Morphogenesis and regulated gene activity are independent of DNA replication in *Xenopus* embryos

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Summary

*Xenopus* embryos were transferred into media containing aphidicolin at late blastula, mid-gastrula, and early neurula stages. In each case, embryos continued to differentiate in the absence of DNA replication. When the inhibitor was added at late blastula, embryos continued to develop for about 8 h. However, when aphidicolin was added at the early neurula stage, development could be seen for up to 40 h after addition. The influence of replication on embryonic gene activity was studied by RNA blot analysis. Of the genes we examined only histone gene expression was down regulated by the addition of aphidicolin. The expression of various embryo-specific genes was unaffected by the lack of DNA synthesis. Even after several hours of treatment with aphidicolin, replication-inhibited tailbud and tadpole stages showed the same levels of specific mRNAs as control embryos containing 4–5 times more DNA. We conclude that morphogenesis and embryo-specific gene activity are independent of both DNA replication and a precise amount of DNA per embryo.

Key words: aphidicolin, replication, *Xenopus*, embryogenesis.

Introduction

The first 6 h of embryonic development in the frog *Xenopus laevis* is characterized by several rounds of rapid synchronous cell divisions. During this period, each cell cycle lasts about 30 min and consists of DNA synthesis and mitosis without discernible G1 and G2 phases (Graham and Morgan, 1966). At the 4000-cell mid-blastula stage, there is an abrupt change to a more complex cell cycle, as G1 and G2 phases are measurable and the S and M phases elongate during an event called the mid-blastula transition (MBT). After the MBT, cleavages become less synchronous, transcription is initiated (Brown and Littna, 1964), and cells become motile (Johnson, 1976). Evidence suggests that this transition from an early embryonic to a somatic type cell cycle is triggered by an increased DNA-to-cytoplasm ratio resulting from the preceding twelve division cycles (Newport and Kirschner, 1982a and b).

Experiments in which DNA synthesis has been inhibited by the drug aphidicolin have produced evidence for a difference in the control of the cell cycle in early embryos relative to that in somatic cells (reviewed in Hartwell and Weinert, 1989). Aphidicolin is a tetracyclic diterpene tetraol produced by the fungus *Cephalosporium aphidicola* and is a competitive inhibitor of DNA polymerases α and δ (Ikegami et al. 1978; Goscin and Byrnes, 1982). The α and δ polymerases are responsible for chromosomal DNA duplication; DNA polymerase δ functions in leading strand synthesis and DNA polymerase α functions in lagging strand synthesis (Tsurimoto et al. 1990). Aphidicolin does not inhibit DNA polymerases β and γ, which are involved in DNA repair and mitochondrial DNA synthesis, respectively (Ikegami et al. 1978). Inhibition of DNA replication in yeast or vertebrate somatic cells blocks chromosome condensation, formation of the mitotic spindle and cytokinesis (Rattner and Phillips, 1973; Byers and Goetsch, 1974). However, inhibition of DNA synthesis in early embryos from several species shows the lack of a strict coupling between replication and cell division when aphidicolin is added shortly after fertilization. Fertilized eggs from starfish (Nagano et al. 1981) and sea urchins (Sluder and Lewis, 1987) will undergo cell cycle progression in the presence of aphidicolin. Cytoplasmic and nuclear aspects of the mitotic cycle will also continue in *Drosophila* embryos after treatment with the inhibitor (Raff and Glover, 1988, 1989). In the first few hours of *Xenopus* development, inhibition of DNA synthesis has no effect on surface contraction waves or cell division when aphidicolin is added to fertilized eggs (Kimmelman et al. 1987; Newport and Dasso, 1989), although cell division is arrested if the inhibitor is added after embryos have accumulated more than 700–800 cells (Dasso and Newport, 1990).

