Review

Temporal control of gene expression from endogenous and exogenously-introduced DNAs in early embryogenesis of *Xenopus laevis*

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ABSTRACT We review here our studies on temporal control of the expression of genes in zygotic nucleus and of exogenously introduced genes in *Xenopus* embryos. For zygotic gene expression, our studies have revealed that mRNA synthesis, tRNA synthesis and rRNA synthesis initiates at the cleavage stage, MBT stage and late blastula stage, respectively. We also briefly summarize here results of the effects of weak bases on rDNA expression, nucleo-cytoplasmic transport and polysomal mobilization of newly-synthesized RNAs. For exogenously injected genes expression our data have shown the control of the expression by the promoter and configuration (circular or linear) of the injected DNAs but not necessarily by cellular changes that take place during the midblastula transition (MBT).

KEY WORDS: *Xenopus embryonic cells, MBT (midblastula transition), rRNA synthesis, RNA transport and mobilizaton, DNA microinjection*

Introduction

Over thirty years ago Brown and Caston (1962) extracted RNAs by phenol method from *Rana pipiens* embryos and started to handle undergraded, as opposed to alkaline-hydrolyzed PCA or TCA-extracted, RNAs from developing frog embryos. Subsequently, Brown and Littna (1964) used embryos of *Xenopus laevis* which contain a lesser amount of RNase to pursue their studies on RNA synthesis. They found that the presence of impermeable surface coat covering the embryos prevented the uptake of precursors of RNA administered to the culture medium. They overcame the difficulty by injecting ^{32}P-orthophosphate into female *Xenopus laevis*, and examined the pattern of labeled RNAs accumulated in embryos that have been derived from the ^{32}P-preloaded eggs. In doing so they laid the foundations for the studies of gene expression from zygotic nucleus (nuclear DNA) in early amphibian embryos. Since then studies on the pattern of RNA synthesis have been continued by Shiokawa and Yamana (1965) using *Rana japonica*, and by Yamana and Shiokawa (1966), Shiokawa and Yamana (1967), and Woodland and Gurdon (1968) using *Xenopus laevis*.

The original experimental design by Brown and Littna (1964) enabled one only to follow cumulatively labeled RNAs. Yamana and Shiokawa (1966), Shiokawa *et al.* (1967) and Shiokawa and Yamana (1967) used embryonic cells dissociated by EDTA, which incorporated specific radioactive precursors very efficiently. Shiokawa and Yamana (1967) found no appreciable difference in the overall pattern of RNA synthesis between ^{3}H-labeled dissociated embryonic cells and $^{14}CO_2$ labeled whole embryos. It was also found that the pattern of RNA synthesis changed from blastula-type (high 4S RNA or tRNA synthesis) to neurula-type (high rRNA synthesis) in dissociated blastula cells when the cells were cultured until control embryos reached the neurula stage (Shiokawa and Yamana, 1967).

More recent studies have further refined our view of RNA synthetic pattern in *Xenopus laevis*. Such advance is due mainly to the newly developed molecular cloning techniques which allowed one to isolate single genes and inject them into fertilized eggs. The DNA injection pioneered by Gurdon and Brown (1977) provided us with the unique opportunity to follow the developmental expression of exogenous genes of defined structures (Asano and Shiokawa, 1993).

In this review, we first summarize our studies on the temporal changes in RNA synthetic pattern based on zygotic nucleus and then proceed to our more recent data obtained by following the fate of exogenously-injected DNAs during *Xenopus* embryogenesis.

Abbreviations used in this paper: MBT, midblastula transition; PCA, perchloric acid; TCA, trichloroacetic acid; DETA, ethylenediamine tetraacetic acid.

***Address for reprints:** Laboratory of Molecular Embryology, Zoological Institute, Faculty of Science University of Tokyo, Bunkyo-ku, 113 Tokyo, Japan. FAX: 81-3-38161965.

