehringer Mannheim) as carrier, and frozen. Equal volumes of the last wash medium were frozen separately as controls for background.

Pulse-labelling of a complete series of embryonic stages required 2-3 days; however, it was found that embryonic viability during incubation decreased as the [^{32}P]UTP aged. Consequently, freshly ordered [^{32}P]UTP was used prior to its calibration date. All embryos labelled from a given batch of [^{32}P]UTP were counted simultaneously. Each frozen sample was thawed with 100 μl 20 mM EDTA in diethylpyrocarbonate-treated H_2O containing 50 μg salmon sperm DNA, and 5 ml cold 10% TCA was added. After 15 minutes on ice, precipitated nucleic acids were trapped on a Whatman GF/C filter and washed with 30 ml 10% TCA and 5 ml 100% ethanol. Filters were dried, suspended in fluor (Ready-Solv HP, Beckman), and counted in a Beckman Model LS8100 liquid scintillation counter.

RNA extraction methods and gel electrophoresis

For determining radioactivity incorporated into RNA, labelled embryos frozen in 10 μl of GITC buffer with carrier tRNA were thawed in 100 μl additional buffer, and 10 μl of each sample was filter-precipitated as above and assayed for radioactivity. The remainder was extracted twice with phenol:chloroform (1:1) and once with chloroform:octanol (10:1), back-extracting the organic phase each time with an additional 50 μl of buffer mix containing carrier RNA. Potassium acetate was added to 0.3 M, and nucleic acids were precipitated with 2.5 volumes of ethanol. The samples were dissolved in 40 μl TE with RNAsin (Promega, 25 U/ml). To determine the fraction of radioactivity attributable to RNA, each of four 10 μl aliquots was treated according to one of the following procedures: (1) One aliquot was TCA-precipitated and counted directly to determine recovery. (2) One aliquot was mixed with an equal volume of 2× RQ1 buffer (80 mM Tris, pH 7.9; 20 mM NaCl; 12 mM $MgCl_2$;0.2 mM $CaCl_2$) and digested with RQ1 DNAse (Promega; 50 units/ml for 15 minutes at 37 °C). (3) One aliquot was mixed with an equal volume of 2× proteinase K buffer (200 mM Tris pH 7.8; 25 mM EDTA; 300 mM NaCl; 2% SDS) and digested with proteinase K (400 μg/ml, 15 minutes at 37 °C). (4) One aliquot was mixed with an equal volume of 4× SSPE (600 mM NaCl; 80 mM Na_2HPO_4; 1 mM EDTA) and digested with RNase A (10 μg/ml, 30 minutes at 37 °C).

For analysis by gel electrophoresis, nucleic acids were extracted and precipitated from staged and labelled embryos by the procedures above, redissolved in 12 μl TE, and digested with RQ1 DNAse after adding an equal volume of 2× buffer and RNAsin (25 U/ml). Aliquots

representing equal numbers of nuclei (calculated from the age and number of embryos in each sample) were electrophoresed in formaldehyde gels, which were dried and exposed to X-ray film (Kodak X-OMAT AR).

^{3}H-labelling and autoradiography

5,6-[^{3}H]UTP or uridine (NEN, 35 Ci/mmol, 1 mCi/ml, in 50% ethanol) was evaporated in a microfuge to near dryness, resuspended in 1/10 volume H_2O, and added to 9 volumes of 1× EGM, ([^{3}H]UTP molarity approximately 30 μM). Embryonic labelling was done in the same manner as with ^{32}P, using embryos of up to 20 cells in these experiments and incubating for 15 or 30 minutes. Each batch of early embryos was divided in two, and one aliquot was labelled in the presence of 200 μg/ml α-amanitin.

Embryos were washed twice in nonradioactive EGM (wash time approximately 3 minutes) and immediately placed in a drop of fix (2.5% paraformaldehyde; 0.1% glutaraldehyde; 125 mM phosphate, pH 7.2) on a gelatin-subbed slide and flattened under a coverslip. Coverslips were floated off with additional fix (total time 1 minute), and the slides were then fixed for 10 minutes in 3:1 methanol:acetic acid at 4°C, followed by 3 minutes in 100% methanol and air dried. Slides were then rinsed in distilled water, dipped in photographic emulsion (Kodak NTB2 diluted 1:1 with distilled H_2O), dried and exposed for autoradiography at 4°C. Control slides were digested 30 minutes at 37°C with 100 μg/ml RNAse A in PBS before dipping in emulsion. After 5-10 days exposure, slides were developed in Kodak D19 (2.5 minutes at 17°C), rinsed in dH_2O, fixed 5 minutes in Kodak Rapidfix, washed and dried. Slides were stained for 5 minutes with DAPI (Boehringer Mannheim, 1 μg/ml in PBS) to visualize nuclei and mounted in Gelvatol (Monsanto). Microscopy was performed with a Zeiss Photomicroscope at 400×; individual embryos were photographed with bright-field illumination for counting silver grains and with low-level bright-field plus fluorescent illumination for nuclear counting. Several background areas away from the embryos were also photographed on each slide for counting silver grains.