We are interested in the role of DNA replication in controlling the specificity and timing of gene expression during vertebrate embryogenesis. Experiments with cells in culture have shown that differentiation can
occur without DNA replication in cells that have been inhibited with cytosine arabinoside (ara-C) (Nadal-Ginard, 1978; Pinset and Whalen, 1985). Experiments with heterokaryons in which DNA replication is inhibited with ara-C have also shown that DNA synthesis is not necessary for induction of muscle gene expression in a cell normally not expressing those genes (Chiu and Blau, 1984). We asked if DNA replication has an influence in acquiring the differentiated phenotype during Xenopus development. Our experiments stem from the observation that visible morphogenesis continues when DNA synthesis is inhibited by aphidicolin (Andrews and Brown, 1987). We have since found that developing Xenopus embryos carry on extensive differentiation in the absence of new DNA synthesis for periods as long as 1–2 days. In this paper, we describe experiments in which this differentiation is studied at both the morphological and molecular level in an attempt to determine the dependence of organogenesis and regulated gene activity on the process of DNA replication.

Materials and methods

Preparation of experimental embryos

Xenopus eggs were fertilized in vitro and maintained in 0.1× OR-2 medium (Wallace et al. 1973). Fertilized eggs were dejellied in 2% cysteine (pH 8.0), rinsed thoroughly with 0.1× OR-2, and kept in 0.1× OR-2 at either 18°C or 23–25°C until they developed to the appropriate stage. Embryos were staged according to Nieuwkoop and Faber (1956). At appropriate stages the embryos were divided into two groups, one developing in 0.1× OR-2 medium with 125 μg ml⁻¹ aphidicolin (Boehringer Mannheim), and the other in 0.1× OR-2 medium alone. The initial aphidicolin stock solution was made 20 mg ml⁻¹ in dimethyl sulfoxide (DMSO); therefore, control OR-2 medium contained the same amount of DMSO (0.63%) as the aphidicolin-containing medium. Both groups developed at 23–25°C. Embryos were collected from each group at selected time points for the purpose of DNA and RNA analysis. Photographs were taken with Kodak Ektachrome Tungsten 160 film on a Wild stereomicroscope and camera or with Kodak TMax 100 film on a Zeiss stereomicroscope with a Canon camera.

Preparation and analysis of embryonic DNA and RNA

Groups of 2 or 3 embryos used for DNA analysis were homogenized by a micropipet in 0.3 ml SETS (150 mm NaCl, 10 mm EDTA, 50 mm Tris [pH 7.5], 0.5% SDS) and stored at −20°C until all collections were completed. Homogenates were incubated for 30 min at 55–60°C with 250 μg ml⁻¹ protease K, extracted once with phenol, once with 1:1 phenol:chloroform, and precipitated from 0.3 m sodium acetate with 2 volumes cold ethanol. One-fourth or one-eighth embryo equivalents of total nucleic acid were analyzed by electrophoresis on a 0.5% agarose gel. For slot blot analysis of specific DNA sequences, the DNA was denatured and the RNA degraded by a 1 h incubation at 60–70°C in 0.3 m NaOH. Ammonium acetate was used to adjust pH and ionic strength, and the DNA was immobilized onto nitrocellulose using a Schleicher and Schuell Minifold II slot blot apparatus. The filter was baked in a vacuum oven at 80°C for 2–3 h. Probes were made from gel-purified plasmid inserts, since vector sequences such as pBR322 showed some hybridization with Xenopus genomic DNA. The insert was labeled by random priming (Feinberg and Vogelstein, 1983, 1984) using α[³²P] dCTP (3000 Ci mmol⁻¹). Occasionally RNA probes were made using SP6 RNA polymerase with α[³²P] UTP (400 Ci mmol⁻¹). In order to monitor [³H]thymidine incorporation in living embryos, 6-[³H] thymidine (20–30 Ci mmol⁻¹) was diluted to a concentration of 0.1 mCi ml⁻¹ in control and aphidicolin-containing media. Embryos were incubated in these solutions and collected at appropriate time points. Total nucleic acid was prepared as previously described, one-half embryo equivalents were spotted onto Whatman GF/C filters, and TCA precipitable counts were measured by scintillation counting.