0214-6282/94/$03.00
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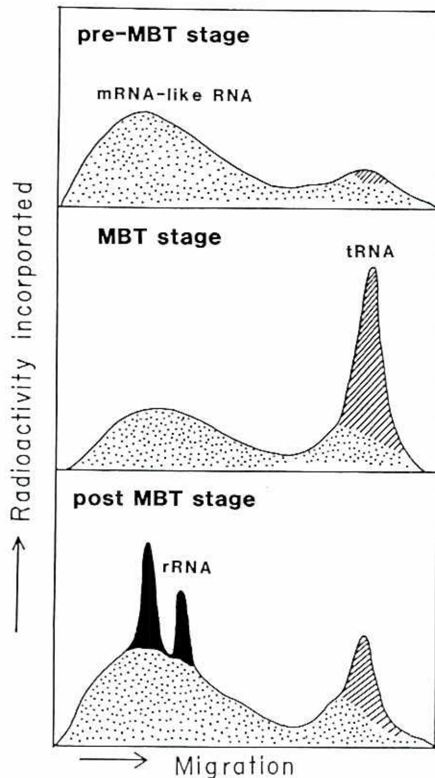

Fig. 1. Three characteristic profiles of RNA synthetic patterns in *Xenopus* early embryos. *Dotted, shaded and black areas are for the product of RNA polymerase II, III and I, respectively. Distance of migration and amount of radioactivity are in arbitrary units. From Shiokawa (1991).*

General pattern of RNA synthesis in early embryogenesis

A general view obtained from earlier studies with ^{32}P-preloaded embryos (Brown and Littna, 1964), dissociated embryonic cells (Shiokawa and Yamana, 1967) and embryos injected with radioactive precursors (Woodland and Gurdon, 1968) is that in *Xenopus laevis* embryogenesis there are at least two characteristically distinct phases with respect to the RNA synthetic pattern (Shiokawa *et al.,* 1981c): the first phase involving very active synthesis of 4S RNA (mainly tRNA) and no apparent rRNA synthesis (blastula-type) and the second phase involving active rRNA synthesis accompanied by moderate synthesis of 4S RNA (neurula-type).

In the above experiments, little RNA synthesis was observed during cleavage stage, and apparently active RNA synthesis (per embryo) occurs only at and after the midblastula stage. In such studies, however, the majority of the experiments were performed using the same number of embryos at different stages. This meant that the comparisons of RNA synthetic activities were made among embryos at different stages on a per-embryo basis, although cell number, and hence nuclear number, per embryo increases tremendously as development proceeds.

About 10 years ago, it was proposed that three major changes in the cellular activities take place at the midblastula stage (just after 12 cleavages). This transition was assumed to involve the coordinated initiation of transcription, acquisition of cell motility, and lengthening (or slowing) of cell cycle giving rise to G_1 and G_2 phases. The phenomenon was termed midblastula transition (MBT) (Newport and Kirschner, 1982).

According to the notion of MBT, RNA synthesis is said to initiate after the midblastula stage (MBT) but never prior to it. Yet in our studies we observed methylation of mRNA CAP as early as in cleavage stage embryos (Shiokawa *et al.,* 1981a, 1981c). We labeled here RNA of preblastula embryos with (methyl-^{3}H) methionine which were dissociated within the vitelline membrane by cultivation in a phosphate buffer (Takeichi *et al.,* 1985). Furthermore, we detected the synthesis of both heterogeneous mRNA-like RNAs and a relatively small amount of low-molecular-weight RNAs (U3, U2 and U1 snRNAs, 5S ribosomal RNA, 4.5S pre-tRNA and 4S RNA) in the dissociated cleavage-stage embryos by labeling them with ^{3}H-uridine. These findings were consistent with those of Kimelman *et al.* (1987).

From these experiments, we came to conclude that pre-MBT-stage embryos synthesize heterogeneous mRNA-like RNA (already at the 64-cell stage), and in addition, small amounts of small-molecular-weight RNAs at the middle of the pre-MBT stage (Nakakura *et al.,* 1987). The kinetics of the accumulation of the RNAs was roughly proportional to the cell number before and after the MBT. However, as for 4S RNA, the rate of the synthesis was found to increase approximately by 100-fold on a per-cell basis at the MBT stage, although the increase in the activity per cell of the synthesis of heterogeneous mRNA-like RNA and other RNAs (5S RNA and snRNAs) was only 2- to 3-fold.