Lineage studies and microscopy

Lineage of permeabilized embryos was followed and timed using a Zeiss inverted microscope with differential interference contrast (DIC) optics at 400×. Embryos were incubated in sealed slide chambers in 30 μl drops of EGM with or without 200 μg/ml α-amanitin at 22°C. Kodak TMax400 film was used for photography.

RESULTS

^{32}P-UTP uptake in cultured devitellinized embryos

In preliminary experiments, small numbers of permeabilized embryos incubated in embryonic growth medium (EGM, see Materials and Methods) containing either [^{3}H]uridine or [^{32}P]UTP readily incorporated radioactive counts detectable after TCA precipitation. Incorporation was linear for at least 60 minutes. When samples of 80 embryos of approximately 100 cells each were labelled for 30 minutes with [^{32}P]UTP, >90% of the TCA-precipitable radioactivity was recovered in the nucleic acids fraction following phenol extraction. Aliquots of this preparation were treated with DNAse, proteinase K and RNAse A to determine the distribution of the incorporated label. As shown in Table 1, most of the incorporation was into RNAse-sensitive material.

To characterize this product further, groups of embryos staged at <20 cells, 30 to 60 cells, and 250 to 350 cells were labelled for 15 minutes with [^{32}P]UTP, and their nucleic acids were extracted as above. Analysis on agarose gels after DNAse digestion showed labelled material in the molecular weight range characteristic of cellular RNA, including bands of the appropriate sizes for rRNAs and rRNA precursor (Fig. 1). Incorporation into mRNA was substantially reduced in the presence of α-amanitin at 150 μg/ml. These results indicated that incorporation of label into RNA could be measured by counting of TCA precipitates and that mRNA synthesis was effectively inhibited by α-amanitin.

Table 1. Nuclease and protease sensitivity of TCA-precipitable counts in devitellinized cultured embryos labelled with [^{32}P]UTP

Fraction	Total cpm	% of initial cpm
Pre-extraction	1708	100
Extracted nucleic acids	1650	97
DNase treated	1725	101
Proteinase treated	1437	84
RNase treated	117	6.8

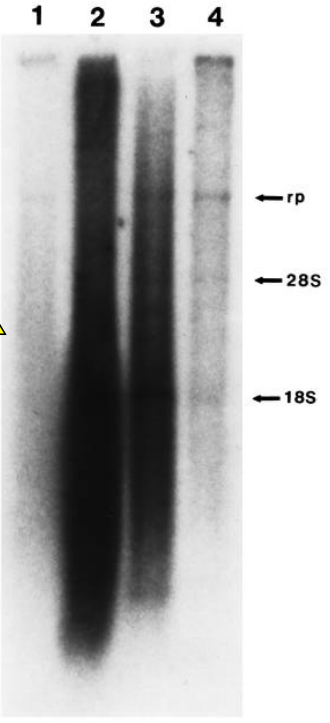

Fig. 1. Autoradiography of RNA separated by gel electrophoresis after extraction from [^{32}P]UTP-labelled embryos. Lane 1, embryos of 8-20 cells at the end of the 15-minute labelling period; lane 2, 30-60 cells; lane 3, 250-350 cells; lane 4, 250-350 cells, labelled in the presence of 150 μg/ml α-amanitin. Each lane contains RNA from approximately 3200 nuclei. Arrows indicate rRNA precursor, 23S and 18S bands. Exposure was 14 days.

To determine the rate of incorporation as a function of developmental stage, labelling was carried out either in the absence or presence of α-amanitin on embryos staged to within a single cell cycle throughout the first half of embryogenesis, from two cells to the 550 cells present when morphogenesis begins. Fig. 2 shows incorporated TCA-precipitable counts per nucleus for each group of staged embryos, as a function of embryonic age (time after first cleavage) and number of nuclei per embryo. Results are shown for three of several experiments which gave similar results. In each paired set of embryos, the uptake of label was without exception lower in the presence of α-amanitin. Between 30% and 50% of the incorporation was estimated to be α-amanitin-sensitive at all stages. The first incorporation above background was detected in the period between 40 and 60 minutes after first cleavage, when the embryos have between 8 and 12 cells at the end of the labelling period.