Depending on the experiment, anywhere from 5–25 embryos were collected at each time point for RNA blot analysis. These embryos were homogenized in 2 ml SETS with a Brinkmann Polytron homogenizer and stored at −20°C until all collections were completed. Usually, an additional 2 ml SETS was added to the homogenate before extraction. Homogenates were extracted once with phenol, twice with 1:1 phenol:chloroform, and precipitated with 2 volumes cold ethanol. Total nucleic acid was pelleted, resuspended in 0.2× SETS, distributed into aliquots equivalent to 5 embryos each, and re-precipitated with ethanol. A single aliquot was pelleted and resuspended in 10 μl of 0.1× Tris [pH 7.5], 0.1% SDS, and was then divided into two tubes containing 4 μl each (or 2 embryo equivalents). MOPS buffer, formaldehyde, and formamide were added to each tube and samples were heated to 65°C, followed by the addition of dyes. RNA was separated on a 1.5% agarose gel containing formaldehyde and transferred to nitrocellulose (Maniatis et al. 1982). Probes for RNA blots were made from linearized plasmids containing specific Xenopus sequences. Typically, 0.1 μg of DNA was labeled by random priming (Feinberg and Vogelstein, 1983, 1984) using α[³²P] dCTP (3000 Ci mmol⁻¹). The blots were prehybridized and hybridized at 42°C in 50% formamide, 5×SSPE, 0.1% SDS, 2×Denhardt's reagent, 100 μg ml⁻¹ denatured DNA and 200 μg ml⁻¹ tRNA. After hybridization, filters were washed twice at 42°C with 0.1×SSPE, 0.1% SDS for 15 min each. If needed, they were also washed at 65°C.

Results

Duration of development is dependent on the time of aphidicolin addition

At the beginning of each experiment, developing Xenopus embryos were divided into a control group and an aphidicolin group. Embryos in the aphidicolin group were placed in media containing 125 μg ml⁻¹ (0.37 mm) aphidicolin. We used this relatively high concentration of inhibitor based on the observation that lower concentrations (such as 75 or 100 μg ml⁻¹) failed to completely inhibit DNA replication in many of the groups that we examined. Embryos were transferred into media containing aphidicolin at three different developmental stages: late blastula (stage 9), mid-gastrula (stage 11), and early neurula (stage 13). Stage numbers are according to Nieuwkoop and Faber (1956). For comparison purposes, embryos in both the control group and the aphidicolin group were from the same
batch of siblings. During the course of each experiment, embryos treated with aphidicolin would eventually develop more slowly than the control group. An experiment was terminated when further development of the inhibitor-treated embryos could not be detected based on their external appearance. At the end of an experiment aphidicolin-treated embryos were often misshapen and fragile compared to the control group.

Genomic DNA was prepared from control and inhibitor-treated embryos beginning at the time of transfer into aphidicolin (0 h) and at various time points throughout the course of the experiment. Fig. 1A shows the normal increase in genomic DNA content (on a per embryo basis) during the first seven hours after the late blastula stage (stage 9). Densitometric analysis indicates a five- to six-fold increase in DNA per embryo during this entire period. On the same gel the DNA content of the aphidicolin-treated siblings is significantly less at each time point. Any apparent increase in the DNA content of replication-inhibited embryos is small and could be a result of the procedure for extracting embryonic DNA. Embryos that are transferred into aphidicolin at the late blastula stage will continue to differentiate for 7–9 h. At the end of this period, the control group has begun to develop neural folds (stage 14/15). When embryos at the middle gastrula stage (stage 11) are transferred to aphidicolin, development will proceed for up to 26 h in the presence of inhibitor. Therefore, by adding aphidicolin just 4 h after the late blastula stage, differentiation will continue for a full day rather than 7–9 h. Fig. 1B shows little or no increase in DNA during the 26 h period when embryos are developing in the presence of aphidicolin; during the same time period there is a two- to three-fold increase in the DNA content of the control group. When development of the inhibitor-treated group ceases, the control embryos have reached the tailbud stage (stage 29/30). Embryos transferred into media containing aphidicolin at the onset of neurulation (stage 13) will continue to develop for a period of greater than 40 h. In this case, addition of the inhibitor only 3 h after the middle gastrula stage results in 44 h of development before the aphidicolin-treated embryos cease differentiation. Fig. 1C shows the expected increase in DNA per embryo in the control lanes. During this 44 h period, the control embryos increase their total DNA content approximately four- to five-fold, whereas aphidicolin-treated embryos show little change in the amount of genomic DNA. When the experiment concludes, the control embryos have developed into swimming tadpoles (stage 39).

