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Gene expression in *Xenopus* embryogenesis

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SUMMARY

This article considers some aspects of the storage of macromolecules in the oocyte of *Xenopus laevis* and the activation of previously unexpressed genes during early embryogenesis. The large quantity and complex nature of poly(A)$^+$ RNA accumulated in the egg provides the cleavage embryo with a supply of mRNA sufficient to sustain protein synthesis for several hours of development. Onset of gene activity at the midblastula transition (MBT) leads to the synthesis and accumulation of molecules of various RNA classes, including tRNAs, rRNAs, mRNAs and mitochondrial RNAs. At gastrulation the poly(A)$^+$ RNA population is still qualitatively similar to that of the egg but some sequences not present in egg RNA have accumulated by this time. Through the use of a subtractive cDNA cloning procedure we have prepared a library of sequences that represent genes activated for the first time between MBT and gastrula. A study of several of these cDNA clones suggests that genes in this class are restricted in their activity to embryonic and tadpole stages.

INTRODUCTION

The regulation of gene expression during early embryogenesis has been a subject of study in many animals for some years. This question can be looked upon as a subset of the problems of gene regulation in general. Many basic questions arise in embryos in similar ways as in any other cells: Granting, as we may, that differential gene activity occurs and is critically important to development of any organism, what are the major mechanisms regulating such activity? One issue that arises is at what level differential gene activity is controlled, i.e., at the transcriptional or post-transcriptional level. The post-transcriptional mode includes rather different mechanisms, including RNA splicing and transport and, almost certainly of considerable importance, translational control. These mechanisms can be studied in many cell types, including embryos. While the study of mechanisms *per se* may often find cultured cells or differentiated tissues a more convenient object for analysis, the rapid changes in the activity of many genes make embryos a system of particular interest. There are additional incentives for research on gene activity in embryogenesis beyond the study of mechanisms of gene regulation. To learn something about the way in which the activity of various genes affects embryo

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development one must by necessity study embryos. In particular, any search for genes that are directly required for embryonic development must use either genetics or molecular studies of embryos.

Our studies on gene expression in *Xenopus laevis* are based on the general points raised above. In vertebrate embryos the genetic approach to mechanisms of development, so effective in some species like *Drosophila*, is quite limited in its applicability. Therefore, a major effort in studying gene regulation in early development as well as identifying genes whose products are critical during embryogenesis must proceed primarily by the application of molecular methods. The basis for choosing *Xenopus* embryos for such a study is manifold: this vertebrate embryo is accessible in adequate numbers all the year, its biology has been well studied, established methods for manipulating the embryo exist, and a considerable amount of molecular work has been done on *X. laevis*, providing a conducive environment for further studies. In addition, the specific timing of RNA synthesis in this embryo is favourable to an analysis of the activation of gene activity.

THE *XENOPUS* EGG STORES A LARGE AMOUNT OF MRNA

The accumulation of many substances in the egg for later use by the embryo is a general feature of oogenesis (see Biggers & Schuetz, 1972; Davidson, 1976; Dawid, Kay & Sargent, 1983). Some of the stored substances, like yolk, will be broken down during embryogenesis into monomers which are employed as building blocks or as energy source; other macromolecules and cell organelles are maintained intact and are used as such at a later time. Among the stored materials ribosomes and mitochondria have been studied extensively, as discussed later in this article. Messenger RNA, the class of stored materials with the largest information content, is of primary interest in our context. This RNA population is synthesized in the oocyte during its early growth period. It has been shown that most if not all of the poly(A)$^+$ RNAs in the egg have reached their final abundance levels in previtellogenic oocytes (Rosbash & Ford, 1974; Cabada, Darnbrough, Ford & Turner, 1977; Dolecki & Smith, 1979; Golden, Schafer & Rosbash, 1980). During the following weeks of oocyte growth large amounts of ribosomes, mitochondria including mitochondrial DNA and RNA (mtDNA and mtRNA) (Webb & Smith, 1977), and many other substances, especially yolk, continue to accumulate, but the poly(A)$^+$ RNA population remains largely unchanged.