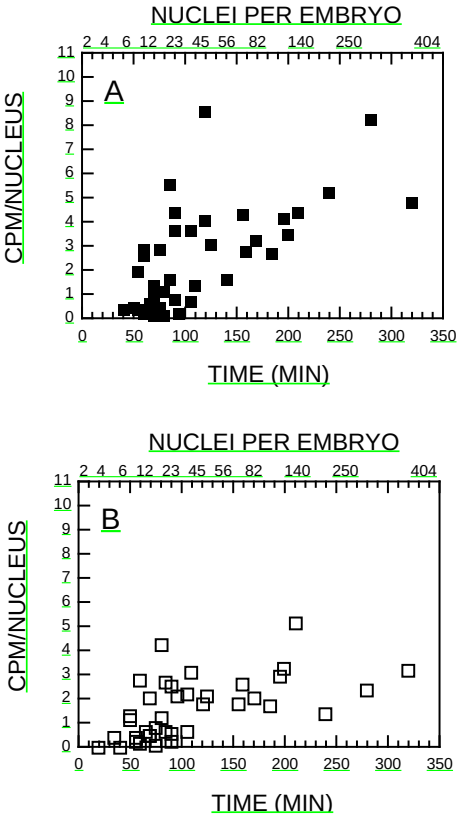

Fig. 2. Rates of [^{32}P]UTP incorporation by permeablized embryos at different stages in the absence (A) or the presence (B) of α-amanitin. Groups of embryos were staged at 2, 4 or 8 cells, permeabilized after various incubation times and then divided into two aliquots that were incubated with [^{32}P]UTP for 20 minutes (approximately one cell cycle) in EGM (A) or EGM containing 200 μg/ml of α-amanitin (B). The embryos were then washed twice and frozen, and incorporated ^{32}P was counted on filters following TCA precipitation. The graph shows cts/minute calculated per nucleus as a function of embryonic age at the end of the labelling period (minutes after first cleavage) and nuclei per embryo. To determine average nuclear numbers, groups of embryos (20 for each time point) cultured in a parallel experiment were fixed, flattened, stained with DAPI and photographed for counting. For the incorporation measurements, each point represents data from a single group of 10 to 40 embryos. Total counts per sample ranged from 35 cts/minute (2σ error=±34) to 36,000 (2σ=±2); counts for all but the youngest samples were >100 cts/minute (2σ=±20). Background ranged from 25 to 63 cts/minute and was subtracted from experimental cts/minute before calculations. Differences in viability were observed between preparations, between different batches of label and with age of label; preparations that looked unhealthy or did not divide during the labelling period were discarded.

[^{3}H]UTP uptake detected by autoradiography

To assess transcription in individual embryos, preparations permeabilized as above were labelled for 15 minutes with either [^{3}H]uridine or [^{3}H]UTP, washed, fixed and autoradiographed (Fig. 3). Both the nucleoside and nucleotide were incorporated, although the rate of labelling was higher with [^{3}H]UTP. RNase digestion prior to autoradiography eliminated almost all of the cytoplasmic and a large fraction of the nuclear grains (data not shown). Data for early embryos, grouped by number of nuclei and labelled with or without α-amanitin, are shown in Fig. 4. Each group of embryos corresponds to one early round of cell division. By the fourth cell cycle (9 to 16 cells), there is an increase in incorporation and the difference in means with and without α-amanitin is statistically significant. At the following cell cycle (17 to 24 cells), mean incorporation both with and without α-amanitin differs significantly from that in the preceding cycle, at the 95% confidence level. Since the labelling period for each embryo covered the preceding 15 minutes, or cell cycle, and the means include embryos spread across their particular age group, the earliest increase in incorporation in these experiments is estimated to begin somewhere between 8 and 16 cells. Therefore, these results agree with those presented for ^{32}P labelling in the previous section.

Cleavage in α-amanitin inhibited early embryos

Early cell divisions in *C. elegans* are strikingly invariant from embryo to embryo with respect to timing, inequality of daughter-cell sizes in some divisions, asynchrony and orientation of cleavage planes (Sulston et al., 1983). To investigate whether any aspects of this pattern depend on early embryonic transcription, early cell divisions were examined in the presence of α-amanitin, beginning with the earliest possible time of permeabilization.

To assess effects on the extent of cell division, the final nuclear number was determined in embryos staged to within 5 minutes and cultured with α-amanitin from specific ages. Fig. 5A shows the average final nuclear number plotted against the age and cell number at which exposure to the drug commenced. In Fig. 5B, these data are replotted to show the number of cell cycles completed after drug addition at various ages. The final number of nuclei is consistent with the interpretation that maternally supplied gene products are sufficient to support the first 6 to 7 rounds of cleavage. Subsequent to this point (128 nuclei), cell division stops less than one cycle after addition, indicating dependence on embryonically transcribed message. The additional cleavage in embryos permeabilized very early (<4 cells) was seen in several experiments and is statistically significant by paired *T* tests.

To determine whether embryonic transcription is necessary for the asymmetric pattern of the early divisions which differentially segregate maternal information, the pregastrulation cell lineages were followed with DIC microscopy in permeabilized embryos, both in the absence and in the presence of α-amanitin. Because the eggshell has been removed, permeabilized embryos take on a somewhat different form than untreated embryos (Fig. 6); some of the normal cell contacts are missing and division is about 25% slower than in intact embryos (data not shown; for intact embryos see Deppe et al., 1978). Nevertheless, the relative timing of divisions within different lineages (Fig. 7), their distinctive cleavage planes and the characteristic inequality of daughter cell sizes in several of the early cleavages were observed to be fairly normal through the first 5 to 6 cell cycles. No differences were found between embryos cultured with or without α-amanitin (200 μg/ml) and observed up to 87 cells (Figs 6,7). These observations were made to within one cell cycle before drug-treated embryos stop division. At about 32 cells, permeabilized embryos characteristically undergo cell movement in the P lineage in which the C cells at the end of the embryo contact the AB and MS cells,

