RNA species whose transcription is totally silent in pre-MBT stage are not mRNA but rRNA and possible involvement of weak bases (ammonium salts and/or amines) in the transcriptional silence of rRNA genes during the pre-MBT stage in *Xenopus* early embryos

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ABSTRACT

In *Xenopus laevis* embryogenesis, fertilized eggs undergo 12 cycles of synchronous divisions and reach the stage called midblastula transition (MBT). It has long been believed that during the first 12 cycles of cleavage (pre-MBT stage), transcriptional activity of the zygotic nuclei is totally absent. However, heterogeneous mRNA-like RNA is synthesized in pre-MBT stage embryos, and exogenously-injected bacterial CAT genes with SV40 promoter are expressed from the cleavage stage. Nevertheless, the synthesis of rRNA as detected by rRNA-specific 2'-O-methylation does not take place in pre-MBT embryos and starts only from the latter half of the MBT stage, corroborating the fact that formation of definitive nucleoli as well as the transcription of microinjected rRNA genes starts only at and after MBT stage. Thus, while mRNA-like RNA synthesis occurs from pre-MBT stage, synthesis of rRNA is controlled in the way that transcription of rRNA genes is totally silent during pre-MBT stage and is initiated only at the latter half of MBT stage. Once initiated, the rate of the synthesis of rRNA is constant throughout later stages on a per-cell basis. We searched substances which are responsible for the transcriptional silence of rRNA genes during the pre-MBT stage. Weak bases such as ammonium ion and amines selectively inhibited rRNA synthesis at the transcriptional level in post-MBT stage embryo cells. Since we found that the level of ammonia extracted from embryos is much higher in pre-MBT embryos than in post-MBT embryos, we suggest that weak bases like ammonium ion could be responsible for the transcriptional silence of rRNA genes by slightly increasing intracellular pH during the pre-MBT.

Keywords: Pre-MBT Transcription; Absence of rRNA Synthesis; Initiation of rRNA Synthesis; Nucleolus Formation; Weak Bases; Amines; Ammonium Ion; *Xenopus* Embryogenesis

1. INTRODUCTION

MBT is the important time point of transition from the phase of cleavage division to the phase of morphogenetic cell interactions. After 12 rounds of cleavage cell cycles, *Xenopus* fertilized eggs reach midblastula stage (4096 cells/embryo), or the stage of so-called midblastula transition (MBT), when G_1 and G_2 phases reappear in the cell cycle [1,2]. During the cleavage stage, translation of maternal mRNA takes place actively [3,4]. At MBT, cell division shifts from synchronous to asynchronous one [5, 6], cell cycles shift from a checkpoint-unregulated to checkpoint-regulated state [7,8], and cells, especially those at the dorsal marginal zone, acquire motility [9]. Also, strongly activated, at least on a per-embryo basis but not necessarily on a per-cell basis, is the transcriptional activity from zygotic nuclear genes [10-17]. Also, the per-embryo activity of the transcription of exogenously-introduced bacterial CAT (chloramphenicole acetyltransferase) genes with pan-expression promoter of SV40 virus starts to increase from cleavage stage on [18,19].

Since the reports of Newport and Kirschner [5,6], it has long been believed that there is no transcription at all before MBT stage. However, recent results about this

issue led to acceptance of the view that some transcription takes place during the pre-MBT stage. In our old studies, for instance, we noticed that heterogeneous non-mitochondrial RNA is pulse-labeled during the pre-MBT stage [12], and recently Peter Klein and his group [17] reported that there is a transcription of TGF-β family member mRNAs, which is important for the post-MBT morphogenesis. If these are all correct, it follows that transcription of RNA polymerase II-dependent RNAs occurs even before the MBT. In the original paper by Newport and Kirschner [5,6], it is described that cleavage embryos make a leaky transcription, since overexposure of autoradiogram to X-ray film made faint signals in the high-molecular-weight RNA region visible. In the reports of Newport and Kirschner [5,6], however, this was not interpreted as indicating the transcription of small amount of heterogeneous mRNA-like RNA in pre-MBT stage. The idea that transcription takes place even during the pre-MBT stage is similar to that in sea urchin embryogenesis in which new transcription starts right after the initiation of development or even before the fertilization. If we think this way, we encounter the new idea that MBT in *Xenopus* embryogenesis is not a remarkable stage of the development at least concerning the transcriptional activation of zygotic genome on a per-cell basis.

