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DNA Synthesis and the Control of Embryonic Gene Expression in C. elegans

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Summary

DNA synthesis in each cell lineage of the early C. elegans embryo was measured using microspectrofluorimetry. Aphidicolin was shown to inhibit DNA synthesis almost instantly and completely. Aphidicolin was then used to investigate how DNA synthesis controls expression of two biochemical markers that appear at different times during gut development: gut granules and a carboxylesterase. We show that marker expression is controlled neither by reaching the normal DNA:cytoplasm ratio, by counting the normal number of rounds of DNA synthesis, nor by a simple lengthening of the cell cycle. Instead, expression of both gut markers requires a short period of DNA synthesis in the first cell cycle after the gut has been clonally established.

Introduction

The synthesis of DNA is often invoked as a mechanism for controlling and timing gene expression during embryonic development. A number of different experimental systems have been investigated, and a number of different models have been suggested. For example, Satoh and coworkers (Satoh, 1979; Satoh and Ikegami, 1981a, 1981b; Satoh, 1982a, 1982b; Mita-Miyazawa et al., 1985; Nishikata et al., 1987) have proposed that a definite number of rounds of DNA synthesis are required for acetylcholinesterase expression in the muscle lineage of ascidian embryos. In other systems, the DNA:cytoplasm ratio has been implicated in timing the onset of the embryonic midblastula transition (Kobayakawa and Kubota, 1981; Newport and Kirschner, 1982a, 1982b; Mita, 1983; Mita and Obata, 1984; Edgar et al., 1986), an event associated with the first major wave of transcription from the zygotic genome. More recently (Edgar and Schubiger, 1986; Kimelman et al., 1987), it has been shown that transcription can be induced prematurely by artificial elongation of the cell cycle, as if the DNA:cytoplasm ratio in the embryo only indirectly controls the onset of gene expression.

Much of the work relating DNA synthesis to the differentiation of cultured cells has been interpreted in terms of the quantal cell cycle model (Holtzer et al., 1975, 1983), which states that cells can only alter their state of commitment or differentiation by passing through the S phase of a special or "quantal" cell cycle. On a more molecular level, perhaps it is only when DNA is transiently nucleosome-free during replication that control proteins can take their place at their specific sites of action (see, e.g., Weintraub, 1979; Brown, 1984). Over the last few years, a number of studies have shown that the quantal cell cycle model cannot be strictly true, since cells can change (or can be made to change) their pattern of gene expression in the complete absence of DNA synthesis (Nadal-Ginard, 1978; Turo and Florini, 1982; Chiu and Blau, 1984; Temple and Raff, 1985; Pinset and Whalen, 1985). The results of Blau and coworkers (Chiu and Blau, 1984; Blau et al., 1985) show particularly clearly that *trans*-acting factors do not need DNA replication to initiate the expression of new genes. However, it could be argued that the experimental systems used to test the quantal cell cycle model have all been to some extent artificial, and the question remains whether critical rounds of DNA synthesis do indeed occur during development.

Because of its highly defined cell lineages, the nematode Caenorhabditis elegans is an excellent organism in which to study how DNA synthesis influences lineage-specific gene expression during development. The gut lineage is particularly convenient to study, since it is clonally descended from only one cell of the 8-cell embryo; this clonal progenitor of the gut is called the E cell (Deppe et al., 1978; Sulston et al., 1983). Development of the gut appears to depend, at least in part, on the cytoplasmic localization of maternally derived factors (for discussion, see Davidson, 1986).

In the present paper we use the DNA polymerase inhibitor aphidicolin (Ikegami et al., 1978; Lee et al., 1984) to investigate how inhibition of DNA synthesis influences gene expression in the developing gut of embryos of the nematode C. elegans. We follow the expression of two biochemical markers localized to the gut lineage: a carboxylesterase (McGhee, 1987) and a collection of autofluorescent, birefringent products of tryptophan breakdown known as gut granules (Babu, 1974; Siddiqui and von Ehrenstein, 1980). We have shown previously that esterase expression in the C. elegans gut is lineage-autonomous and does not require cytokinesis (Edgar and McGhee, 1986). Embryos that are homozygous for an (apparent) null mutation in the esterase structural gene show little, if any, esterase activity during early development (McGhee et al., 1987). Thus the esterase staining seen in the present work derives from a single genetic locus, *ges-1* (McGhee and Cottrell, 1986). In contrast, there may be as many as four genes involved in producing gut granules (Siddiqui and von Ehrenstein, 1980). Although gut granules represent localized enzyme products rather than localized enzyme activities, gut granule appearance is also lineage-autonomous and independent of cytokinesis (Laufer et al., 1980; Edgar and McGhee, 1986), suggesting that the enzymes themselves are gut-localized. Both esterase expression and gut granule production are inhibited by α-amanitin (Edgar and McGhee, 1986, and see below), indicating that these genes are transcribed from the zygotic genome.

Our results show that the relation between DNA synthesis and marker expression during C. elegans develop-

ment differs in a number of ways from results in the other organisms cited above. We find that gut markers are expressed only if embryos are allowed to enter the first round of DNA synthesis after the gut has been clonally established, i.e., when the embryo has a total of only 8 cells. Subsequent rounds of DNA synthesis, which normally occur before marker gene transcription, can be inhibited without preventing marker expression. Thus expression of these genes is controlled neither by reaching the normal DNA:cytoplasm ratio nor by counting the normal number of rounds of DNA synthesis. Moreover, a simple elongation of the embryonic cell cycle does not appear to be sufficient to cause marker expression. Rather, an early period of DNA synthesis appears to be crucial, after which the timing of gene expression is independent of both DNA synthesis and cytokinesis. Some implications of these results are discussed below.

Results

Time Course of DNA Synthesis in Early Embryos

In early C. elegans embryos, each of the stem cell lineages shows a distinctive and invariant schedule of divisions, occurring at roughly 15–20 min intervals (Deppe et al., 1978; Sulston et al., 1983). To measure the time course of DNA synthesis in each embryonic lineage, embryos of known age (±1 min) were permeabilized and then incubated with the DNA-binding dye 4′,6-diamidino-2-phenylindole (DAPI), and the DNA-induced fluorescence was measured using a microspectrofluorimeter. The data shown in Figure 1 cover the 2 hr period between fertilization and the 24-cell embryo. The following observations can be made:

—Following sperm entry, the DNA content of the oocyte nucleus drops from 4C to 1C as the oocyte completes meiosis. Shortly thereafter, both male and female pronuclei decondense and DNA synthesis begins immediately; synthesis is completed as the nuclei move toward each other. The 4C fusion nucleus is short-lived and divides almost immediately. (See Albertson [1984] for a more complete description of pronuclear movements).

—In all early divisions, DNA synthesis is rapid and begins immediately after decondensation of the anaphase chromosomes. S phase does not overlap with the previous mitosis, as has been suggested by Satoh and Ikegami (1981a, 1981b) for ascidians. Half of the DNA synthesis in each 15–20 min cell cycle takes place in the first 3–5 min following the completion of mitosis, and no period that could be called a G1 phase can be detected.

—DNA synthesis occupies the entire intermitotic period. The second half of each round of DNA synthesis is markedly slower than the first half, and chromosomes begin to condense as DNA synthesis slows. However, it is doubtful whether any G2 phase can be assigned.