Further, it has been shown here that while the labeling of 4.5S pre-tRNA is relatively active during the pre-MBT stage, it is reduced

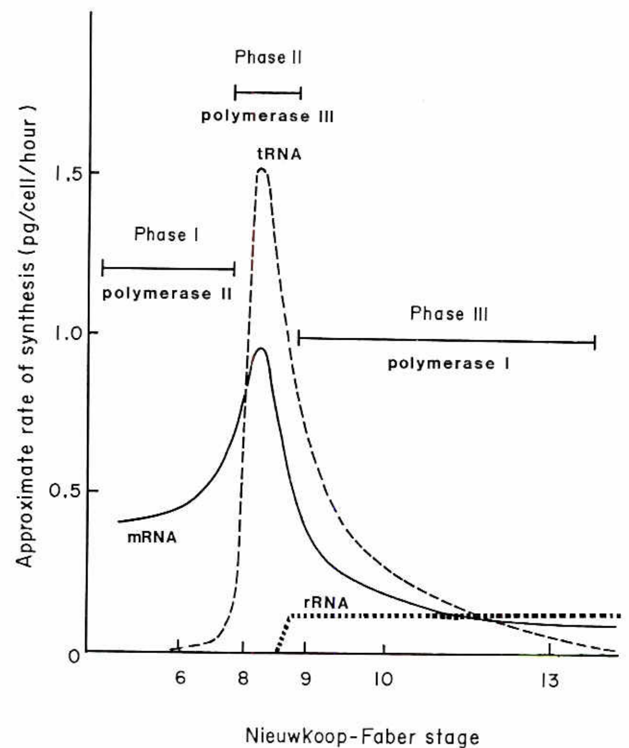

Fig. 2. Changes in approximate rates per cell of RNA synthesis in *Xenopus* embryos. *Xenopus early development is divided into three phases. From Shiokawa (1991).*

greatly during and after the MBT stage. Therefore, we suggested that the processing of pre-tRNA may be accelerated at the MBT stage, when a large increase in the rate of 4S RNA takes place (Shiokawa *et al.,* 1989a).

The distinct peaks of 18S and 28S rRNA labelings were initially detected at the gastrula stage (Brown and Gurdon, 1964). However, since mRNA synthesis per cell is extremely high at the blastula stage (10-fold or more than in gastrula and neurula stages) (Sagata *et al.,* 1978; Shiokawa *et al.,* 1981c, 1989a), it was suspected that rRNA may be synthesized at the stages earlier than the gastrula stage, although its occurrence might have been covered by the active mRNA synthesis

Therefore, to specify the stage when rRNA synthesis initiates, dissociated embryonic cells were labeled with (methyl-^{3}H) methionine (Shiokawa *et al.,* 1981a, 1981c), and labeled high-molecular-weight RNAs were analyzed on DEAE-Sephadex columns after complete digestion with RNases A and T_2. Morula RNA labeled for 3 h gave rise to methylation peaks of charge -2 (nucleosides) and -5 (mRNA CAP) nucleotides, but practically no charge -3 (2'-0-methylated dinucleotides; NpmNp) or charge -4 (two consecutively 2'-0-methylated trinucleotides; NpmNpmNp) nucleotides. Since charge -3 and -4 nucleotides were derived from rRNA, it was concluded that morula cells do not synthesize rRNA, although as noted above they actively add cap structure to mRNA (Shiokawa *et al.,*1981a, 1981c).

When early blastula cells were labeled for 4 h until the late blastula stage we obtained a very large peak of rRNA-specific charge -3 component (NpmNp), and in addition, a small peak of charge -4 component (NpmNpmNp). When we divided the blastula stage into two 2 h-periods, we obtained results which led us to conclude that embryos start to synthesize rRNA not in the former half but in the latter half of the blastula stage (late blastula stage) (Shiokawa *et al.,* 1981a,c, 1989a). The timing of the first appearance of definitive nucleoli studied cytologically (Nakahashi and Yamana, 1976) coincided with this result.

Based on the results, the average rate of rRNA synthesis was estimated as about 1 ng/embryo/h at the late blastula stage. As expected from the previous data the rate per embryo of rRNA synthesis increased greatly throughout development, but the rate per cell increased only slightly and was negligible during the following 15 h (Shiokawa *et al.,*1981c).