In our studies, however, based on ^{3}H-uridine-labeled RNA labeling profiles we previously proposed our working hypothesis that *Xenopus* early development consists of three characteristic phases of RNA synthesis: The first one is pre-MBT stage which is characterized by the synthesis of heterogeneous mRNA-like RNA and low level of small-molecular-weight RNA probably due to the activity of DNA-dependent RNA polymerase II, and the second phase is MBT stage which is characterized by additional activation of tRNA synthesis due to the activity of DNA-dependent RNA polymerase III, and the third phase is post-MBT stage which is characterized by additional active synthesis of rRNA due to the activity of DNA-dependent RNA polymerase I (**Figure 1**). In 4 - 5 hrs after MBT, embryos reach early gastrula stage and enter the phase of extensive morphogenesis including the neural inductions due to invaginating Spemann organizer, which results in the establishment of mesodermal and neural structures [20,21]. These induction processes are results of various cellular cross-talk involving interactions of various growth factors and their receptors [2,22,23].

In the present article, we describe that rRNA is probably the only RNA species which is totally not transcribed before MBT (or during pre-MBT stage) but starts to be synthesized just after the MBT stage. Therefore, rRNA fits the RNA whose gene was first described to be totally silent during pre-MBT stage but is activated only after MBT stage. In this sense, the general idea of the tran-
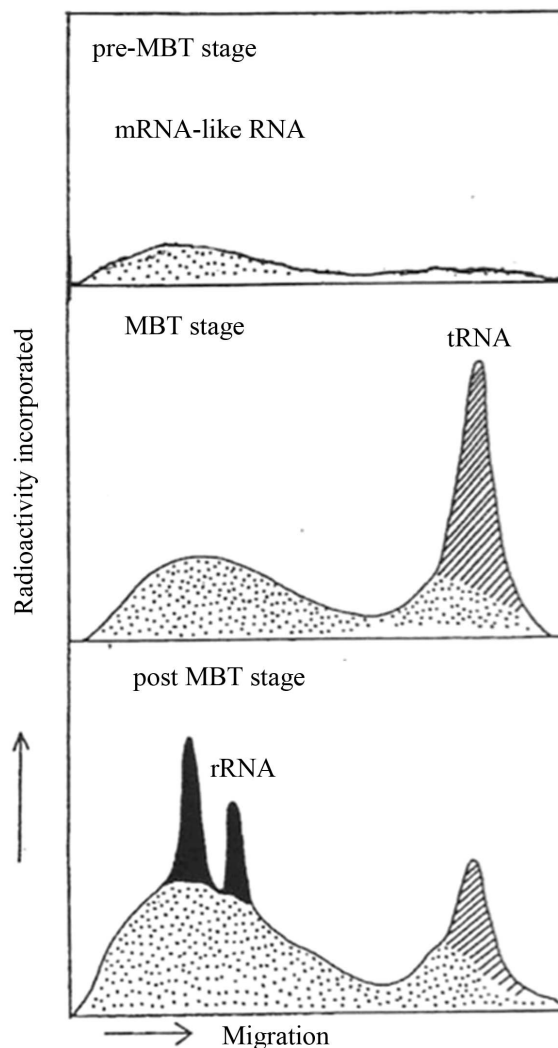

Figure 1. Characteristic patterns of RNA synthesis in early *Xenopus* development. It is shown that there are three major developmental stages, with respect to changes in RNA synthetic pattern before and after MBT. From Shiokawa *et al*. [13].

scriptional silence of zygotic nuclear DNA in *Xenopus* development before MBT is still valid as far as the transcriptional control of rRNA genes is concerned. It seems to be valid that MBT is the stage when G_1 phase appears in the cell cycle and is the stage when the maternal program of apoptosis is first executed as a fail-safe mechanism of development but not before. However, we propose here additionally that MBT is the stage when rRNA genes are first transcribed but not before.