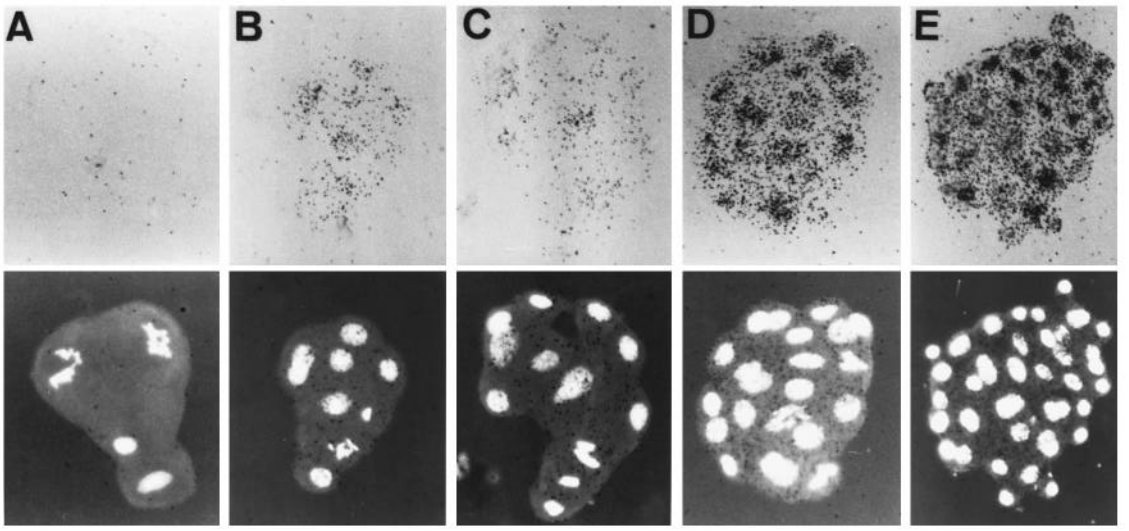

Fig. 3. Autoradiography of [^{3}H]UTP-labelled embryos. Early embryos were permeabilized and labelled with [^{3}H]UTP for 15 minutes, fixed, flattened and exposed for 5 days. Top panels, silver grains; bottom panels, nuclei stained with DAPI. (A) 4 nuclei; (B) 8 nuclei and a polar body; (C) 14 nuclei; (D) 22 nuclei; (E) 37 nuclei.

resulting in a compact round embryo. These movements appear to originate in the two E cells (the gut progenitors), and may reflect the cell movements of early gastrulation in a normal embryo within the egg shell. The movements occur even in the presence of α-amanitin (Fig 6M-O). Thus maternal information appears sufficient to control at least the patterns of the early determinative cleavages that lead to establishment of the founder cell lineages.

DISCUSSION

When does embryonic transcription start?

Earlier run-on transcription experiments showed that extracts of pregastrulation *C. elegans* embryos, averaging about 8 cells each, exhibited a high rate of α-amanitin-sensitive nucleotide incorporation (Schauer and Wood, 1990). However, since the embryos used to prepare these extracts ranged from the 2-cell to the 30-cell stages, the results indicated only that transcription must initiate before gastrulation onset at the 30-cell stage. In the experiments reported here, we have determined the stage of transcription initiation more precisely using staged, permeabilized early embryos to assay incorporation of [^{32}P]UTP by scintillation counting and incorporation of [^{3}H]UTP by autoradiography.

Our results show that transcription is initiated before the 16-cell stage, but probably not before the 8-cell stage. The precise time of initiation is difficult to pinpoint, because of scatter in the data resulting from at least three possible sources of error. First, only small numbers of embryos were available for each age sample after staging, permeabilization and division into two portions for labelling in the presence and absence of α-amanitin. Therefore, in the earliest samples where incorporated counts are close to background, the sampling error is high. Second, the chitinase permeabilization is a difficult procedure to standardize and differences in viability and corresponding rates of labelled UTP uptake were observed in different preparations of embryos. Third, the assay of a complete set of developmental stages required several days of experiments, and both embryonic viability during labelling and rate of [^{32}P]UTP uptake decreased with age of the labelled substrate.

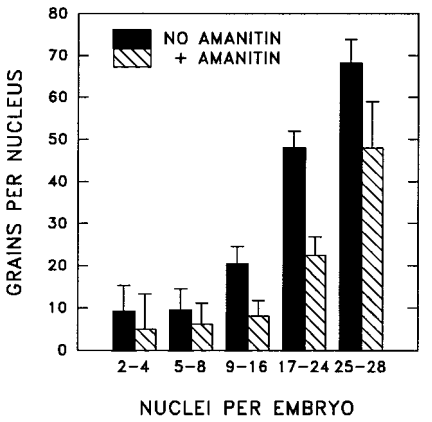

Fig. 4. Incorporation of [^{3}H]UTP by staged embryos. Silver grains and nuclei were counted on enlarged photographic prints for four sets of embryos totalling 110 labelled in EGM and 92 labelled in EGM plus 150 μg/ml α-amanitin. Insufficiently flat embryos and a few embryos showing nuclear abnormalities that suggested damage during permeabilization were not included. Solid bars represent the mean number of grains per nucleus in groups corresponding to each early cell cycle, labelled without α-amanitin; hatched bars show the means for the α-amanitin-treated samples.