Some of the oocyte's mRNAs are undoubtedly translated in the oocyte itself to support its own metabolism and to synthesize many of the stored products, e.g., ribosomal proteins (Bozzoni *et al.* 1982; Pierandrei-Amaldi *et al.* 1982). Yet most of the oocyte poly(A)$^+$ RNA is probably never translated in the oocyte and will be used only during embryogenesis, some weeks or months after its synthesis. This mRNA storage is one of the clearest examples of translational control known in any system.

The oocyte poly(A)$^+$ RNA set is a large one, both in absolute quantity and,

more importantly, in its complexity. There are some 20,000 different poly(A)$^+$ RNA species in the egg (Davidson & Hough, 1971; Perlman & Rosbash, 1978), a value that is on the high side of the range of RNA complexity found in a variety of somatic cell types. While some of these poly(A)$^+$ RNAs do not appear to be functional mRNAs (Richter, Anderson, Davidson & Smith, 1984), others certainly are. The accepted view of the function of these stored mRNAs is to assume their utilization during early embryogenesis before the zygotic genome has taken over the control of protein synthesis. While the details of recruitment of various maternal mRNAs into polysomes during embryogenesis is not known, it is clear that different RNAs may be recruited at different times, providing evidence for the functioning of translational regulation in the embryo (see Woodland, 1980; Dworkin & Hershey, 1981).

RNA SYNTHESIS IN THE *XENOPUS* EMBRYO BEGINS AT MIDBLASTULA

During the first 12 cleavage divisions, which proceed synchronously and rapidly, there is no detectable RNA synthesis in the embryo. The first RNA synthesis can be observed at the midblastula stage (Brown & Littna, 1966*a*; Bachvarova & Davidson, 1968; Shiokawa, Tashiro, Misumi & Yamana, 1981*a*; Newport & Kirschner, 1982*a, b*). The specific time at which this and several other changes in embryo behaviour occur has been termed the midblastula transition (MBT). There has been considerable interest in the nature of the genes that are activated at this stage. While different authors have at various times suggested that the synthesis of some classes of RNAs is initiated earlier than of others, it now appears that many different classes of RNA begin to accumulate at the same or a closely similar time point. It is clear that several small nuclear RNAs are among the most-rapidly synthesized RNAs immediately after the MBT (Newport & Kirschner, 1982*a*). The accumulation of tRNAs also starts at or shortly after this time (Brown & Littna, 1966*b*). Some divergence of opinion persisted over the question at what time rRNA (18S and 28S RNA) is first synthesized. Shiokawa, Misumi & Yamana (1981*b*) have provided evidence for the synthesis of rRNA during the late blastula stage in experiments in which dissociated embryo cells are labelled with [^{3}H]methionine. These experiments might be criticized since the embryos were severely disturbed during the labelling period, possibly affecting synthetic behaviour. In preliminary experiments we have obtained supportive evidence for the synthesis of 18S and 28S rRNA within the first hour after MBT by a different method which involves injection of labelled precursors into the embryo.

Poly(A)$^+$ RNAs undoubtedly begin to accumulate soon after MBT, as shown by labelling experiments (Shiokawa *et al.* 1981*a*), and by the accumulation of novel RNAs by the gastrula stage, as will be discussed below. The rate of poly(A)$^+$ RNA synthesis in the blastula and gastrula embryo has been estimated by Shiokawa, Misumi & Yamana (1981*c*), with the conclusion that about 40 % of the mass of gastrula poly(A)$^+$ RNA is newly synthesized. Since the total amount of

poly(A)$^+$ RNA does not appear to vary between the egg and gastrula embryo one must suggest turnover of pre-existing RNA and its replacement by new molecules.

Because just a small fraction of the newly synthesized molecules is different in sequence from egg RNA, it appears that RNA synthesis in the blastula and early gastrula embryo may function to a large extent to replace maternal RNA molecules with new molecules of the same sequence. Those new RNA molecules that are qualitatively distinct are particularly interesting, as will be discussed further below.