Inhibition of DNA synthesis at the late blastula stage
The usual pattern of differentiation for the first 7–9 h following the late blastula stage includes gastrulation followed by the formation of the neural plate and the neural folds during early neurulation. Fig. 2 shows that *Xenopus* embryos will attempt to undergo the same processes in the absence of DNA replication. 3.5 h after adding the inhibitor at the late blastula stage, the control embryos have formed a blastopore and yolk plug characteristic of gastrulation. The same structures are slightly larger in the aphidicolin-treated embryos, indicating a slower rate of development. After 6 h, the blastopore in the control group is reduced to a slit; however, the blastopore and a small yolk plug are still visible in the inhibited embryos. At 8.5 h, the control embryos have a well-defined neural plate surrounded by neural folds at the anterior end. Embryos treated
with the inhibitor show far less definition in the anterior region, with only a pigmented streak of cells at the location of the future neural groove. At this time, embryos developing in the presence of aphidicolin will no longer differentiate and eventually fall apart, releasing cells from their interior.

The extent of development during the 7–9 h period after adding the inhibitor indicates that many of the events of gastrulation and early neurulation can occur in the absence of DNA replication. This suggests that certain patterns of developmentally regulated gene expression may occur in the absence of new DNA synthesis. To test this idea, we examined levels of gene activity from control and aphidicolin-treated embryos by RNA blot analysis. Total RNA was prepared from embryos collected at various time points beginning at the late blastula stage. Four cDNAs isolated from the *Xenopus* DG library (differentially expressed in gastrula; Sargent and Dawid, 1983) were used as probes. These clones were gifts from I. B. Dawid and T. D. Sargent and were especially useful for our purposes since their respective mRNAs are abundant and the level of each message is developmentally regulated.

Fig. 3 shows the expression of five different genes during the first seven hours of post-blastular development where 0 h is the late blastula stage (stage 9). The steady-state level of mRNAs complementary to cDNA clones designated DG56 and DG70 are shown in

The top panel. The DG70 mRNA encodes an epidermal cytokeratin (Winkles et al. 1985), whereas the DG56 gene product is unknown. The onset of DG70 expression in the aphidicolin-treated embryos occurs at the same time as the controls. Inactivation of the gastrula-specific DG56 gene also occurs at approximately the same time in both groups. The bottom panel shows the steady state levels of transcripts probed with DG42 and a genomic clone containing the genes for *Xenopus* histones H3 and H4.

The bottom panel shows hybridization of mRNAs complementary to the cDNA clone, DG 42, and to a genomic clone containing the genes for *Xenopus* histones H3 and H4. However, the same is not true for histones H3 and H4. At the first collection, 2 h after transfer into...
aphidicolin, the levels of the H3 and H4 mRNAs are greatly reduced when compared to the control lanes. Expression of the histones is often tightly coupled with DNA synthesis (for review see Schümperli, 1986; Marzluff and Pandey, 1988), and histone mRNAs are typically degraded when cells in S phase are treated with inhibitors of DNA replication (Heintz et al. 1983; Sittman et al. 1983). If inhibition with aphidicolin blocks the embryonic cell cycle in S phase or at the G1/S boundary, then it is not surprising that embryos treated with aphidicolin show a reduction in their histone mRNA levels. This suppression of H3 and H4 mRNAs serves as a convenient internal control since it continues for the duration of the experiment, whereas the concentration of the stage-specific DG42 mRNA increases in the same embryos.

Inhibition of DNA synthesis at the middle gastrula stage
During the first 8–9 h after aphidicolin is added at the middle gastrula stage (stage 11), external differentiation of the inhibitor-treated embryos appears to continue at the same rate as the control group (Fig. 4). This includes closure of the blastopore, formation of the neural plate and neural folds, convergence of the neural folds, and a slight curvature and elongation along the anteroposterior axis. At 10 h the control embryos have reached the late neurula stage (stage 19), showing a distinct curvature and presence of the dark cement gland. Replication-inhibited embryos also form the cement gland but individual embryos appear less concave in the mid-lateral region. 7 h later the control embryos (stage 24/25) have elongated and show the formation of future eyes and ears. At the same time point (17 h), embryos treated with aphidicolin have also elongated but are relatively featureless in the head area with the exception of the prevailing cement gland. At the end of the experiment, 23–26 h after addition of inhibitor, the control embryos have reached the tailbud stage (stage 29/30) and will occasionally display a twitching sort of movement. Embryos that have developed in the presence of aphidicolin for the same period are not as long, have splotchy pigmentation, and are missing the tailbud altogether. Their exterior is featureless; eye cups, myotomes and the translucent dorsal fin are missing. These embryos cease differentiation and ultimately break open, spilling cells from their interior. DNA isolated from the embryos in Fig. 4 is shown on the agarose gel in Fig. 1B.