—When the cell cycle begins to slow, for example in the P lineage, the overall rate of DNA synthesis decreases, not the fraction of the cell cycle occupied by S phase.

—In the gut lineage, a G2 phase of the cell cycle makes its first appearance at gastrulation. DNA synthesis in the gut lineage resumes immediately after the fourth cleavage (Figure 1B), and the DNA content reaches 4C within 20 min (data not shown). Thus the 2 gut cells have completed S phase shortly before they migrate into the embryo; this is about 1 hr before the next cell division. We do not know when the G1 and G2 phases first become established in other cell lineages.

Aphidicolin Inhibition of DNA Synthesis

With the information of Figure 1 as a background, the next step was to determine the minimal dose of aphidicolin required to inhibit DNA synthesis completely and immediately.

As described in more detail in Experimental Procedures, embryos were treated with hypochlorite and chitinase and then permeabilized in one of two ways: gentle pressure on the coverslip ("pressure-permeabilized"), or gentle pipetting to strip off both the egg shell and the vitelline membrane ("pipette-permeabilized"). For pressure-permeabilized embryos, an aphidicolin dose of 7.5 μg/ml inhibited DNA synthesis almost completely over the first 30 min, and more than 90% over a 3 hr period following drug addition. An example is shown in Figure 2A. For pipette-permeabilized embryos, an aphidicolin dose of only 5.5 μg/ml gave equivalent results, i.e., essentially 100% inhibition over the first 30 min, followed by a gradual increase over the next several hours. This is shown quantitatively in Figure 2B.

We also verified that, within the gut lineage, aphidicolin addition causes immediate and complete inhibition of DNA synthesis, whether the drug is added at the end or the beginning of a cell cycle (Figure 2C, taken from McGhee et al., 1987). In the majority of experiments, cytochalasin D was also added at the same time as aphidicolin; this prevented problems arising from attempts at cytokinesis without nuclear division and gave a rapid check that individual embryos had indeed been permeabilized.

Expression of Gut-Specific Markers following Inhibition of DNA Synthesis

Collections of early embryos (2–8 cells) were permeabilized and their ages determined (±1 min) by noting cell number and the time of a particular cell division. Aphidicolin was added, and the embryos were incubated overnight at 16°C; more than 90% of such embryos remain viable as determined by exclusion of trypan blue, by the noncoagulated character of their cytoplasm, and by direct observation over the first several hours following drug addition. Gut granules were scored by birefringence, and esterase activity was scored by histochemical staining, as described previously (Edgar and McGhee, 1986).

The primary finding of the current paper is the following. Embryos that had 4 cells or fewer at the time of inhibition of DNA synthesis express neither esterase nor gut granules. Conversely, embryos that had 8 cells or more at the time of aphidicolin addition express both esterase and gut granules with close to the same intensity and probability as do control embryos that received cytochalasin D alone. Examples of individual embryos that document this observation are shown in Figure 3A. The striking and abrupt

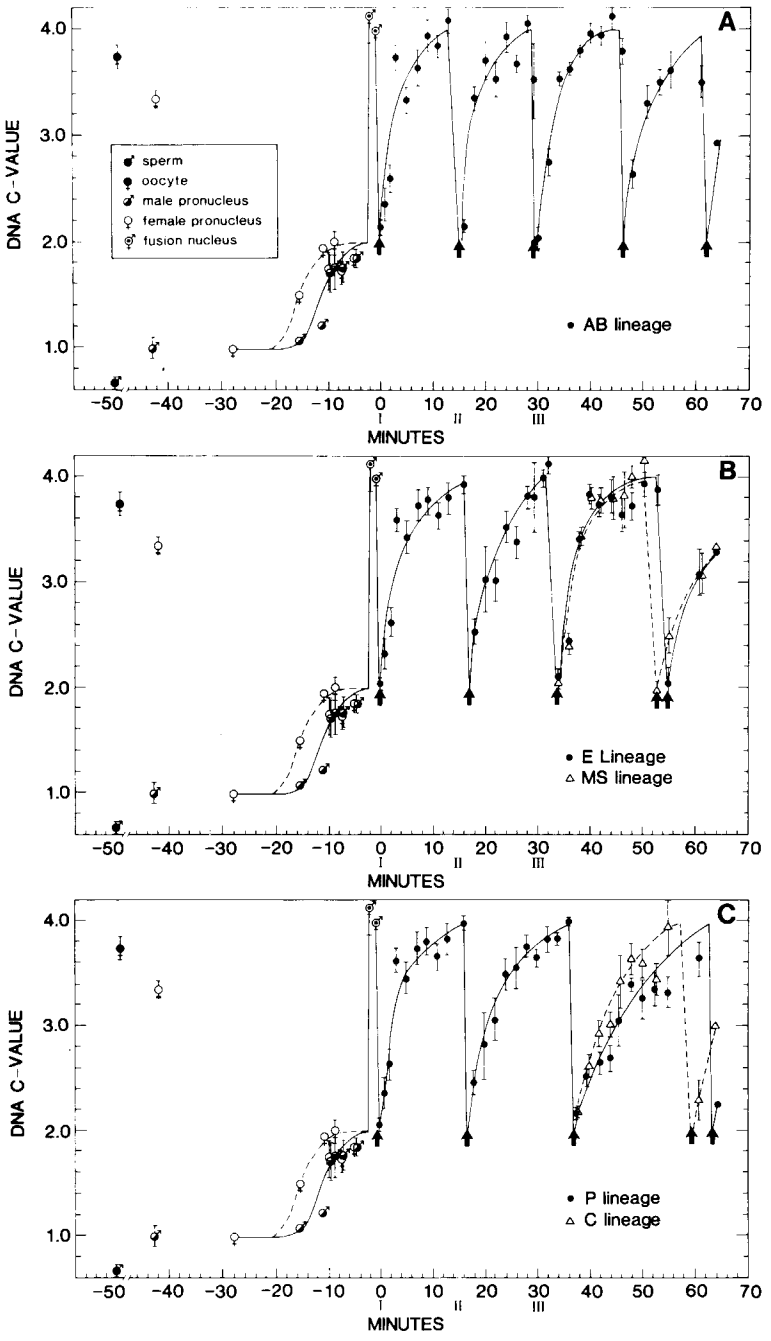

Figure 1. DNA Synthesis in Early C. elegans Embryos.

(A) AB lineage. (B) EMS lineage. (C) P lineage. Ordinate, average C values; abscissa, minutes after first cleavage (22°C). Roman numerals indicate AB divisions. Each point represents an average of at least 20 embryos. Bars indicate standard errors. Arrows indicate observed times of anaphase (see text), when nuclei should have a DNA content of 2C.

nature of the transition from aphidicolin sensitivity to aphidicolin resistance is shown in Figure 3B, in which a total of 506 pressure-permeabilized embryos were scored as either positive or negative for marker expression as a function of the time of aphidicolin addition.