ACTIVATION OF RNA SYNTHESIS IN MITOCHONDRIA

Xenopus eggs, as those of other animals, contain large numbers of mitochondria and consequently large amounts of mtDNA and mtRNA (Dawid, 1965, 1966, 1972). These mitochondria are distributed among the dividing cells of the early embryo in the same way as various other materials that had been stored in the egg. Given this reserve of mitochondria it appears reasonable to assume that the frog embryo does not need to synthesize additional ones for some time in embryogenesis, and this is in fact the case. Chase & Dawid (1972) have measured the accumulation of cytochrome oxidase, a typical mitochondrial component, in the *Xenopus* embryo and found that a net increase in the amount of enzyme could be measured only after gastrulation. Likewise, net increases in the amounts of mtDNA and mitochondrial rRNA were found only in postgastrula development. The synthesis of mtRNA was also studied by the incorporation of radioactive precursors into the two rRNA molecules; a very low rate of incorporation of uncertain significance was observed in pregastrula embryos while a higher rate of incorporation was apparent thereafter.

More recently we have studied the question of initiation of RNA synthesis in mitochondria during blastula stages at higher sensitivity by labelling embryos by injection of [^{32}P]GTP into the fertilized egg, and analysing the RNA by methyl mercury hydroxide agarose gel electrophoresis. In experiments not shown here we confirmed the earlier conclusion of others (see above) that no RNA synthesis of any kind can be detected before the midblastula (4000 cell) stage. This includes mtRNA, with the reservation that we have not shown explicitly that labelled GTP injected into the fertilized egg cytoplasm can enter the mitochondria. Shortly after the MBT, RNA synthesis is detected both in total RNA and in RNA prepared from isolated mitochondria. The gel in Fig. 1 illustrates the results obtained with RNA from embryos 1 h after MBT that had been injected with [^{32}P]GTP before first cleavage. The two major bands visible in the gel are the mitochondrial rRNAs, known to be about 960 and 1600 nucleotides in length (Ramirez & Dawid, 1978; Rastl & Dawid, 1979). At least 9 other bands are visible in this gel. It is known that mammalian mitochondria encode 13 polypeptides corresponding to 5 identified and 8 unidentified open reading frames (Anderson *et al.* 1981; Bibb *et al.* 1981; Michael *et al.* 1984). In *Xenopus*, 10 mRNA molecules have been identified and mapped onto the mtDNA (Rastl & Dawid, 1979). While the bands in Fig. 1 have not been

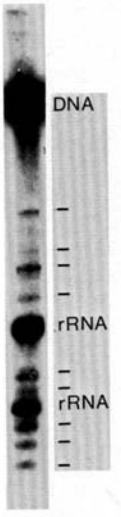

Fig. 1. Radioautograph of gel eletrophoretic separation of mitochondrial RNA. *X. laevis* eggs were fertilized *in vitro*, dejellied with 2 % cysteine, washed, and each egg was injected with about 30 nl [^{32}P]GTP at 10 mCi ml^{-1} and 3000 Ci mmole^{-1}. After 7 h of development the stage-8 embryos were collected and mitochondria were prepared by differential centrifugation as described (Dawid, 1966). RNA was extracted and separated on a methyl mercury hydroxide/agarose gel (see Maniatis, Sambrook & Fritsch, 1982). The radioautograph of the dried gel shows at least 12 bands. The slow running band is due to DNA, presumably of nuclear rather than mitochondrial origin, which is rapidly synthesized during this period of development and may be expected to contaminate mitochondrial preparations that have not been treated with DNase (see Dawid, 1966). The two major RNA bands are mitochondrial rRNAs, about 1600 and 960 nucleotides in length (Rastl & Dawid, 1979). Other RNAs are marked and are likely mitochondrial mRNAs (see text).

sufficiently well characterized to allow us to identify them directly with the previously described mtRNAs, the total number and size distribution suggests that these bands correspond to mitochondrial mRNAs, suggesting that most of them are synthesized and accumulated very soon after MBT.

The activation of mitochondrial transcription at MBT suggested by the results described above is important for the general hypothesis that has been proposed to explain timing of the initiation of RNA synthesis in the *Xenopus* embryo in general. Newport & Kirschner (1982*b*) proposed that timing could be explained by the presence of a substance in the egg which is used up or bound by a newly synthesized substance during embryogenesis. When the original material, thought of as a suppressor of gene activity, is bound or otherwise eliminated, RNA synthesis could start. As the simplest hypothesis the general suppressor of transcription was suggested to be histones, known to be stored in the egg (Woodland, 1980); the material binding the histones and thus neutralizing their effect was suggested to be nuclear DNA. Thus, activation of RNA synthesis would only depend on nuclear replication but not on cell division or absolute time, as found. This attractive hypothesis fails

to explain the timing of initiation of mtRNA synthesis, since typical histones do not occur in mitochondria (Caron, Jacq & Rouviere-Yaniv, 1979) and thus could not be responsible for the lack of transcriptional activity in mitochondria during the first 12 cleavages. Furthermore, mtDNA does not increase in quantity during pregastrula development (Chase & Dawid, 1972) and thus would not be able to bind a putative inhibitor. It is possible, though, that the original hypothesis holds for nuclear activity, while a separate signal activates mitochondrial transcription at a similar time.