To analyze patterns of gene expression during the same experimental period, we probed blots of total RNA with radiolabeled DNAs encoding six different proteins. The top panel of Fig. 5 shows that inactivation of the blastula/gastrula-specific DG56 gene occurs in the aphidicolin-treated embryos at the same time as the controls. In these same embryos, the ectoderm-specific DG81 cytokeratin mRNA (Jonas et al. 1985; Miyatani et al. 1986) is detected 2 h after transfer into medium containing aphidicolin. The level of DG81 message in the aphidicolin-treated embryos parallels the controls, showing a steady increase during the entire 24 h period. This was an unexpected result since the amount of genomic template in the inhibited embryos is a fraction of that found in the controls (see Fig. 1B). Yet both groups show approximately the same level of DG81 mRNA per embryo. RNA that hybridizes to the DG70 probe (Fig. 5, middle panel) is already present when the experiment begins at the mid-gastrula stage (0 h) and also accumulates to about the same level in both groups.
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Fig. 5. Northern blot analysis of total RNA prepared from control and aphidicolin-treated embryos beginning with aphidicolin addition at mid-gastrula (stage 11). Control (CON) and aphidicolin-treated (APH) *Xenopus* embryos were collected at the indicated times (hrs) after transfer of the APH embryos into media containing aphidicolin at stage 11 (0h). Total RNA from 2 embryo equivalents was separated on an agarose gel containing formaldehyde, transferred to nitrocellulose and hybridized with a mixture of labeled DNA probes. The top panel shows hybridization of RNA with cDNA clones DG56 and DG81. The middle panel shows hybridization of mRNAs complementary to the cDNA clone, DG 70, and to a genomic clone containing the gene for *Xenopus* histone H1A. The bottom panel shows hybridization of mRNAs to a genomic clone containing the genes for *Xenopus* histones H3 and H4.

The northern blot showing DG70 mRNA levels was simultaneously probed for histone H1A mRNA (a gift from M. Perry; Perry et al. 1985) to investigate whether the message for this linker histone is affected by the inhibition of DNA synthesis. The level of H1A mRNA drops significantly within 2h of aphidicolin treatment. This level remains low, showing no increase for the duration of the experiment. Again, the same is true with histones H3 and H4 (Fig. 5, bottom panel). We conclude that when the *Xenopus* middle gastrula stage embryo differentiates in the absence of DNA replication, the timing and levels of three developmentally regulated DG mRNAs (DG56, DG70, and DG81) are unchanged by the lack of replication or by the abnormally low level of DNA per embryo. However, in the same embryos, the mRNAs encoded by three genes whose expression is probably coupled to replication (histones H1A, H3, and H4) are greatly reduced.

**Inhibition of DNA synthesis at the onset of neurulation**

The most striking feature of blocking DNA synthesis at the onset of neurulation (stage 13) is the length of time for which the embryos continue to differentiate without a significant increase in DNA content. Fig. 6 shows that differentiation continues for over 40h after the addition of inhibitor. At the completion of the experiment the control embryos have developed into swimming tad-
poles (stage 39). For the first 16 h of development the external appearance of the aphidicolin-treated embryos is indistinguishable from that of the controls. Both groups have elongated along their anteroposterior axis and appear to be at stage 24, although the aphidicolin group has only a fraction of the DNA found in a control stage 24 embryo (see Fig. 1C). Over the next three hours (from stage 24 to stage 26, 19 h after addition), one can detect subtle morphological differences that tend to vary with each sibling group. Characteristic differences seen in the inhibited embryos include a slight curvature of the embryo and a rounded tail which fails to form the typically pointed tailbud. Another difference is a poorly defined, less pigmented eye cup that tends to have a more rostral location than the controls. As development continues, the aphidicolin group will eventually develop myotomes and begin to show some movement.