We therefore conclude that *Xenopus* early embryogenesis consists of at least three different phases with respect to the pattern of RNA synthesis (Fig. 1) (Shiokawa *et al.,* 1989a; Shiokawa, 1991). The first is the pre-MBT stage, which is characterized by a relatively high activity (per cell but not per embryo) of mRNA-like RNA synthesis and also by a relatively low activity of low-molecular-weight RNA synthesis. The second is the MBT stage, which is characterized by a great activation of the synthesis of 4S RNA. The third is the post-MBT stage, which is characterized by a gradual activation (per embryo) of the synthesis of rRNA. Thus, RNA synthetic patterns at pre-MBT, MBT and post-MBT phases are characterized by predominant activity of RNA polymerases II, III and I, respectively (Fig. 2).

Regulation of rRNA synthesis in early embryos

The expression of rDNA is temporally and spatially regulated. Thus, at the gastrula stage, cells at animal or dorsal half initiate rRNA synthesis earlier than those at the vegetal or ventral half (Wada *et al.,* 1968; Woodland and Gurdon, 1968; Shiokawa and Yamana, 1979). Since anucleolate mutant embryos which are unable to synthesize ribosomes can survive until the tadpole stage but never beyond (Brown and Gurdon, 1964), the onset of rRNA synthesis at the late blastula stage seems to be an extremely safe device to provide the new ribosome supply for later stages.

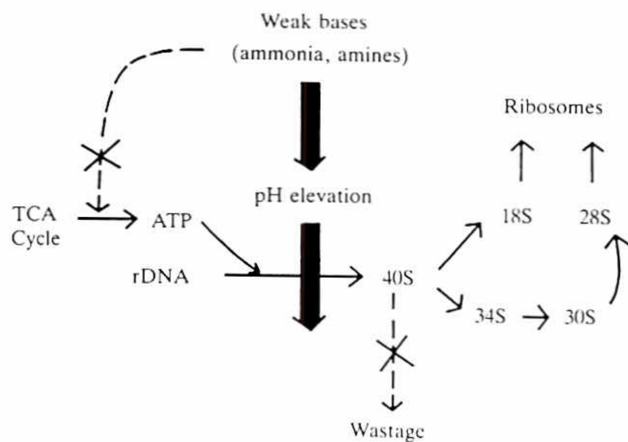

Fig. 3. A working hypothesis on the mode of action of weak bases on rRNA synthesis. *Weak bases do not interfere with ATP generation (upper X) and do not induce degradation of 40S pre-rRNA (lower X), but block the transcription of 40S pre-rRNA (lower large arrow) probably via a slight elevation of intracellular pH (upper large arrow). From Shiokawa et al. (1987b).*

In a classical experiment by Gurdon and Brown (1965), neurula nuclei which actively synthesize rRNA stop doing so when they are transplanted into the cytoplasm of unfertilized eggs. The cessation of rRNA synthesis was not due to nuclear damage on transplantation since the transplants synthesized rRNA when they reached the neurula stage. These observations suggested that there may be some negative regulative mechanism of rRNA synthesis in early stage embryos.

Genomic clones of rDNA have been employed in many experiments to elucidate the mechanism of its transcriptional regulation in *in vitro* systems (Reeder, 1984). However, the mechanism of the onset of rDNA expression at the particular developmental stage (the late blastula stage) in normally developing embryos is still unknown, although several mechanisms such as the involvement of transcription-stimulators (Crampton and Woodland, 1979; Russell, 1983), transcription factor xUBF (Leblanc *et al.,* 1993), chromatin configuration changes (Reeves, 1978; Spadafora and Riccardi, 1985), demethylation (Bird *et al.,* 1981) and histones (Dimitrov *et al.,* 1990) have so far been proposed.