POLY(A)$^+$ RNA ACCUMULATION AFTER MBT: MATERNAL AND EMBRYONIC SEQUENCES

Among genes encoding mRNAs that are activated at or shortly after MBT we can distinguish two classes, those encoding RNAs with sequences homologous to RNAs stored in the egg and those that are expressed for the first time in the embryo. While explicit evidence is lacking for the activation at MBT of individual genes whose cognate RNAs also occur in the egg there are good reasons to believe that such molecules exist. The rate of poly(A)$^+$ RNA accumulation in the early embryo (Shiokawa *et al.* 1981*c*) and the fact that egg RNA and gastrula RNA are qualitatively similar (see below) suggest that many transcripts in the late blastula are homologous to maternal RNAs. However, qualitatively new mRNAs are also synthesized immediately after MBT, and it is this class of RNAs and the genes that encode them which have been the focus of our interest. We reasoned that genes activated at this time might be expected to encode products that are important for the processes of gastrulation and for the formation of germ layers and tissue anlagen during the immediately ensuing periods. Thus, we proceeded to isolate this class of gene sequences and study the molecular and biological properties of a subset of this class.

For experimental purposes we defined the sequences that we wished to study as those poly(A)$^+$ RNAs that are present in gastrulae but absent from eggs. The selection of this class of sequences is a specific case in the general problem of obtaining a group of genes active in one stage, tissue or cell line and inactive in another. At least three general methods are available for this purpose, all depending on the manipulation and analysis of cDNA clones. The simplest approach is to make a cDNA library from stage A poly(A)$^+$ RNA and screen a number of colonies or plaques with labelled RNA samples from stages A and B. Those colonies that react with the A probe but not the B probe represent differentially expressed genes. The method has been applied in many cases including *Xenopus* embryos (Dworkin & Dawid, 1980*a, b*), and has led to the detection of certain differentially expressed genes. However, the approach has serious limitations. If the desired mRNAs are relatively rare in the population in which they occur one must screen many colonies to find them and, more importantly, the signal obtained is very low because the probe, which is a mixed probe of all of stage A poly(A)$^+$ RNAs, contains each

individual sequence at low concentration. Below a level variously estimated around 0·05 % of total poly(A)$^+$ RNA, the signal cannot be detected over the unavoidable filter background. Thus, the direct differential screening approach is suited primarily for the detection of differentially expressed moderately abundant mRNAs.

The second method relies on the preparation of an enriched probe. A cDNA library from stage A is prepared as above, but it is screened with a subtracted probe. For this purpose, labelled cDNA is prepared from stage A RNA and is annealed with an excess of RNA from stage B. The hybrid is removed and the remaining material, now highly enriched for A-specific sequences, is used in a screen of cDNA colonies or plaques. A substantial increase in sensitivity may be obtained in this way, depending on the extent of similarity between the A and B RNA populations and the quality of the subtractive hybridization. This approach has been used successfully in recent experiments on T cell-specific mRNA (Davis *et al.* 1984).

The third method, the one we and others have devised, involves the preparation of a subtracted cDNA library (Sargent & Dawid, 1983; Davis *et al.* 1984). Figure 2 shows the steps we used in the construction of a library that represents the desired sequences from *Xenopus* embryos, i.e., copies of those poly(A)$^+$ RNAs that are present in the gastrula but absent from the egg. The critical points are the use of an excess of driver RNA over cDNA, in our case 30-fold by mass; hybridization to kinetic completion; removal of small DNA molecules that could be breakdown products or initially small cDNAs that failed to hybridize because of their size; and finally, high efficiency second strand synthesis and transformation to obtain an adequate number of clones from limited amounts of material. This method of generating a subtractive cDNA library is the most effective but also technically most demanding and time consuming of the approaches available to isolate differentially expressed genes. A subtractive library, once it is generated, provides a valuable source of clones for the study of tissue- or stage-specific genes. The technique allows one to focus attention on a biological event of importance and to cast a wide but highly selective net for molecular species whose expression is regulated during the event in question.