By 30 h the controls are at stage 33/34. They are streamlined in appearance with a well-developed tail, myotomes and melanophores on their lateral sides. The aphidicolin embryos appear bumpy and rough around the edges, and most have developed curved bodies. Their myotomes are not as obvious, but movement can still be seen. At 44 h the controls are swimming tadpoles (stage 39). In contrast, the bodies of the aphidicolin-treated embryos are shorter, severely curved, and retain a rounded tail. Their eyes are invariably smaller, and they fail to develop the same distribution of melanophores seen in the controls. This is when the experiment ends. As shown in Fig. 1C, the amount of DNA extracted from embryos that have developed in the presence of aphidicolin for the full 44 h is actually less than the first few aphidicolin time points. This is a trend that we saw at the conclusion of several experiments and may be due to the action of endogenous nucleases.

As with the previous experiments, total RNA was prepared from control and inhibitor-treated embryos at various time points. Again we probed for expression of the two epidermal cytokeratins, DG70 and DG81. Unlike DG42 and DG56, both DG70 and DG81 are active during the entire 43 h period in which embryo collections were made (Jonas et al. 1985; Winkles et al. 1985). The top panel of Fig. 7 confirms that levels of DG70 mRNA increase in control embryos after the onset of neurulation and then decrease significantly by the early tadpole stage (43 h). When the same sibling group is treated with aphidicolin, DG70 levels are similar to the controls, showing the same large increase from 0 to 19 h followed by a more gradual decline at 32 and 43 h. Probing RNA from the same individuals with the gene for histone H1A shows that H1A mRNA levels are below those found in the controls and never exceed the initial concentration seen at 0 h. The bottom panel of the same figure shows, again, that the pattern of DG81 expression is generally unchanged by the lack of replication and the low levels of DNA per embryo. However, in the same embryos, the amount of histone H5 and H4 message shows a steady increase in the control group but fails to show a similar increase in the replication-inhibited embryos. Therefore, in an experiment that lasts nearly 2 days, we conclude that the steady-state levels of DG70 and DG81 mRNAs show no significant change in embryos that develop in the absence of DNA synthesis.

Analysis of sequence-specific replication
We have been able to show inhibition of DNA synthesis during early *Xenopus* development by examining the total amount of genomic DNA at various time points (Fig. 1). In other experiments, [3H]thymidine was added to control and aphidicolin-containing media at 0 h and was present during the entire incubation period. In aphidicolin-treated embryos incorporation of [3H] into TCA-precipitable counts was reduced by an average of 78%. The remaining label precipitated by TCA could be due to DNA repair, mitochondrial DNA synthesis, or the metabolic conversion of labeled thymidine into substrates for RNA synthesis. Since the replication-inhibited embryos are clearly alive and developing, we cannot discount any of these possibilities. Another
Fig. 8. Densitometric analysis of autoradiographs of *Xenopus* genomic DNA isolated from control and aphidicolin-treated embryos. Genomic DNA was isolated from control embryos and embryos that were transferred into media containing aphidicolin at either late blastula (stage 9) or at the onset of neurulation (stage 13). The DNA was alkali-denatured, immobilized onto nitrocellulose using a slot-blot apparatus, and probed with either radiolabeled DNA or RNA. Films were scanned with a laser densitometer to determine the relative concentration of specific coding sequences.

(A) Embryos were placed into aphidicolin-containing media at stage 9. One embryo equivalent of genomic DNA isolated at various developmental time points was probed for the genes encoding histone H1A with an *in vitro* derived 32P-labeled RNA made in the sense orientation with respect to the H1A gene. Densitometric scans show relative optical density of immobilized control embryo DNA on the left of the graph and DNA from aphidicolin-treated embryos on the right. The exaggerated peak at 6 h is due to background radioactivity on the nitrocellulose filter. (B) Embryos were placed into aphidicolin-containing media at stage 13. Two embryo equivalents of genomic DNA were isolated at various developmental time points and hybridized with a gel-purified insert containing the DG81 cDNA labeled with 32P by random priming. Densitometric scans show the relative optical density of immobilized control embryo DNA on the left of the graph and DNA from aphidicolin-treated embryos on the right.

 limitation of monitoring DNA synthesis by the incorporation of labeled precursor or by ethidium bromide staining is that these methods fail to detect isolated replication of specific DNA sequences which could locally alter gene expression.