In our studies of rDNA expression in isolated *Xenopus* neurula cells we happened to find that various ammonium salts and amines added at the concentration of 1 to 5 mM to the culture medium selectively inhibit the synthesis of rRNA (Shiokawa *et al.,* 1985, 1986a,b). Furthermore, we found that ammonia exists in unfertilized eggs at concentration of about 3 mM and its level decreases to a half or a third during the late blastula stage (Shiokawa *et al.,* 1986a). Therefore, we suggested that ammonium ion may be a negatively-controlling factor (Shiokawa *et al.,* 1986a). We also showed that the inhibitory effect of weak bases totally disappeared when Na^+ was replaced by choline$^+$ in the medium (Shiokawa *et al.,* 1987b). The removal of Na^+ from the medium inhibits a slight

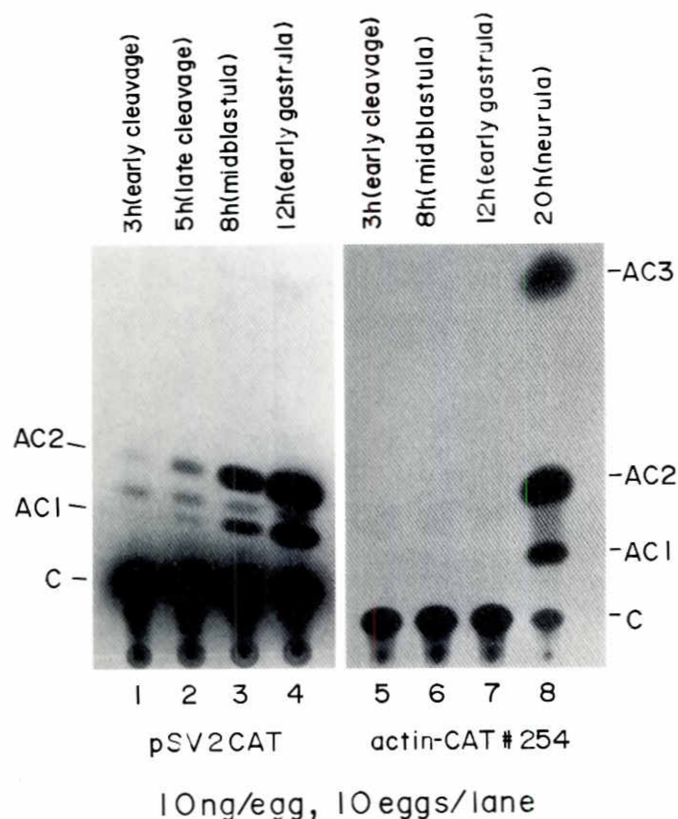

Fig. 4. CAT enzyme assays using embryos injected with circular CAT genes. *Fertilized eggs were injected with 10 ng of either circular pSV2CAT or actin-CAT fusion gene #254, and 10 embryos each were harvested for CAT enzyme assay. Autoradiography was carried out for 2 months. From Shiokawa et al. (1990b).*

elevation of intracellular pH that normally takes place in the presence of added ammonium ion (Stith and Maller, 1985). Therefore, we postulated that the inhibitory effect caused by weak bases may be mediated by a slight increase in the intracellular pH.

As in other systems, the expression of rDNA in *Xenopus* embryonic cells involves complex processing steps: rRNA is transcribed as a 40S precursor, trimmed to a 38S rRNA intermediate, cleaved to 18S rRNA and 34S RNA, and then the latter is finally converted to 28S rRNA via a 30S intermediate (Wellauer and Dawid, 1974). We examined the rate of the processing of 40S pre-rRNA into 18S and 28S mature rRNAs in *Xenopus* embryonic cells by pulse-chase experiments using actinomycin D (Shiokawa *et al.*, 1986c, 1987a,b; Shiokawa and Fu, 1987). The length of the time needed for complete disappearance of pulse-labeled 40S pre-rRNA was 65, 62, 38, and 30 min, and the half-life of 40S pre-rRNA was 19, 16, 12, and 9 min, for late gastrula, yolk plug, neurula, and muscular response stages, respectively (Shiokawa *et al.*, 1986c, 1987z). These results show that rRNA processing is accelerated as development proceeds.

Following these studies, we tested the effect of weak bases on the processing of 40S pre-RNA. A relatively high dose (5 mM) of ammonium salts and amines totally and selectively inhibited the labeling of not only 18S and 28S mature rRNAs but also of 40S pre-rRNA. Further experiments were carried out under the conditions of partial (50%) inhibition (0.5-1 mM of weak bases) of rRNA synthesis, and results were obtained that the extents of the inhibition of the labeling of 40S pre-rRNA, 30S intermediate rRNA and 18S and 28S mature rRNAs were much the same. These results showed that the inhibition of rRNA synthesis by ammonium salts and amines is due to the inhibition of rDNA transcription, but neither to the induction of its breakdown (wastage), aberrant processing, nor to formation of unstable mature rRNAs (Shiokawa *et al.*, 1987b).