Despite these difficulties, the results of [^{32}P]UTP experiments were quite consistent, showing no incorporation over background during the first three cell cycles and a significant increase over background during the fourth cell cycle, between the 8- and 16-cell stages. Because embryos were synchronized only to the extent of having the same number of cells at the beginning of the labelling period, we could not determine when in this cell cycle transcription begins.

We obtained similar results from [^{3}H]UTP incorporation assayed by autoradiography, which showed that grains over nuclei above the background level were first visible in 8- to 12-cell embryos. The considerable variation in grain count observed between embryos of a given stage in the same preparation could reflect either transient developmental delays due to damage during permeabilization or differences in transcription rate at different points in the cell cycle. The degree of synchrony in these experiments was not sufficient to test the

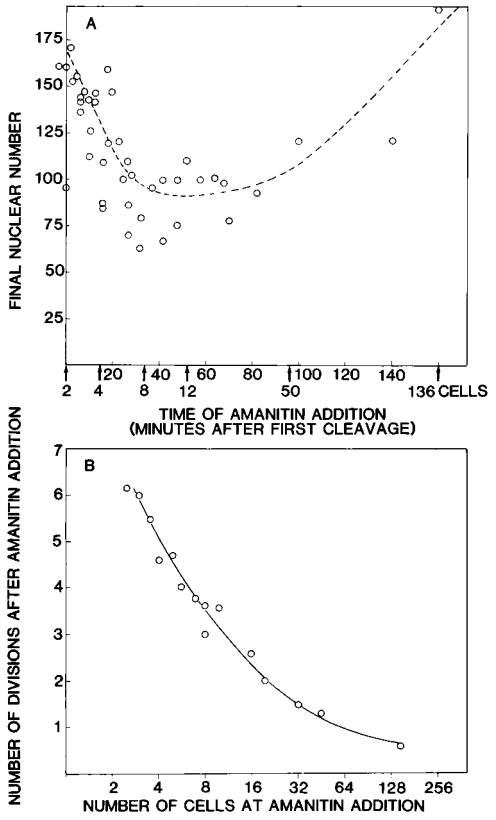

Fig. 5. Effects of α-amanitin inhibition on early cell division. (A) Terminal nuclear number in early embryos incubated with α-amanitin. Embryos were staged at 5-minute intervals, permeabilized and incubated overnight in the presence of 200 μg/ml α-amanitin to assess the extent of cell division. After overnight incubation, embryos were fixed, flattened, stained with DAPI and photographed. The final number of nuclei was then counted from the negatives. Control embryos without amanitin reached >400 cells. Each point represents an average for 8-40 embryos. One experiment had a consistently lower final number of nuclei, probably due to general conditions of permeabilization or the medium. (B) Number of cell cycles completed after addition of α-amanitin. The number of divisions after drug addition was calculated as (x-y), where 2^x is the mean final nuclear number, and 2^y is the nuclear number at time of α-amanitin addition.

latter possibility; however, we did not see labelling over nuclei in which the chromosomes were visibly condensed, indicating that transcription ceases during mitosis, as would be expected. We also did not observe any obvious differences in labelling between cells of different lineages in those embryos where the chromosomal morphology allowed us to assign lineage identities.

By the 16- to 20-cell stage, embryonic transcription appears to be proceeding at a high level, consistent with the earlier ma-on transcription results (Schauer and Wood, 1990). Thus both