The DG library

The result of the subtractive cDNA cloning procedure summarized in Fig. 2 was a library of cloned DNAs whose developmental properties were expected to include early embryonic expression. We named this collection of clones the DG library, signifying *D*ifferentially-expressed-in-*G*astrula. A group of 84 randomly picked cDNA clones were studied. As expected from the method of isolation the DG clones did not react significantly with labelled RNA probes from the egg or blastula stages, but about half the cloned DNAs hybridized with a gastrula probe. This result shows that the method of preparation of the library had effectively selected for the desired DG sequences.

A set of eight DG clones were studied further with respect to the developmental

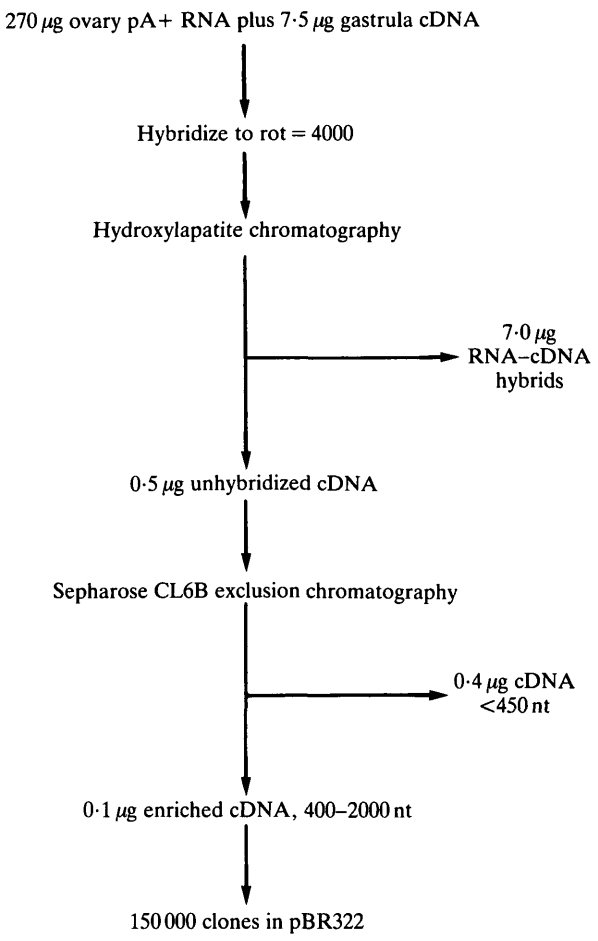

Fig. 2. Flow diagram for the production of the subtracted DG library, as described in the text. From Sargent & Dawid, 1983.

profile of their expression (Sargent & Dawid, 1983). Figure 3 shows a series of RNA dot blots in which RNA from different developmental stages was hybridized with labelled DNA from the eight selected DG clones and from clone r5, which represents a RNA that is present in eggs and all other stages and tissues tested. Panel A, which was exposed ten times longer than panel B, illustrates the absence of any of the DG RNAs from the egg sample and their presence in the gastrula preparation. Some of the RNAs decrease in concentration already by neurula and become undetectable in 3-day tadpoles; others are still present in the tadpole, and DG81 RNA is most abundant at this stage. Unpublished observations show, however, that this latter RNA decreases in concentration during later development and becomes rare after metamorphosis.

The results shown above and additional unpublished experiments suggest that few if any of the genes activated at or shortly after MBT remain active during later life. These early genes may encode substances that are specifically required in