It is known that when the level of the intracellular ammonia is increased, oxaloacetic acid is transaminated into aspartic acid, and TCA cycle is inhibited. However, there was no decrease in the level of ATP in the weak base-treated neurula cells (Shiokawa *et al.*, 1987b). Therefore, we concluded that the effect of ammonium salt is not via inhibition of energy supply. This is consistent with the fact that various amines, which do not bind to oxaloacetic acid, are also effective (Shiokawa *et al.*, 1986b, 1987b). Based on these results, we proposed a working hypothesis to explain the mechanism of the inhibition of rDNA transcription by weak bases (Fig. 3) (Shiokawa *et al.*, 1987b).

Nucleo-cytoplasmic transport and polysomal mobilization of newly synthesized RNAs

There has been little information concerning when and how newly transcribed RNAs are transported to the cytoplasm and then mobilized to polysomes in early embryonic cells. To answer these questions, embryonic cells dissociated at several developmental stages were labeled with ^{3}H-uridine, and nuclear and cytoplasmic RNAs were analyzed (Shiokawa *et al.*, 1979, 1981b).

When 1h-labeled blastula nuclear non-poly(A) RNAs were analyzed, heterogeneous nuclear RNA and small-molecular-weight RNA were found to be labeled. In the cytoplasm, little label accumulated except for one large 4S RNA peak. When labeled for 3 h, labeled blastula nuclear non-poly RNA consisted of 4S RNA, 4.5S tRNA precursors, 5S RNA and U1, U2 and U3 snRNAs. After labeling for another 2h, 40S rRNA precursor appeared in the nucleus and at the same time, 18S rRNA appeared in the cytoplasm (Shiokawa *et al.*, 1977, 1979). Newly synthesized 28S rRNA was transported to the cytoplasm only after another 2h and this happened together with the transport of 5S RNA.

In the nuclear fraction of 1 h-labeled tailbud embryo cells, 40S rRNA precursor, several rRNA intermediates, heterogenous nuclear RNA, snRNA and 4SRNA were labeled. At this time, transport of 4S RNA and 18S rRNA took place in the cytoplasm. After labeling for 3 h, in addition to a large peak of 18S rRNA a small peak of 28S rRNA appeared in the cytoplasm. At 5 h, the amount of the labeled cytoplasmic 28S rRNA increased and the ratio of the newly synthesized 28S rRNA:18S rRNA became very close to the steadystate value (2:1), indicating the transport of equimolar amount of the two RNAs (Shiokawa *et al.*, 1977, 1979). Thus, the rates of the transport of 18S and 28S rRNAs and 5S RNA appear to be accelerated in embryos at later stages.

Blastula nuclear poly(A)$^{+}$RNA labeled for 1 to 3 h migrated on denaturing formamide gels quite heterogeneously, with the main component at around the 40S RNA region and these RNAs contained poly(A) sequences of about 150 nucleotides long (Sagata *et al.*, 1976, 1980). On sucrose density gradients, these blastula nuclear poly((A)$^{+}$RNA distributed mainly between the 28S and 18S regions, whereas newly-arrived cytoplasmic poly(A)$^{+}$RNA appeared

between the 18S and 4S RNA, with the main component at 10S region (Sagata *et al.*, 1976). At the tailbud stage, the average size of labeled poly(A)⁺RNA became slightly smaller than at the blastula stage both in formamide gel (Shiokawa *et al.*, 1979) and on sucrose density gradient (Sagata *et al.*, 1978). Thus, poly(A)⁺RNAs are transported to the cytoplasm immediately after their synthesis both at the blastula and neurula stages. These results show that the rates of the transport of newly synthesized 4S and mRNA do not change greatly depending on the stages.