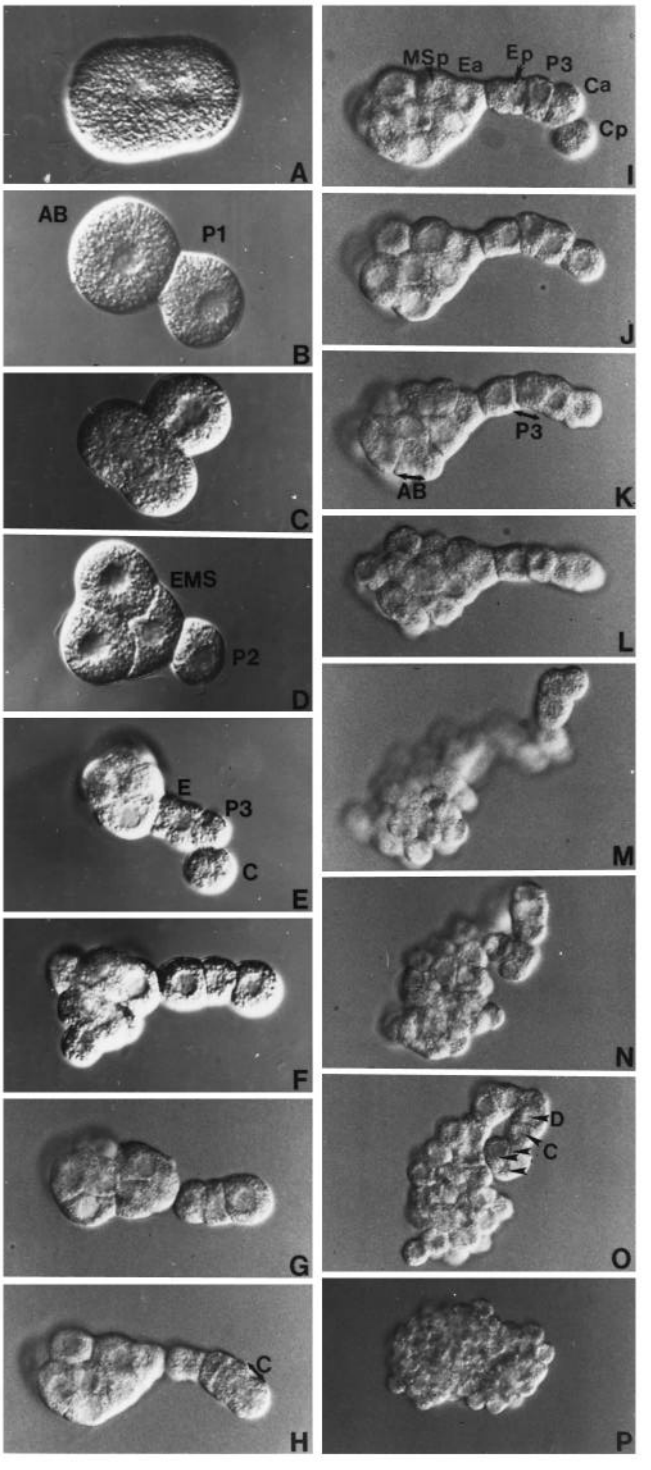

Fig. 6. Early divisions in permeabilized cultured embryos. (A-F) Development of an embryo cultured to the 12-cell stage without α-amanitin; (G-P) the 12-cell and subsequent stages of an embryo cultured in the presence of 200 μg/ml α-amanitin from the 2-cell stage onward. Embryos cultured with or without α-amanitin behaved indistinguishably up to about 100 cells, exhibiting the same division pattern as well as timing of divisions (see Fig. 7). Founder cells and their progeny are labelled in some of the panels; double-ended arrows indicate cleavage axes. (A) First cleavage, t=0 (approximately 40 minutes after fertilization); (B) 2-cell stage, 10 min; (C) second cleavage, AB cell dividing, 20 min.; (D) 4-cell stage, 25 min; (E) 8-cell stage, 57 min; (F) 12-cell stage, with eight AB cells, four P lineage cells, 73 min; (G) 12-cell stage, α-amanitin present from two cells onward, 81 min; (H) C division, 93 min; (I) 15-cell stage, 97 min; (J-L) 16- to 24-cell stages with P_3 and the 8 ABs dividing, 105, 110 and 120 min, respectively; (M) 187 min, (N) 200 min, (O) 230 min, note gastrulation-like movements beginning (in M) at 46 cells (32 AB, 4 C; D, P_4, 4 E, 4 MS cells), in which P-lineage cells move to contact MS and Eaa; (P) same embryo after 16 hours showing α-amanitin arrest at approximately 100 cells.

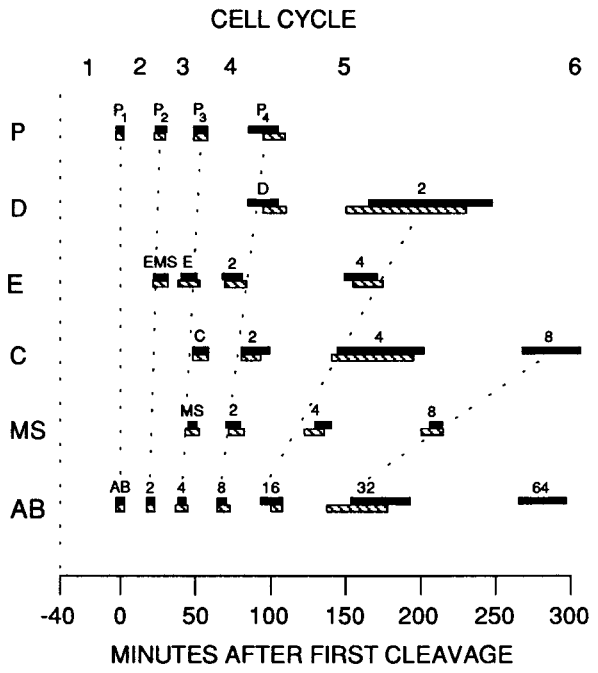

Fig. 7. Timing of divisions in permeabilized cultured embryos with and without α-amanitin. The six founder cell lineages are listed on the vertical axis. Horizontal lines indicate division times of cells in the various lineages at each cell cycle in the presence (solid bars) and absence (hatched bars) of α-amanitin. Their lengths indicate the spread in division times between five observed embryos. Embryos without α-amanitin were not observed beyond 250 minutes. Dotted vertical and oblique lines connect the divisions at the end of each cell cycle. Cell cycle periods vary between lineages, with the most rapid divisions in the AB descendants. For example, at the end of the fifth cell cycle, the AB, MS and C descendants begin to divide at about 100, 125 and 140 minutes, respectively.