The Transition to Aphidicolin Resistance Occurs Much Earlier Than Transcription of the Marker Genes

We now show that the apparent requirement for early DNA synthesis is distinct from the actual transcription of the esterase gene and of the genes that produce gut granules. In Figure 4, the data from Figure 3B (closed circles, solid lines) as well as similar data from 550 pipette-permeabilized embryos (open triangles, dashed lines) are expressed as the percentage of esterase-positive or gut-granule-positive embryos in a 4–5 min age span, plotted as a function of time of aphidicolin addition. As just noted, the transition to aphidicolin resistance occurs during the first cell cycle after the gut becomes clonally established. The transition is about 2 min later in pipette-permeabilized embryos than in pressure-permeabilized embryos. Since the aphidicolin could well permeate more quickly into pipette-permeabilized embryos, which lack the vitelline

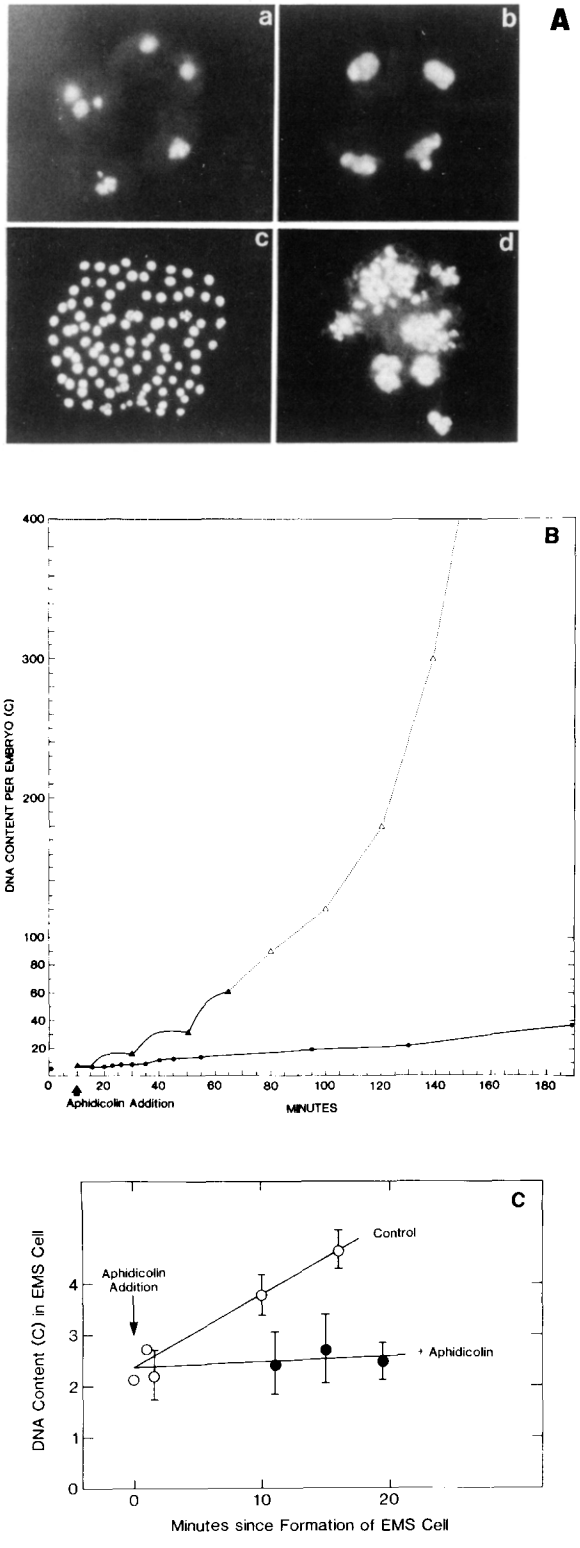

Figure 2. DNA Synthesis in Aphidicolin-Blocked Embryos.

(A) Permeabilized late 4-cell embryos were blocked with either cytochalasin D (1.5 μg/ml) or cytochalasin D plus aphidicolin (5.4 μg/ml). (a) Untreated embryo at t = 0. Note that the two AB nuclei have already divided and that a polar body (DNA content 1C) is visible. Total DNA content is ~16C (4C per cell × 4). (b) Embryo treated with cytochalasin D and aphidicolin, at 3 hr after blocking both cytokinesis and DNA synthesis. Note polar body. Total DNA content quantitated in similar embryos averaged 29C (1.8 × original DNA content). (c) Embryo permeabilized at 4 cells and cultured for 3 hr without drugs; 92 nuclei can be counted, giving an estimated DNA content of 276C. (d) Cytochalasin D–treated 4-cell embryo at 2.5 hr after blocking cytokinesis (no added aphidicolin). DNA synthesis and nuclear division have continued even though cytokinesis was blocked.

(B) Pipette-permeabilized embryos were placed in a medium containing 5.5 μg/ml aphidicolin at 10–12 min after first cleavage (DNA content 4C per nucleus; see Figure 1). Total DNA per embryo was determined at various times thereafter by DAPI fluorescence (lower curve); each point represents an average of 3–14 embryos. The upper curve shows total DNA content in untreated embryos: the filled triangles represent actual measured DNA content (Figure 1); the open triangles (dashed line) represent DNA content estimated from the total number of nuclei during embryogenesis at 25°C (Sulston et al., 1983), assuming an average DNA content of 3C per nucleus.

(C) Pipette-permeabilized embryos were placed in 5.5 μg/ml aphidicolin and 1.5 μg/ml cytochalasin D (closed circles), or cytochalasin D alone (control, open circles) at second cleavage (±1 min), then fixed and stained for DNA quantitation at intervals over the following 20 min (22°C). DAPI fluorescence levels of the EMS nuclei were converted to C values by normalizing to the DNA fluorescence of polar bodies on the same slide. Each point represents 5–8 embryos; bars indicate standard deviations. The figure is taken from McGhee et al. (1987).

membrane, we assume that the later transition time is the more accurate. After the transition, 90%–100% of pressure-permeabilized embryos and 75%–80% of pipette-permeabilized embryos express the markers.

Figure 4A shows that the time at which esterase expression becomes resistant to aphidicolin is considerably earlier than the time at which it becomes resistant to α-amanitin. In this experiment, DNA synthesis was blocked by adding aphidicolin to embryos that had 2 gut cells (16–32 total cells) and in which esterase expression had consequently become aphidicolin-resistant. The RNA polymerase II inhibitor α-amanitin was then added to these embryos at various times, and esterase expression was scored after overnight incubation (Figure 4A, open circles, solid line). In reasonably good agreement with previous results obtained without aphidicolin (Edgar and McGhee, 1986), esterase expression starts to become α-amanitin–resistant (and we assume that the esterase mRNA starts being transcribed) at the late 4-gut-cell stage; it becomes completely α-amanitin–resistant when the embryo has 8 gut cells. This is 150–200 min after first cleavage, at a point when the embryo has a total of 100–150 cells.

We conclude that esterase gene transcription occurs two to three cell divisions and about 2 hr after esterase expression becomes resistant to aphidicolin. We also conclude that inhibiting several rounds of DNA synthesis does not markedly change the time of esterase gene transcription or the time at which esterase activity can first be detected (data not shown). This latter observation shows that aphidicolin does not prevent mRNA capping, as has been suggested in other systems (see, e.g., Caldwell and Emerson, 1985).

Figure 4B shows the same experiment using gut granules as a marker. Gut granule expression becomes α-amanitin–resistant at a point when the embryonic gut has 2 cells; this is one cell division (and roughly 30 min) after the expression becomes resistant to aphidicolin.