The next question we asked was when and how such newly synthesized RNAs are used for protein synthesis. To answer this, we studied the mobilization of newly synthesized RNAs (rRNA, 4SRNA and poly(A)+RNA) into polysomes (Shiokawa *et al.*, 1981b). We found here that throughout the stages, 4S RNA and poly(A)+RNA are mobilized to polysomes immediately after their appearance in the cytoplasm. However, 18S rRNA, which is in 40S ribosomal subunit, was found to stay ca. 30 min in the soluble fraction until 28S rRNA which is in 60S ribosomal subunit reaches the cytoplasm. Then, two rRNAs were mobilized together into polysomes in the form of 80S ribosomes (Shiokawa *et al.*, 1981b).

The contribution of newly synthesized 18S and 28S rRNAs to the total polysomal rRNA remains very small (ca. 0.4%) throughout early stages as expected from the study of Woodland (1971). About 3% of newly synthesized cytoplasmic 4S RNA was polysomal and this percentage was valid also for total unlabeled 4S RNA, implying that there seems to be no discrimination for utilization between tRNAs of maternal and zygotic origins. From cleavage to blastula stages less than 10% of the newly synthesized cytoplasmic poly(A)⁺RNA was mobilized into polysomes, but in later stages the percentage increased to 20-25%. Again the efficiency of the mobilization was not quite different between maternal and newly synthesized mRNAs. These results show that throughout the stages newly synthesized 4S RNA, poly(A)⁺RNA and rRNA are mobilized into polysomes shortly after they reach the cytoplasm (Shiokawa *et al.*, 1981b).

Expression of exogenously-injected genes

To help understand the mechanism of gene expression in early *Xenopus* embryos, exogenous genes were injected into the fertilized egg and their expression followed in the course of early embryogenesis. For instance, Busby and Reeder (1983) injected pXlr101A, a plasmid that contains *Xenopus laevis* rDNA single repeat, into fertilized eggs of *Xenopus borealis,* and showed that the injected rDNA expression initiates at the late blastula stage at about the time when endogenous rRNA expression starts (Shiokawa *et al.*, 1981a,c). These results show that the mechanism controlling the expression of endogenous (or chromosomal) rDNA may also be effective on the exogenously-introduced rDNA.

When we injected closed circular (c.c.) DNAs such as pBR322 and pXlr101A which do not contain viral promoter, we found that the injected DNAs were once converted to open circular (o.c.) form and then converted again to c.c. form. These circular DNAs remained within the embryos without being replicated appreciably (at most 2-fold) (Fu *et al.*, 1989; Shiokawa *et al.*, 1989b). However, when circular plasmids like pSV2CAT, which consists of bacterial chloramphenicol acetyltransferase (CAT) gene and SV40 early promoter that contains viral replication origin were injected, they frequently replicated (ca. 10 fold) as circular DNA. Therefore, it appears that replication of circular DNAs depends on the presence of viral replication origin especially in later stages. When we injected linearized DNAs, they always replicated quite actively (by about 50- to 100 fold), and at the same time, formed concatemers of large sizes (by 10 to 20 times the initial size) (Fu *et al.*, 1989), probably after ligated in all the possible combinations (head to head, head to tail, and tail to tail). However, the majority of injected DNAs, both circular and linear, disappear by the tailbud stage, although some of the DNAs, especially concatemers, are preserved even in 1-month-old tadpoles either as episomal or chromosome-integrated DNA (Fu *et al.*, 1989).

There was no appreciable abnormality in cleavage and in later morphogenesis, when the amount of the injected DNA was less than 1-2 pg/egg. However, when a relatively large amount (10 ng/egg or more) of either circular or linearized DNA was injected, it was assembled in the form of nucleus-like structures (Shiokawa *et al.*, 1986d, 1987c, 1992; Trendelenburg *et al.*, 1986), mostly in the animal region, after being complexed with maternal histones (Shiokawa *et al.*, 1992). These nucleus-like structures were unequally partitioned into descendant blastomeres, thereby interfering with normal cleavage (Shiokawa *et al.*, 1986d, 1992).