these studies indicate clearly that embryonic transcription in *C. elegans* embryos begins well before gastrulation, in contrast to the conclusion of Hecht et al. (1981) that message transcription begins after gastrulation at 80 to 100 cells. The discrepancy may be due to the differences in measurement techniques, as the earlier studies measured in situ hybridization to nuclear poly(A)$^+$ mRNA, detecting the first increase in embryos of >80 cells, whereas we have measured nucleotide incorporation directly. If early transcription includes a high proportion of poly(A)-mRNA, or if message is rapidly transported to the cytoplasm in early embryos, the hybridization methods might not be sensitive enough to detect nuclear-cytoplasmic differences in a high background of cytoplasmic maternal message.

Embryonic transcription is initiated as early as the 4- to 8-cell stage in the large parasitic nematode *Ascaris lumbricoides* (Cleavinger et al., 1989), which has an early cleavage pattern very similar to that of *C. elegans*, but a much slower rate of cleavage. It has been argued that in *Xenopus* (Newport and Kirchner, 1982a,b; Takeichi et al., 1985; Kimelman et al., 1987) and *Drosophila* (Edgar et al., 1986) transcription does not occur during the rapid divisions in which S-phase occupies most of the cell cycle because transcription complexes cannot form until the lengthening cell cycles include a G_2 phase. However, this explanation is not supported by results from *C. elegans* or from the leech *Helobdella*, and is not likely to account for the difference in time of transcription onset between *C. elegans* and *Ascaris*. Presence of a G_2 phase is clearly not sufficient for embryonic transcription: in embryos of both *Helobdella* and *Ascaris*, which at all stages have extended G_2 phases, no transcription is detected before the 4-cell stage (Cleavinger et al., 1989; Bissen and Weisblat, 1991); neither does presence of a G_2 phase appear necessary for transcription. Blast cells in *Helobdella* embryos at stages 7-8 synthesize RNA during S phase as well as during G_1 and G_2 (Bissen and Weisblat, 1989; 1991). In *C. elegans* the first G_2 phase occurs in the E lineage at the 20-cell stage (Edgar and McGhee, 1988), but we detect transcription earlier than that in most if not all lineages by autoradiography. Thus we conclude that, as in the leech, the S phase block to transcription is not absolute.

RNA polymerase II transcription

Inhibition of RNA synthesis by α-amanitin provides an estimate of the relative contributions to transcription of mRNA synthesis by RNA polymerase II, which is sensitive to the drug, versus synthesis of other RNAs by polymerases I and III, which are relatively insensitive. In our incorporation experiments, significant inhibition by α-amanitin is already seen by the 16- to 20-cell stage, indicating that RNA polymerase II contributes significantly to overall transcription from the time when it is first detectable. At all stages tested, we find that 30% to 50% of total transcription is α-amanitin sensitive. This could be an underestimate of the fraction of transcription by RNA polymerase II if inhibition were incomplete. We cannot rule out this possibility. However, the method of permeabilization by complete removal of the vitelline membrane allows maximal access of the drug; no significant mRNA synthesis is seen by RNA blot analysis in α-amanitin-treated embryos (Fig. 1), and the doses used (150-200 μg/ml) are well above the 100 μg/ml that produces the maximal effect on limitation of final cell number as well as loss of expression of gut granules, gut esterase and several antigenic markers of differentiation.

The 30% to 50% α-amanitin inhibition that we observe differs from results obtained earlier in run-on transcription experiments. In early embryo extracts, Schauer and Wood (1990) reported 85-90% inhibition of incorporation by 1 μg/ml of α-amanitin. A similar study of embryonic run-on transcription in *Ascaris* found 25% inhibition at the 4- to 16-cell stages, 55% at the 30-cell stage, and >80% at the 60-cell stage (Cleavinger et al., 1989). A possible explanation for these differences is that in run-on assays, initiated transcripts are completed but reinitiation does not occur (Schauer and Wood, 1990). If RNA polymerases I and III initiate at a higher rate than II, more α-amanitin-resistant transcripts would accumulate during the labelling period of our experiments than in the run-on assays. Thus our direct labelling experiments may reflect the in vivo ratios more accurately.