The clear separation between the transition to aphidico-

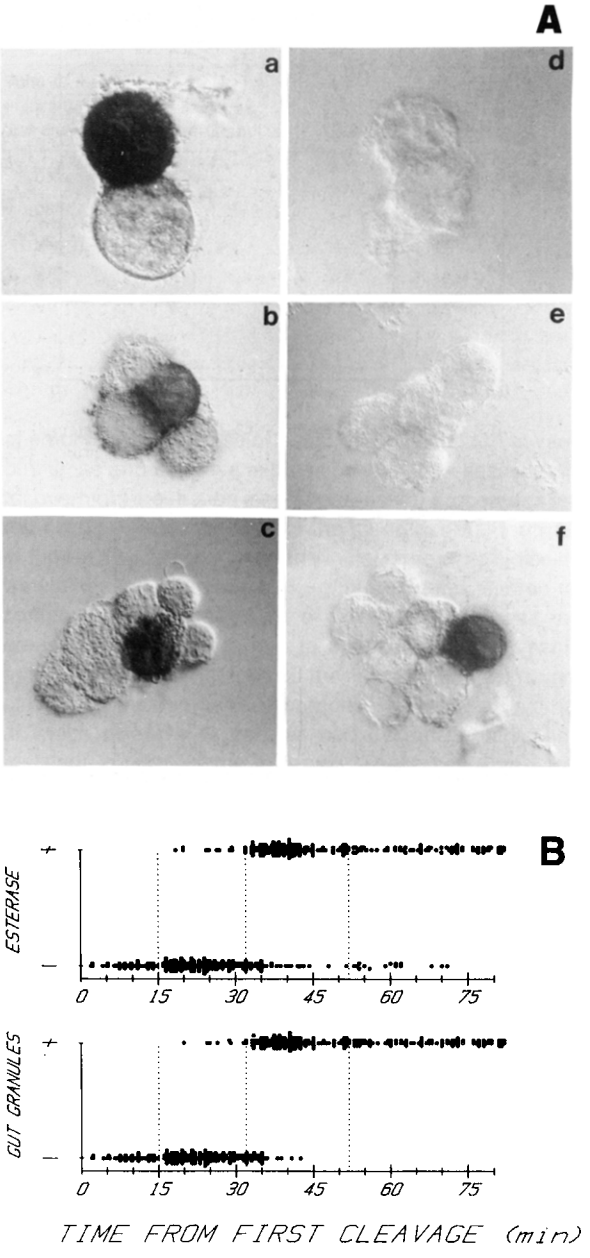

Figure 3. Expression of Gut Differentiation Markers by Embryos Blocked in DNA Synthesis at the 2-Cell to 16-Cell Stages

(A) C. elegans embryos at early cleavage stages were treated with 1.5 μg/ml cytochalasin D (a–c) or with 1.5 μg/ml cytochalasin D plus 5.5 μg/ml aphidicolin (d–f), incubated for 12–18 hr at 16°C, and stained for esterase. Shown are 2-cell (a and d), 4-cell (b and e), and 8-cell (c and f) embryos. In virtually all embryos, gut granules were detected in the same cells that stained for esterase.

(B) Ordinate: expression of esterase (top) or gut granules (bottom) scored as either (+) or (–). Abscissa: time of aphidicolin addition in minutes after first cleavage (22°C). Each dot represents an individual embryo (506 embryos total). Cleavage times for the EMS lineage are shown with dashed vertical lines. A clear transition from nonexpression to expression occurs at the 8-cell stage. A small number of embryos do not fit this pattern: expressing embryos treated at 4 cells may be due to erroneous timing; embryos not expressing after the 8-cell stage are possibly damaged.

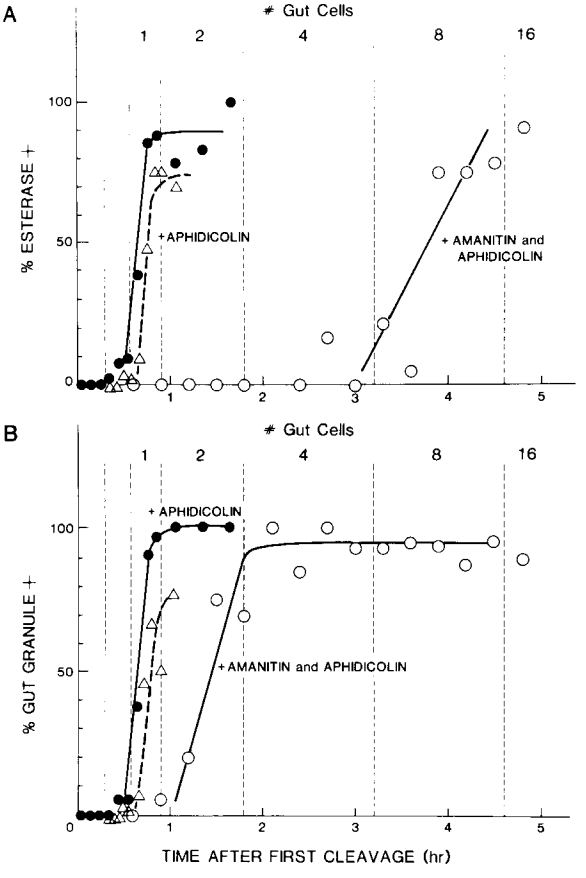

Figure 4. Expression of Gut Differentiation Markers in the Presence of both Aphidicolin and α-Amanitin

(A) Esterase expression. (B) Gut granule expression. Shown are accumulated data from 506 pressure-permeabilized embryos (closed circles) and 550 pipette-permeabilized embryos (open triangles) plotted on an extended time scale to show the time of transition to aphidicolin resistance relative to the time of transition to α-amanitin resistance. Ordinate: percentage of marker-positive embryos within a 4 or 5 min age interval. Abscissa: time of drug addition (hours since first cleavage). Dashed vertical lines indicate cleavages of the E lineage. Open circles show the percentages of embryos expressing the marker if α-amanitin (150 μg/ml) was added at the indicated times; in this experiment embryos were first blocked with aphidicolin at 16–32 cells, and so would be expected to express gut granules and esterase in the absence of further drug treatment. Each point represents an average of 10–40 embryos.

The transition from α-amanitin sensitivity to insensitivity (implying that transcription has occurred) for gut granule expression coincides with that observed without aphidicolin treatment (Edgar and McGhee, 1986). Esterase expression shows a lengthened period of α-amanitin sensitivity (+30 min) in the presence of aphidicolin compared with its absence (Edgar and McGhee, 1986). This apparent slowing of development may be due to the permeabilization procedure rather than the drug since we have observed that, after 2–3 hr, permeabilized embryos divide more slowly than do normal embryos.

lin resistance and the transition to α-amanitin resistance rules out the following trivial explanation of our results: addition of aphidicolin halts development and the only embryos to express the markers are those in which marker gene transcription has already occurred.

In C. elegans, nuclear poly(A) can first be detected by in situ hybridization shortly after gastrulation, and is observed to increase rapidly thereafter (Hecht et al., 1981).

As can be seen from Figure 4, transcription of the gut granule genes is probably occurring during the actual cell migrations of gastrulation, and transcription of the esterase gene occurs shortly after gastrulation has been completed. Thus the genes used here as markers must be among the earliest to be transcribed during C. elegans development.