We injected various circular plasmids that carried CAT gene at 1 ng/egg (ca. 10^7 copies/egg) and carried out CAT assay using 10 embryos at each stage. The plasmids injected were above-noted pSV2CAT, pAl0CAT2 which includes only the GC-rich 21-bp repeats and TATA box of the SV40 early promoter, pAd12.E1aCAT which carries the relatively strong promoter of E1a protein of type 12 adenovirus, pSVOCAT which contains no viral promoter, and pAl0CAT3m which is a polylinker-containing derivative of pSVOCAT. When pSV2CAT was injected, it was found to be expressed from the blastula stage on (Etkin and Balcells, 1985; Fu *et al.*, 1989). Such CAT enzyme expression accompanied the appearance of CAT mRNA (ca. 1.6 Kb). pAdl2.E1aCAT was also expressed at a level comparable to that of pSV2CAT. However, pAl0CAT2 was expressed relatively far more weakly and two other plasmids without promoters were expressed only at background levels (Fu *et al.*, 1989). Then, we concluded that the extent of the expression depends on the strength of the promoter included in the plasmid (Fu *et al.*, 1989, 1990).

Etkin and Balcells (1985) who injected 1 ng/egg of pSV2CAT into *Xenopus* fertilized eggs reported that CAT gene expression takes place only after the 12th cleavage at the timing of MBT. However, since we previously observed the synthesis of heterogeneous mRNA-like RNA during cleavage stage (Nakakura *et al.*, 1987; Shiokawa *et al.*, 1989a), we expected the expression of CAT gene even before the MBT. Then, we repeated the experiment of Etkin and Balcells (1985) after increasing the amount of circular pSV2CAT by 10-fold (10 ng/egg) using 10 embryos at all stages or after increasing the number of embryos per sample by 10-fold (100 embryos/sample) without increasing the dosage (1 ng/egg) (Shiokawa *et al.*, 1990b). We detected here CAT enzyme activity not only at and after the MBT but also at 3h (early cleavage) and 5 h (late cleavage stage) after fertilization (Fig. 4). Therefore, we concluded again that it is not the changes associated with the MBT, but the nature of the promoter within the plasmid that determines the timing of the expression of exogenous genes. It was found here that the increase in the CAT enzyme signal during the early development roughly paralleled the increase in the number of cells per embryo. This observation suggests that the increase in the CAT gene expression during development may be due to the increase in the number of nuclei that had contained the CAT gene.

When we tested the expression of a CAT gene #254, which contains the promoter of *Xenopus* alpha-actin gene at doses of 1 ng/egg and 10 ng/egg, we found that injected genes are expressed always at and after the neurula stage (Fig. 4) (Shiokawa *et al.*, 1990b) just like its endogenous counterpart (Mohun *et al.*, 1986). These results indicate that under our conditions the expression of the gene which has the promoter of a temporally controlled *Xenopus* gene was expressed at the correct timing during development.

We then tested the expression of pSV2CAT and pSV0CAT which were linearized at various restriction sites (Fu *et al.*, 1989). It was found here that all the linearized pSV2CAT DNAs were expressed at levels more or less similar to that of circular pSV2CAT. Furthermore, similar, or slightly lower, levels of expression were observed also with linearized pSVOCAT that carries no promoter. Therefore, we concluded that linearized CAT genes are expressed, irrespective of whether the DNAs contain promoter or not (Fu *et al.*, 1989). It is known that for exogenous DNAs to be expressed, torsional stress is needed within the molecules (Harland *et al.*, 1983). Since injected linearized DNAs were converted to concatemers of extremely large sizes, we assume that such concatemer formation may result in the formation of promoter-like structures within the molecule. Another possibility is that such concatemers are bound to intranuclear structural organization such as nuclear matrix and generate torsional stress which may be necessary for transcription.

When we injected the linearized fusion gene #254, we found that it was expressed as early as at the blastula stage (Shiokawa *et al.*, 1990a). We suggested, therefore, that when linearized, the timing of the expression of the injected genes deviates from the normal control, even though the genes possessed developmentally-controlled promoter in an intact form.

Finally we injected circular form of *Drosophila* amylase genes into fertilized *Xenopus* eggs. We found here that the injected genes were expressed at the gastrula and neurula stages as *Drosophila* amylase (Shiokawa *et al.*, 1989b). These results indicate that genes with heterologous promoter can also be transcribed, and furthermore, processed, and translated in *Xenopus* embryonic cells.

Future problems

Mechanisms of the regulation of RNA synthesis in early amphibian embryogenesis are still not well understood. We continue our studies in this line, searching for the hidden, new framework of the regulation, with special reference to polyamine metabolism in embryonic cells.

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