The roles of maternal gene products and embryonic transcripts in early embryonic patterning

It is clear that at least a few maternal determinants are partitioned during early cleavage to the appropriate cell lineages, where they participate in the determination of gut, germ line and other cell identities (Laufer et al., 1980; Strome and Wood, 1983; Cowan and McIntosh, 1985; Bowerman et al., 1992; Mello et al., 1992; Bowerman et al., 1993). However, the

demonstration of early embryonic transcription raises the possibility that some critical early events in embryogenesis require embryonically synthesized gene products. To investigate the extent of such requirements, we used α-amanitin to assess the effects of eliminating or greatly reducing RNA polymerase II mediated transcription on early cell division, cell lineage patterns and morphogenesis.

When α-amanitin is added to the culture medium of permeabilized embryos, the final cell number achieved is relatively constant, approximately 100 cells, whether exposure to the drug begins at 4 cells or at close to 100 cells. Addition after the 100-cell stage blocks division within approximately one cell cycle. This result is consistent with the earliest arrest stage described for a nonmaternal embryonic lethal mutation; an *emb-29* mutant arrests at approximately 140 cells (Hecht et al., 1987). A reasonable model for our results is that components of the cell-cycling machinery, maternally supplied as either mRNA or proteins, can support division to approximately 100 cells, at which point these components must be supplemented or replaced by embryonically produced gene products for cell division to continue.

Somewhat surprising, however, is the finding that exposure of embryos to α-amanitin before 4 cells results in a significantly higher final number of nuclei, close to the 140 seen in *emb-29* mutant embryos. One possible explanation is that early products of embryonic transcription, not made in the presence of α-amanitin, destabilize or degrade maternal messages or proteins required for cell division. A similar phenomenon of an extra cell division has been observed in *Drosophila* embryos injected with α-amanitin before the onset of embryonic transcription (Edgar and Schubiger, 1986).

Not only does early cell cycling in *C. elegans* embryos proceed in the presence of α-amanitin, but the lineage-characteristic patterns of cell-cycle timing, cleavage orientations and unequal cleavages also appear to be unchanged. Earlier work has shown that normal early cell fate determination depends on these patterns; when they are changed by manipulation or by mutation, cell fates are also affected (Priess and Thomson, 1987; Kemphues et al., 1988; Schnabel and Schnabel, 1990; Wood, 1991; Bowerman et al., 1992; Mello et al., 1992). Therefore, it is possible that the apparently normal early cleavages in α-amanitin-treated embryos are also accomplishing normal partitioning of maternal components and normal early cell fate determination, although we have no evidence other than cell appearance to support this possibility. If we assume that α-amanitin is effectively blocking RNA polymerase II transcription in these experiments, none of this early pattern, up to at least 100 cells, would require embryonic transcription, even though transcription demonstrably starts during this period. Our results differ in this regard from those obtained with the leech embryo, in which the divisions forming the teloblasts at about 25 cells are abnormal in the presence of α-amanitin, generating supernumerary yolk-filled blastomeres rather than yolk-free teloblasts (Bissen and Weisblat, 1991).

An analysis of early lineages in *C. elegans* embryos homozygous for any one of several deficiencies, representing embryonic losses covering nearly half the genome, also identified no chromosomal regions required for correct early patterning (Storfer-Glazer and Wood, 1994). All the deficiency embryos showed abnormalities in morphogenesis, but most arrested with the normal number of 550 cells. Only one (carrying a deficiency that included the *emb-29* gene) arrested before 140 cells. In addition, all known mutations affecting the early lineages, including genes known to affect fate specification as well as early cleavage patterns, show strict maternal effects (Priess et al., 1987; Kemphues et al., 1988; Mains et al., 1990; Bowerman et al., 1992; Mello et al., 1992).

However, α-amanitin does affect morphogenesis as well as expression of early tissue-specific markers of differentiation. In α-amanitin-blocked embryos, no organ primordia are observed and the embryos arrest as masses of undifferentiated cells. The earliest tissue-specific markers of differentiation can be blocked by α-amanitin treatment well before the time at which cell division is arrested. Gut granules, normally first detectable at about 100 cells, are sensitive to α-amanitin treatment up to the 16-cell stage but appear if the drug is added subsequently (Edgar and McGhee, 1986; 1988). This implies that the latest transcription leading to gut granule expression occurs before 16 to 20 cells, or during the second E cell cycle after the gut lineage is established. Gut esterase, a second gut-specific marker, appears slightly after 100 cells, but is sensitive to α-amanitin only until the end of the second E cell cycle (Edgar and McGhee, 1986; 1988).

Our results and other recent studies referenced above support the following model for *C. elegans* embryogenesis. The pregastrulation cleavage patterns, including asymmetric partitioning of cytoplasmic components and determination of some but not necessarily all founder cell fates are controlled by maternally supplied gene products. Embryonic transcription, initiated during this period as the founder-cell lineages become established, is required for refinements of fate specification dependent on cell interactions, subsequent cellular differentiation and morphogenesis. An answer to the question of whether embryonically produced gene products are required for founder-cell fate determination must await further molecular and genetic characterization of the earliest transcripts, which we are now pursuing.

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