Aphidicolin Pulses Provide Further Evidence for a Crucial Period of DNA Synthesis

A trivial explanation for the apparent requirement for early DNA synthesis is that embryos younger than 8 cells are somehow damaged by the aphidicolin and consequently never reach the developmental age (50–150 total cells) at which the markers are normally expressed. Experiments in which aphidicolin is presented as a pulse argue against this interpretation. When 4-cell embryos are exposed to aphidicolin for 40 min (during which time control embryos would have completed the 4-cell to 8-cell division) and the aphidicolin is then removed, embryonic DNA synthesis (as quantitated by DAPI fluorescence and increased nuclear number) reaches a normal rate within 15 min, and the embryos eventually reach 120–300 nuclei. Yet neither esterase nor gut granules are ever expressed (0/120 embryos), arguing that our results are not due to nonspecific aphidicolin toxicity.

The pulse experiments also show that our results are not influenced by the slow increase in DNA content that eventually occurs in aphidicolin-treated embryos (e.g., Figure 2B). As long as DNA synthesis is inhibited during the 1E-cell stage, the DNA content can subsequently increase manyfold without allowing marker expression.

The Rate at Which Markers Become Aphidicolin-Resistant Is Directly Related to the Rate of DNA Synthesis in the E Cell

In Figure 5A, the kinetics of the transition in which gut markers become aphidicolin-resistant are compared to the kinetics of DNA synthesis in the E cell, the gut progenitor. The dashed line shows the time course of DNA synthesis in the E lineage (taken from Figure 1B). The data points (taken from Figure 4A) are the percentage of marker-positive (pipette-permeabilized) embryos calculated for a 2 min time span. The ordinate scales are adjusted so that the maximum level of marker expression (80%) coincides with a cellular DNA content of 4C. Within the scatter of the data, the rate at which the expression of both esterase and gut granules becomes aphidicolin-resistant is the same as the rate of DNA synthesis in the E cell. The transition to aphidicolin resistance is completed over an approximately 15 min time period, compared with a ±1 min uncertainty in assigning the ages of individual embryos.

We next show that slowing the rate of DNA synthesis (by low doses of aphidicolin) also slows the rate at which esterase expression becomes aphidicolin-resistant. In this experiment groups of embryos were staged at the second cleavage (taken as t = 0 min) and permeabilized, and then low doses of aphidicolin (0–1.25 μg/ml) were added at t = 15 min, i.e., close to the beginning of the 1E cell cycle. In some groups of embryos, the rate of DNA synthesis was quantitated using DAPI fluorescence. In other groups

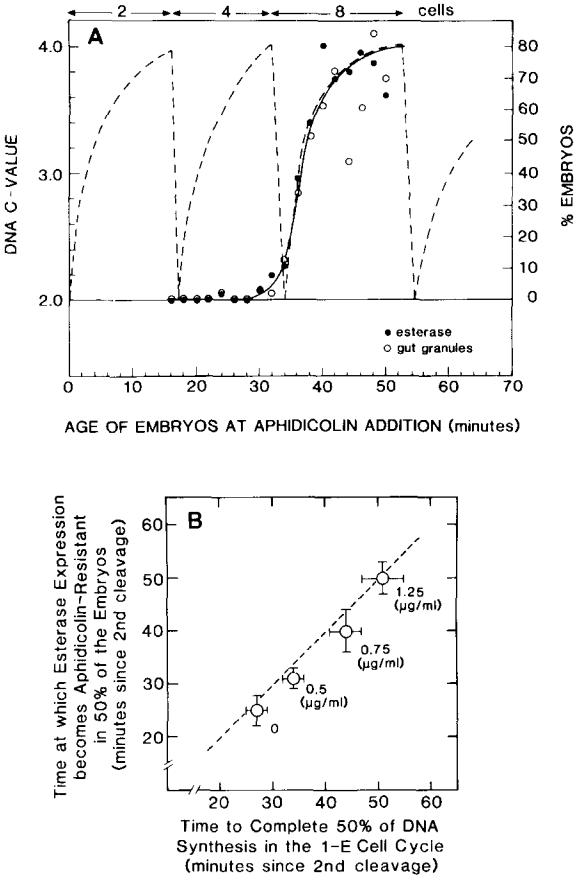

Figure 5. Gut Markers Become Aphidicolin-Resistant at the Same Time as DNA Is Synthesized in the E Cell, the Clonal Progenitor of the Gut

(A) Data from 550 pipette-permeabilized embryos (recalculated from Figure 4) plotted as the percentage of embryos expressing gut granules (open circles) or esterase (closed circles) as a function of embryo age. The dashed curve shows DNA synthesis in the E (gut precursor) lineage, taken from Figure 1B. The total number of cells in the embryo is shown at the top; this representation is only approximate since cleavages are not perfectly synchronous.

(B) Aphidicolin-resistant esterase expression at intermediate rates of DNA synthesis. Experiments similar to those plotted in (A) were performed using doses of aphidicolin that slowed but did not completely block DNA synthesis. (Control experiments showed that 2-cell embryos incubated with up to 2.0 μg/ml aphidicolin eventually produced esterase). Pipette-permeabilized embryos in a single preparation were staged at second cleavage (t = 0 ± 1.5 min), and aphidicolin was added at 15 min (22°C), when DNA synthesis for cycle 2 should be essentially complete and the 1E cell cycle close to starting. After a suitable period (10–45 min depending on the aphidicolin dose), the staged embryos were transferred to a high dose of aphidicolin to block further DNA synthesis and then assayed for esterase production following overnight incubation. Two to five experiments were done for each drug dose, giving a minimum of 10 embryos per point when data were plotted as in (A).

In parallel experiments, the rate of DNA synthesis in the E lineage of staged embryos was quantitated by DAPI fluorescence at the same aphidicolin doses (6–10 embryos per point, over 45 min following drug addition). The time (in minutes since the start of the experiment) for DNA synthesis in the 1E cell to be half-completed is plotted against the time for esterase expression to become resistant to the high dose of aphidicolin in 50% of the embryos. Aphidicolin doses were 0, 0.50, 0.75, and 1.25 μg/ml, as indicated. The dashed line (slope = 1) shows the curve expected if aphidicolin resistance of esterase expression correlated perfectly with DNA synthesis in the 1E cell. The error bars on the data points are estimated as the maximum range of times that could possibly fit the data.

Table 1. Esterase Expression and Gut Granule Expression Are Highly Correlated in Individual Embryos

Time of Assay (min after first cleavage)	Marker		Number of Expressing Embryos
	Gut Granules	Esterase	
33 ± 2	+	+	16
	+	−	2
	−	+	2
	−	−	24
37 ± 2	+	+	45
	+	−	2
	−	+	0
	−	−	35

of embryos, a high dose of aphidicolin was added at various times and esterase expression was then assayed after overnight incubation (see Experimental Procedures for full details). The data were of the same general form as in Figure 5A, except that as the initial dose of aphidicolin was increased from 0 to 1.25 μg/ml, the time required to complete the 1E cell cycle of DNA synthesis was increased from about 15 min to about 45 min. For each aphidicolin dose we estimated the time at which the round of DNA synthesis was 50% complete, as well as the time at which esterase expression had become aphidicolin-resistant in 50% of the embryos. These two times are plotted against each other in Figure 5B; the correlation is obvious. In other words, if DNA synthesis in the 1E cell cycle is slowed, then the transition to aphidicolin resistance of the gut esterase is equally slowed. Within the scatter of the data, curves representing DNA synthesis and marker expression are superimposable.