To control for this possibility, genomic DNA was prepared from control and aphidicolin-treated embryos at various time points. The DNA was alkali-denatured, immobilized onto nitrocellulose using a slot blot apparatus, and probed with the same sequences used to detect messenger RNAs in the previous sections. After autoradiography, each film was scanned with a laser densitometer to determine the relative concentration of specific coding sequences in the genomic DNA from both the control and aphidicolin-treated embryos. Fig. 8 shows the results of two densitometric analyses: the genes for histone H1A from an experiment that began at late blastula, and the genes that hybridize to DG81 from an experiment that began at the onset of neurulation. We show these particular sequences since H1A is an example of a gene that is repressed by the lack of DNA replication, while the level of mRNA that hybridizes to DG81 steadily increases in the same replication-inhibited embryos.

In some sibling groups, our attempt to quantify the relative amount of specific DNA sequences by densitometry has revealed an increase in the amount of DNA between the point when the embryos are transferred into aphidicolin (0 h) and the first collection. We interpret this lag period as the time required for the inhibitor to reach every cell in the embryo. An example of this delayed inhibition is seen in Fig. 8B where the amount of DG81 sequence at the 2 h collection is greater than at the 0 h. Fig. 8A shows that the amount of DNA encoding histone H1A in aphidicolin-treated embryos changes very little during the 9 h following the late blastula stage. At the same time, the amount of DNA from the control embryos shows the anticipated increase in the H1A sequence. We conclude that there is very little or no increase in these specific gene sequences when DNA replication is inhibited with aphidicolin.

**Discussion**

We have investigated the ability of *Xenopus* embryos to differentiate in the absence of DNA replication. Our original observation, that visible morphogenesis occurs in the absence of new DNA synthesis (Andrews and Brown, 1987), suggested that this system could be useful as a model to study the relationship between
replication and gene activity in developing embryos. Our results show that the duration of development without replication is dependent on the time of aphidicolin addition. The later the inhibitor is introduced, the longer development will continue. When aphidicolin is added at the late blastula stage, differentiation can be seen for approximately 8 h. But if the inhibitor is added at the onset of neurulation (about 7 h after the late blastula stage), differentiation will continue for nearly 2 days in the absence of new DNA synthesis. This indicates that many of the normal patterns of morphogenesis, embryonic induction, and differential gene expression can occur independently of a precise DNA content or new DNA synthesis. We have attempted to examine this development at both the morphological and molecular level.

Certain aspects of early development in the absence of DNA replication have been studied in several invertebrate species including Caenorhabditis (Edgar and McGhee, 1988), Chaetopterus (Brachet et al., 1981), Drosophila (Raff and Glover, 1988), starfish (Nagano et al. 1981) and sea urchins (Stephens et al. 1986). In a number of these systems, various mitotic events will continue despite the inhibition of DNA synthesis (Raff and Glover, 1988, 1989; Nagano et al. 1981; Sluder and Lewis, 1987). In Xenopus the addition of aphidicolin to fertilized eggs prevents DNA synthesis but does not arrest the fundamental cell cycle oscillator (Kimmelman et al. 1987). Newport and Dasso (1989) have shown that when fertilized Xenopus eggs are incubated with aphidicolin they will proceed through as many as 14 division cycles in the absence of nuclei. However, if aphidicolin is added after the embryo accumulates a critical amount of DNA (about 700–800 nuclei per embryo), further cell division is blocked. In vitro studies have recently shown that a threshold concentration of unreplicated DNA suppresses mitosis until replication is completed (Dasso and Newport, 1990).