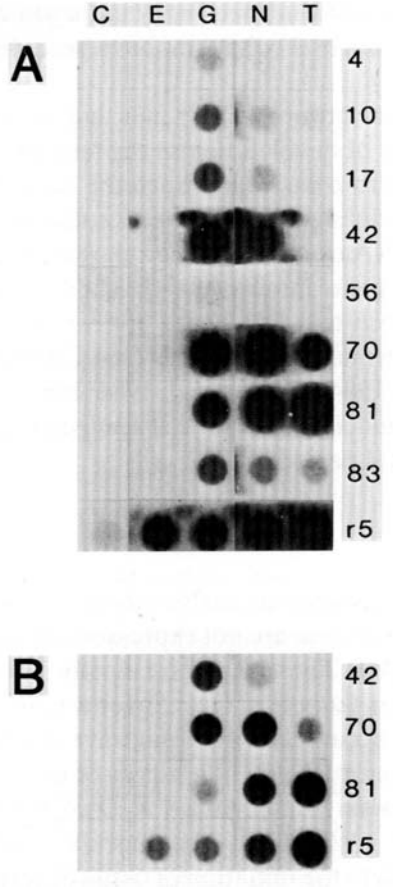

Fig. 3. Developmental profile of DG RNAs. RNA dot blots were prepared by loading total RNA samples onto nitrocellulose filters; the filters were hybridized with nick translated probes from different DG cDNA clones, and cDNA clone r5 which represents a sequence that is present in egg and embryo RNA. Each column contains RNA from a different stage: E, unfertilized egg; G, gastrula (stage 10/11); N, neurula (stage 20); T, tadpole (stage 41). Panel A was exposed for 100 h, panel B for 10 h. After Sargent & Dawid, 1983.

embryonic development and have no counterpart in adult tissues, or they may represent embryonic isoforms of gene families which also have adult representatives. Sequences that appear to correspond to both of these types have been detected in the DG library. The former are likely represented by the clone DG42. This RNA accumulates very rapidly immediately after MBT, peaks in concentration in gastrula to neurula stages, and is no longer detectable in three-day tadpoles (Sargent & Dawid, 1983; see Fig. 3). DG42 cDNA has been sequenced (Sargent *et al.* unpublished observations; Winkles *et al.* 1984) and was found to encode a polypeptide that is not related to any entry in the sequence data base. Because of its very limited time of expression in the embryo it appears that the product of the

DG42 gene must be required for a specifically embryonic function. Several other RNAs with similar developmental behaviour have been detected in the DG library (unpublished observations).

Two other members of the group of DG genes that we studied represent a family of genes with a wide range of developmental expression. These two genes, DG70 and DG81, are expressed from gastrula though the early or the late tadpole stage, but neither RNA persists in significant concentration in the adult. By sequence analysis these two DG cDNA clones have been shown to correspond to two distinct members of the keratin gene family (unpublished observations). The protein molecules encoded by these two DG genes appear to represent embryonic and tadpole forms of the epidermal keratin family (see Crewther, Dowling, Steinert & Parry, 1983; Steinert *et al.* 1984; Geisler *et al.* 1984; Marchuk, McCrohon & Fuchs, 1984) and they might be associated with the shaping of cells and tissues that occurs during these developmental periods.

OUTLOOK

The study of gene expression during early embryogenesis of *X. laevis* has demonstrated that a number of genes that are not expressed during oogenesis are activated within a few hours after MBT. These genes are mostly if not entirely limited in their expression to embryonic or tadpole stages. Several hours after the time at which products of DG genes first accumulate a second wave of gene activation leads to the accumulation of many other mRNAs. In a survey of moderately abundant tadpole RNAs Dworkin, Shrutkowski, Baumgarten & Dworkin-Rastl (1984) found that most begin to accumulate noticeably at the neurula stage, temporally coincident and perhaps causally linked to the initiation of tissue differentiation. One particular example of a lineage-specific mRNA that is not represented in maternal RNA and begins rapid accumulation at neurula is α-actin (Mohun *et al.* 1984 and Gurdon *et al.* this volume). In contrast, cytoskeletal actin mRNAs are stored in the egg and increase from this level during neurulation. Perhaps, many tissue or lineage-specific genes will be found to begin expression during the neurula stage, without being included in the maternal RNA population. Such a suggestion has been made for the sea urchin embryo where several lineage-specific mRNAs have been found to be synthesized *de novo* during embryogenesis (Angerer & Davidson, 1984). If this suggestion is generally valid for different embryos the isolation and characterization of embryonic RNAs will provide molecular markers for cell lineages and tools for the study of determination and differentiation (See Gurdon, Brennan, Fairman & Mohun, 1984; Gurdon, Mohun, Fairman & Brennan, 1985; T. D. Sargent, M. Jamrich and I. B. Dawid, unpublished observations).

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