A further important observation can be made from the above data. In a group of embryos partway through the transition to aphidicolin resistance and in which marker expression is incomplete, individual embryos that will eventually express esterase have a high probability of also expressing gut granules (and vice versa); examples of such data are shown in Table 1. In other words, expression of the two markers in individual embryos is not independent but is highly correlated.

Markers in Different Cell Lineages Also Show Transitions to Aphidicolin Resistance but at Different Times

To determine that the time of the transition to aphidicolin resistance seen in Figures 3–5 is indeed characteristic of gut markers rather than a general feature of all embryonic gene expression, we repeated the aphidicolin addition experiments using two quite different markers of biochemical differentiation: a monoclonal antibody to a hypodermal antigen (presumably expressed in parts of the C and AB lineages) and a polyclonal antibody to body-wall muscle paramyosin (expressed in parts of the C, D, and MS lineages). As shown in Figure 6, we find that expression of both markers can be inhibited by α-amanitin, suggesting the involvement of zygotic transcription. Moreover, both markers show prior transitions from aphidicolin sensitivity to aphidicolin resistance but at quite different times: expression of the hypodermal antigen becomes aphidicolin-

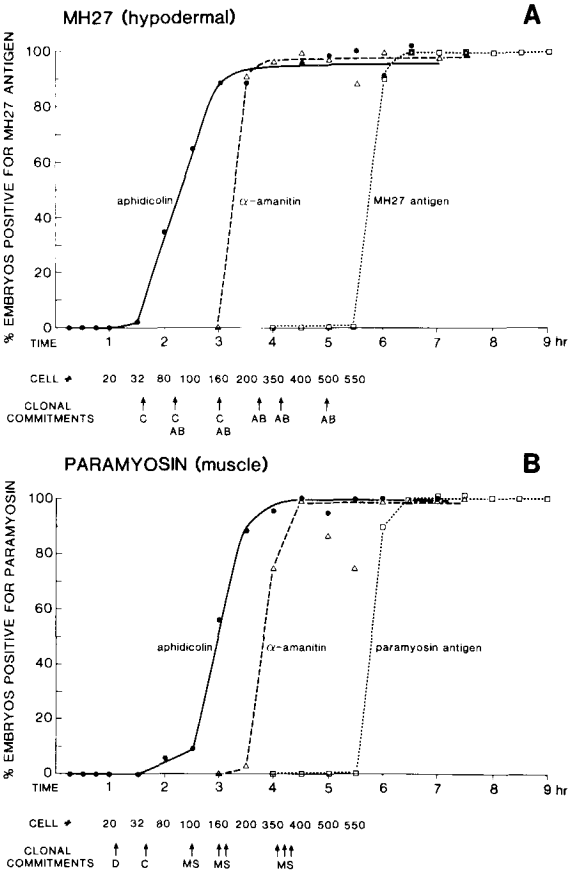

Figure 6. Expression of Nongut Differentiation Markers in the Presence of Aphidicolin or α-Amanitin

(A) Expression of a desmosomal antigen expressed primarily in hypodermal cells and identified by the monoclonal antibody MH27. (B) Expression of body-wall muscle paramyosin. Ordinate: percentage of marker-positive embryos within a 15 min age span. Abscissa: time of permeabilization and drug addition (hours after first cleavage, 22°C). Each point represents an average of 7–65 embryos; embryos were scored simultaneously for both markers. Closed circles represent the percentage of embryos expressing the marker if aphidicolin was added at the indicated time; open triangles represent the percentage of embryos expressing the markers if α-amanitin was added at the indicated time; open squares represent the percentage of untreated embryos in which the antigens were detected at the indicated time, i.e., the normal time of appearance of the marker. Cell divisions giving rise to clonally committed cells are indicated along the abscissa for the several lineages in which these antigens are expressed.

Although MH27 also reacts with intercellular junctions in the gut of late untreated embryos, gut staining with the present methods appears only at 8.5 hr; this is 2 hr after hypodermal staining is observed. Furthermore, gut staining did not appear in any of the embryos treated with aphidicolin, α-amanitin, or cytochalasin D. Thus the staining scored only refers to hypodermal staining.

resistant at about 140 min after first cleavage, and paramyosin expression becomes aphidicolin-resistant about 180 min after first cleavage. Thus expression of both nongut markers has important qualitative features in common with the expression of the two gut markers, namely, a transition to aphidicolin resistance followed by a transition to α-amanitin resistance. Since the lineages expressing these markers are complex, it is difficult to determine

whether the transition times to aphidicolin resistance correspond to clonal establishments.

Discussion

In the first section of this paper, we measured the time course of DNA synthesis during early cleavages of the individual cell lineages of the C. elegans embryo. During this period of early development, cell cycle times are 15–20 min (Deppe et al., 1978) and DNA synthesis occupies most of the time between mitoses. The rate of DNA synthesis is not constant, but, rather, 50% of each round is completed in (approximately) the first 25% of each cycle. When the individual cell lineages slow their cell cycles, the rate of DNA synthesis decreases, not the fraction of the cell cycle occupied by S phase; that is, there is no indication of a G1 or a G2 phase in these early cycles. A G2 phase first appears in the gut lineage shortly before gastrulation.

The most important feature of our results is that, in order to express the two gut-specific markers, esterase and gut granules, embryos must enter (but not necessarily finish) the round of DNA synthesis that takes place in the single E cell of the 8-cell embryo. This is the first round of DNA synthesis after the gut has been clonally established. Judging from the time at which marker expression becomes resistant to α-amanitin, gut-granule-associated transcription occurs about 30 min later, when the embryonic gut has 2 cells; esterase transcription occurs over 2 hr later, at a point when the gut has 4–8 cells. Yet, despite their different transcription times, both markers show identical responses to aphidicolin. Perhaps this crucial period of DNA synthesis is the time when a number of gut-specific genes become "activated" or are somehow given permission for later expression.

Two facts argue particularly strongly that the effects of aphidicolin on marker expression are due to the inhibition of DNA synthesis and not to some nonspecific effects of the drug. First, as shown in Figure 5B, when DNA synthesis is slowed, the rate at which marker expression becomes aphidicolin-resistant also slows in a directly proportional fashion. Second, in other systems aphidicolin resistance is invariably associated with mutations in a single gene associated with DNA metabolism (see, e.g., Sugino and Nakayama, 1980).

As can be seen in Figure 4, there are two (or, in some embryos, possibly as many as three) rounds of DNA synthesis that normally occur between the time at which esterase expression becomes aphidicolin-resistant and the time at which it becomes α-amanitin–resistant. Yet these rounds of DNA synthesis can be inhibited without altering either the timing or the extent of esterase expression. Thus esterase expression is controlled neither by counting the normal number of rounds of DNA synthesis nor by achieving the normal DNA:cytoplasm ratio. An identical statement can be made about gut granule production. Thus our results do not conform to the proposal that the DNA:cytoplasm ratio controls the onset of embryonic transcription (Newport and Kirschner, 1982a, 1982b; Edgar et al., 1986). However, there is the question of whether expression of the two lineage-specific markers described here can really be considered homologous to the generalized transcription that occurs at the midblastula transition in Xenopus and Drosophila.