Since the embryos used in our study began with at least 8000 nuclei, we presumed that aphidicolin inhibition of DNA synthesis would also block cell division. In experiments where we counted embryonic cells that were dissociated in a Ca^{2+}/Mg^{2+}-free medium, we determined that the addition of aphidicolin inhibits cell division; however, it does not appear that division is arrested at the moment the inhibitor is added. This delay is probably due to the time required for the inhibitor to reach every cell in the embryo, along with a population of cells that are already engaged in mitosis. Cooke (1973) showed that, when inhibitors of mitosis were added to Xenopus embryos at the early gastrula stage, differentiation will continue up to a stage 27 tailbud. But when cell division is inhibited prior to gastrulation, development will continue for a much shorter period of time. These results are similar to our own findings that the duration of development is dependent on the time of aphidicolin addition. Cooke suggests that embryos that are inhibited at the earlier stages fail to show advanced development due to mechanical reasons such as low cell number and large cell size.

We have found that, when aphidicolin is added at the late blastula stage (Fig. 2), we can detect a slower rate of development (as judged by closure of the blastopore) within 2 h. However, when aphidicolin is added at mid-gastrulation or at the beginning of neurulation (Figs 4 and 6), differences between control and inhibitor-treated embryos are not apparent for several hours. This could be due to an increased number of smaller cells that more readily perform the processes of cell movement such as epiboly, involution and invagination. As development proceeds these smaller cells can then be arranged to form a variety of organ primordia. This may explain why the eyes and myotomes are visible when aphidicolin is added at the onset of neurulation, but these same structures are not found at the appropriate time when the inhibitor is added only three hours earlier at mid-gastrulation.

Our molecular analysis of development in the absence of DNA replication included an examination of the steady-state levels of embryo and tadpole-specific mRNAs. Two clones, DG70 and DG81, hybridize to mRNAs encoding distinct type I epidermal keratins predominantly expressed in the outer ectodermal layer of Xenopus embryos (Jonas et al. 1985; Winkles et al. 1985; Jamrich et al. 1987). In the normal embryo this particular layer of cells is visibly involved in cell division. Detection of DG70 and DG81 mRNAs at the control levels in aphidicolin-treated embryos indicates accurate gene expression in the absence of DNA synthesis since the majority of these cells would normally undergo at least one round of replication during the course of an experiment. DG42 and DG56 have additional properties making them desirable as probes. The DG42 mRNA is abundantly expressed during gastrulation and neurulation (Sargent et al. 1986). The mRNA that hybridizes to DG56 is specific only to late blastula and gastrula stages and therefore has a very narrow window of expression. Despite what appears to be the initial expression of DG70 in Fig. 3 and DG81 in Fig. 5, it is possible that some of these genes are already active at Oh and simply cannot be detected. However, our morphological data indicate that the expression of previously inactive genes probably occurs in the absence of DNA replication. This is demonstrated by the expression of genes that give rise to previously nonexistent organs such as the cement gland in Fig. 4 and the lens in Fig. 6. The expression of cement gland genes follows the addition of aphidicolin at stage 11, as these mRNAs are first detected in the anterior neural plate of embryos at stage 12 (Jamrich and Sato, 1989). Crystallin proteins in the lens first appear at stage 29/30 (McDevitt and Brahma, 1973), about 20 h after the addition of aphidicolin at stage 13.

Although the amount of DNA in the inhibitor-treated tailbud and tadpole stage embryos is relatively low, the levels of DG70 and DG81 mRNAs are very similar to the controls (Figs 5 and 7). The same observation can be made with DG42 when aphidicolin is added at the late blastula stage (Fig. 3). We conclude that a reduced level of DNA template does not seem to
result in a reduction in the mRNA concentration of these three genes. This could be due to increased transcription of each gene, enhanced stability of their mRNAs, or the expression of these genes in a population of cells that may have escaped inhibition. The sensitivity of our genomic hybridization assay does not allow us to exclude the remote possibility of replication in a small number of cells that could, in turn, produce the correct amount of messenger RNA at the proper time.

Our results show that in the absence of any significant DNA replication, the early *Xenopus* embryo can still undergo the processes of differentiation. At certain stages, an aphidicolin treated embryo that contains only a fraction of the normal amount of DNA can have a nearly identical external appearance as a control embryo at the same developmental time point. Correct patterns of gene expression, cell movement and organogenesis appear to be independent of a precise amount of genomic DNA and new DNA synthesis.

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