The original proposal of Newport and Kirschner (1982a, 1982b) has subsequently been modified (Edgar et al., 1986; Edgar and Schubiger, 1986; Kimelman et al., 1987) to suggest that the DNA:cytoplasm ratio controls the onset of the midblastula transition through its effect on the embryonic cell cycle. The revised model states that embryos can be competent for transcription, but actual transcription only occurs when a G1 or G2 phase appears in the cell cycle. In the present system, if slowing of the cell cycle were the only factor controlling transcription times, both gut granules and esterase should be expressed simultaneously. However, transcription of the esterase gene takes place one cell division and more than 1 hr later than does transcription leading to gut granule production. Thus, in the present system, a simple elongation of the cell cycle does not appear to be sufficient to cause marker expression.

We can also ask whether the time of the transition to aphidicolin resistance (rather than the time of gene transcription) is governed by the DNA:cytoplasm ratio in the embryo. Strictly interpreted, our results do not support this suggestion. As seen in Figure 5, marker expression becomes aphidicolin-resistant very early in the third cell cycle in some embryos but very late in the third cell cycle in other embryos. In other words, aphidicolin resistance of marker expression can occur over a 2-fold range in the DNA:cytoplasm ratio.

Satoh and colleagues have used aphidicolin to study how inhibition of DNA synthesis influences acetylcholinesterase expression during muscle development in ascidians. They made elegant use of naturally occurring muscle sublineages and concluded that cells in the different sublineages must all go through the same number of rounds of DNA synthesis in order to express the marker (Satoh and Ikegami, 1981a, 1981b). Their initial interpretation indicated that this critical replication round did not correspond to the cell cycle following clonal establishment of a sublineage. However, these conclusions were somewhat ambiguous because of complexities in some of the cell lineages (see, e.g., Zalokar and Sardet, 1984) and uncertainty about which replication round was actually inhibited by aphidicolin. One particular sublineage has since been reevaluated (Mita-Miyazawa et al., 1985); if we assume that aphidicolin inhibits DNA synthesis instantly and completely, then the critical round of DNA synthesis required for marker expression indeed turns out to be the first round after the sublineage has been clonally established. This agrees with our findings. However, it is not clear whether this pattern is general since expression of a second muscle marker appears to depend on an earlier round of replication (Nishikata et al., 1987).

Our results can be explained by the presence of an embryonic clock that is independent of DNA synthesis (at least after the gut has been clonally established) and is also independent of cytokinesis. Indeed, since both aphidicolin and cytochalasin D were usually added to-

gether, the time of marker expression is simultaneously independent of DNA synthesis and cytokinesis, i.e., one process does not act as a backup clock for the other. Although transition to aphidicolin resistance occurs when the embryo has 8 cells, we do not know at present whether the preceding two rounds of DNA synthesis are indeed required; for example, a clock could be started at fertilization, count three rounds of replication, and only then become independent of DNA synthesis. The alternative possibility is that markers can ultimately be expressed as long as any DNA synthesis is occurring during this critical time period. We attempted to distinguish between these two possibilities by using aphidicolin pulses, but our results were ambiguous.

Within the accuracy of our data (Figure 5), the probability that a particular embryo will ultimately express either marker appears to increase with the same time course as does overall DNA synthesis in the single E cell of the 8-cell embryo. In any case, it is clear that some embryos obtain permission for marker expression without completing this round of replication. If markers become aphidicolin-resistant by virtue of the replication of only limited regions of the genome, it is unlikely that such regions are the marker structural genes. That is, the structural genes for the markers are dispersed throughout the genome (Siddiqui and von Ehrenstein, 1980; McGhee and Cottrell, 1986), but individual embryos become aphidicolin-resistant for both markers simultaneously (Table 1). This suggests that the replication of a single common locus is required for later gut-gene activation.

If we suppose that only a particular segment of the genome (but nonetheless the same segment in all embryos) need be replicated during the 1E cell cycle for markers to be expressed, the breadth of the transition to aphidicolin resistance implies that this specific genomic region is replicated at different times in different embryos. In other words, during the rapid cleavages of early development, the gene-specific temporal patterns of replication observed in "mature" cells (Furst et al., 1981; Pierron et al., 1984; Goldman et al., 1984) have not yet been established.

In summary, our results suggest that gene expression during development of the C. elegans intestine is not controlled by the DNA:cytoplasm ratio in the embryo nor is it controlled by passage through the normal number of rounds of DNA replication. Elongation of the cell cycle also does not appear to be sufficient for marker expression. Rather, markers seem to be given permission for later expression during a limited period of DNA synthesis that occurs just after the gut has become clonally established. Further DNA synthesis is not required. Perhaps it is during this critical period of DNA synthesis that cytoplasmic determinants migrate to the nucleus, take their place at specific sites on the chromosomes, and in some way bring about later gene expression.

Experimental Procedures

DNA Quantitation

Embryos were treated with sodium hypochlorite for 3 min (0.5% in egg buffer [Edgar and McGhee, 1986]), rinsed in embryonic growth medium (EGM; slightly modified from Edgar and McGhee, 1986; details supplied on request), and digested with chitinase (20 mg/ml) and chymotrypsin (10 mg/ml) in egg buffer for 4–6 min to remove the eggshell, leaving the vitelline membrane intact. With no further treatment, such embryos develop normally and will eventually hatch.

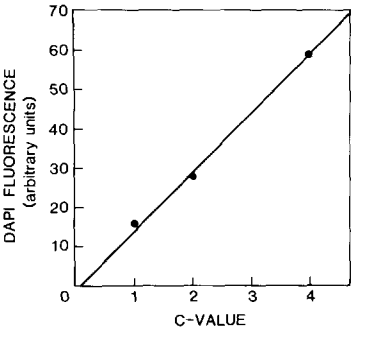

Figure 7. Typical Standard Curve Relating DNA-Induced DAPI Fluorescence to Nuclear DNA Content

Data points correspond to polar bodies (1C), condensed anaphase chromosomes (2C), and a combination of late prophase and metaphase nuclei (4C). The least-squares regression line was then used to calculate DNA content for all nuclei on the same slide.

Individual embryos incubated at 22°C in EGM were staged at 2 min intervals by observing the first, second, or third AB cleavages under the dissecting microscope. At appropriate times, groups of embryos were pipetted into a 3 μl drop of fixative (2.5% paraformaldehyde, 0.1% glutaraldehyde in 125 mM phosphate buffer [pH 7.4]) on a gelatin-subbed slide and flattened immediately with a coverslip to break the vitelline membrane and to convert the embryo to a monolayer of cells. The slide was flooded with additional fixative, fixed a total of 3 min at room temperature, and rinsed in 125 mM phosphate buffer (pH 7.4). For uniformity, 10–20 slides were processed through the following steps together: slides were treated for 5 min in 0.5% Triton X-100 in PBS, washed (25 min in PBS; 15 min in 2× SSC), incubated with RNAase A (100 μg/ml in 2× SSC for 1 hr at 37°), washed (5 min in PBS), stained with the fluorescent DNA-binding drug DAPI dihydrochloride (Boehringer-Mannheim; 1 μg/ml in PBS) for 30 min, and finally washed in PBS (35 min under minimal light exposure). DNA readings were made immediately using a Zeiss Universal microscope equipped with MPM03 photometer. All readings were made at a single lamp field stop (0.25 mm) and measuring aperture (0.32 mm); with the 40× objective, this produced a measuring field 5.0 μm in diameter. The DNA content in all nuclei in all well-squashed embryos (generally 10–30) on a slide was measured, as well as cytoplasmic background for each embryo. Repeated control readings showed that fading of DAPI was negligible (<5%) over the time period of the measurements.

For data analysis, a cytoplasmic background reading was first subtracted from the nuclear readings for each embryo. This background varied considerably for different slides and different embryos but was in the range of 0.3–1.0 × C, where C represents the haploid DNA content. The readings from each slide were converted to DNA content (C values) by a standard curve derived from readings of polar bodies (1C), telophase nuclei (2C), and late prophase or metaphase nuclei (4C); a typical plot is shown in Figure 7. Not all stages were always present on each timed slide (e.g., polar bodies disappear or give obviously low readings during the third cell cycle), but nonetheless a consistent estimate could be made for each slide. For the third and fourth cell cycles, it was generally possible to normalize each embryo to its own metaphase or telophase nuclei.

Nuclei were identified with respect to lineage both by their position in the embryo and by their stage in the mitotic cycle (e.g., all AB nuclei divide synchronously and are easily identified even in later embryos). Where feasible, the accuracy of timing was further improved by ordering embryos according to a particular metaphase or telophase, especially in the third and fourth cell cycles when divisions become more asynchronous in the various lineages. This reduced experimental variation both from inaccuracies in timing and from the inherent spread of

2–3 min observed in living embryos (Deppe et al., 1978; Sulston et al., 1983; data not shown). These stages were then adjusted to cytokinesis times observed in unperturbed embryos under the compound microscope (averaged for 20 embryos at 22°C).

Drug Treatment and Embryo Culture

For drug treatment, early embryos were collected by cutting gravid hermaphrodites and were permeabilized by one of two methods. In the simpler method, embryos were treated briefly with hypochlorite (0.5% in egg buffer for 1 min), rinsed in EGM, and mounted in 3 μl of EGM on gelatin-subbed slides (2%) under a coverslip supported by drafting transfer tape (3M). Pressure was then applied to the coverslip to permeabilize the vitelline membrane and to attach the embryos. Additional medium (20 μl) was added to fill the chamber, which could then be sealed with plastic wrap. Slides with 30–60 embryos were observed by Nomarski optics during one division round, and the time of either the first, second, or third cleavage (22°C) for each embryo was noted on a video tracing or drawing. Medium containing 7.0 μg/ml aphidicolin with or without 1.5–5 μg/ml cytochalasin D (depending on the experiment) was then drawn under the coverslip (three changes of 30 μl), and the embryos were incubated overnight at 16°C. Cytochalasin D effects, indicating drug entry, were apparent as soon as embryos could be observed after drug addition (1–2 min). Nonpermeabilized embryos continued to develop and were not scored.

To allay doubts about slow or incomplete penetration of aphidicolin influencing the narrow time of transition observed, a second permeabilization method, pipette permeabilization, was used to ensure the most rapid and complete drug entry possible. Chitinased embryos (see above) were transferred to EGM and stripped of the inner vitelline membrane by brief pipetting into a small capillary. Groups of embryos timed with respect to a particular cleavage (±1 min) under the dissecting microscope were cultured in chambers containing 30 μl of EGM with appropriate drug additions. Pipette-permeabilized embryos required a slightly lower aphidicolin dose (5.5 μg/ml) than did pressure-permeabilized embryos to give the same inhibition of DNA synthesis.

The experiments shown in Figure 5B used a new stock of aphidicolin that was shown by fluorescence quantitation to inhibit DNA synthesis completely at a level of 3.5 μg/ml. To minimize long-term toxicity effects in these experiments, DNA synthesis was blocked following the low aphidicolin dose (slowdown) period by a pulse of 20 min at 4.0 μg/ml aphidicolin followed by overnight incubation at 2.0 μg/ml aphidicolin. A temperature-controlled stage was used on the dissecting microscope to ensure reproducible cleavage times during these experiments, since long periods of observation and manipulation were required.

In experiments involving treatment with α-amanitin in addition to aphidicolin (Figure 4), pipette-permeabilized embryos were first blocked with aphidicolin at 16–32 cells and then transferred to EGM containing 150 μg/ml α-amanitin at the appropriate times.

After incubation for 12–16 hr in a damp chamber (16°C), embryos were scored for gut granules under polarized light, using a Zeiss IM35 inverted microscope. Embryos were then stained for esterase activity by fixing for 3 min (2.5% paraformaldehyde in 125 mM phosphate buffer [pH 7.4] at 4°C), rinsing 5 min or more in buffer, and staining for 1 hr at 4°C with pararosaniline and α-naphthyl acetate, a slight modification of our previous method. Following a buffer rinse and DAPI staining (3 μg/ml in 125 mM phosphate), embryos were observed and scored using Nomarski optics.

Two nongut markers, muscle paramyosin and hypodermal desmosomes, were scored by immunofluorescence. Groups of staged embryos were chitinased, incubated at 22°C for various times, pipette-permeabilized in medium containing either aphidicolin (5.5 μg/ml) or α-amanitin (150 μg/ml), and finally incubated overnight at 16°C. To determine the normal time of antigen expression, chitinased nonpermeabilized embryos were incubated in EGM and fixed at various times. For immunofluorescence, embryos were fixed and mounted by the same method described above for DNA determination, rinsed in PBS, and then treated for 5 min in 0.5% Triton X-100 in PBS. Following a 5 min PBS rinse, slides were incubated for 30 min at 37°C with normal goat serum (diluted with an equal volume of PBS containing 0.1% Triton X-100) and then incubated for 1 hr at 37°C with primary antibodies. Primary antibodies were a polyclonal rabbit antiserum to C. elegans body-wall paramyosin, generously provided by W. B. Wood, University of Colorado; and MH27, a monoclonal mouse antibody that stains desmosomes of the hypodermal cells and late embryonic gut-cell junctions, generously provided by Ross Francis, Washington University. Primary antibodies were diluted 1:100 and 1:200, respectively, in PBS, 0.1% Triton X-100. Slides were then rinsed (3–5 min) in PBS and incubated for 1 hr at room temperature with fluorescent secondary antibodies (fluorescein-labeled goat anti-rabbit diluted 1:100, and rhodamine-labeled goat anti-mouse diluted at 1:120 in PBS, 0.1% Triton X-100; both obtained from Jackson Laboratories), rinsed again with PBS, stained with DAPI (0.5 μg/ml in PBS) for 15 min, and mounted in Gelvatol. Observations were made on a Zeiss Universal microscope equipped with epifluorescence illumination and Nomarski optics.

Stock solutions were as follows: cytochalasin D (Sigma) at 2.5 mg/ml in ethanol; aphidicolin (Sigma) at 10 mg/ml in dimethyl sulfoxide and diluted on the day of use; and α-amanitin (Sigma) stored frozen at 1.5 mg/ml in water.

Photography

General photography used Kodak Technical Pan film (ASA 125). Photographs for Figure 2 were taken with Tri-X film (ASA 400) at a constant exposure of 3 sec, and were developed and printed identically.

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