2. TECHNICAL DIFFICULTIES OF STUDYING RNA SYNTHESIS IN *XENOPUS* EMBRYOS DURING PRE-MBT STAGE OF DEVELOPMENT

Embryos of *Xenopus laevis* were first utilized as materials

for molecular biological studies of developmental changes in nucleic acid metabolism, especially in the field of the control of RNA synthesis by Brown and Littna [24, 25]. These authors first isolated ^{32}P-labeled RNA by phenol treatment. Gurdon and Brown [26] proved that embryos, which do not contain rRNA genes and therefore do not form nucleoli, do not synthesize rRNA, and thus showed that nucleoli formation is the cytological manifestation of the functioning of ribosomal RNA genes. Shiokawa and Yamana [27,28] and Shiokawa *et al*. [29] introduced dissociated embryonic cell system as a new experimental system for studies of *Xenopus* embryonic RNA synthesis, and after comparing the pattern of RNA synthesis between $^{14}CO_2$-labeled whole embryos and (^{3}H) uridine-labeled dissociated embryonic cells, Shiokawa and Yamana [28] showed that developmental activation of rRNA synthesis is not affected by the artificial cell dissociation and proceeds normally in the dissociated cell system just as in the whole embryos.

The utilization of the dissociated cell system is to overcome the presence of impermeable surface coat which prevents uptake of various radioactive RNA precursors into the whole embryo. The inability of the whole embryo to uptake radioactive precursors made detection of newly synthesized RNA very difficult [28,29]. Furthermore, the previous idea of the total transcriptional silence of pre-MBT stage embryos also partly came from the situation in which cleavage stage embryos contain only a very small number of nuclei. This latter situation of the occurrence of only a small number of nuclei at the stages before MBT (pre-MBT stage) tends to lead to estimation of apparently undetectable or extremely low transcriptional activity per embryo even though transcription per nucleus may not be so small as compared with that in cells in post-blastula stage embryos. Also, it is a very characteristic feature of *Xenopus* fertilized eggs that they contain several 1000-folds amount of ribosomes per cell pre-formed by active transcription of extra-chromosomally amplified ribosomal DNA [30], and this tends to make it difficult to detect new formation of ribosomes in early stages.

3. DEVELOPMENTAL CONTROL OF RRNA GENE EXPRESSION AS STUDIED USING DISSOCIATED XENOPUS EMBRYONIC CELLS

Ribosomes are supramolecular structures which are large cellular machinery to produce proteins. Cellular activity to produce ribosomes sensitively reflects the cellular physiological conditions for cell growth. In eukaryotic cells ribosomes consist of 60S and 40S particles, and RNA moiety of 60S particles consists of 28S rRNA and

two smaller RNAs, 5.8S RNA and 5S RNA, and that of 40S particles consists only of 18S rRNA. In *Xenopus* oocytes rRNA producing genes, rDNA, are specifically amplified at stage III of oogenesis [30] and mature oocytes accumulate ca. 10^{12} ribosome particles which are 10^3 times larger than that of the adult-type cell and is enough for supporting protein synthesis for development up to the stage 42 feeding tadpoles [26].

Gurdon and Brown [26] proved that anucleolate *Xenopus* mutant embryos which do not have rDNA [31,32] neither synthesize rRNA nor form nucleoli, and thus showed that nucleoli formation is the cytological manifestation of the functioning (transcription) of rDNAs. Shiokawa and Yamana [27,28] and Shiokawa *et al*. [29] used dissociated embryonic cells, instead of whole embryos, as an experimental system for studies of *Xenopus* embryonic RNA synthesis. Embryos were dejellied by treatment with sodium thioglycolate (pH 8.3), and then dissociated in the Ca-free medium containing 0.02 M EDTA [28]. During culture isolated cells formed aggregates of various sizes depending on the cell density, but the relative rate of rRNA synthesis was the same, irrespective of the size of the aggregates finally formed [28]. Dissociated embryonic cells were labeled very extensively by simply adding radioactive precursor of RNA into their culture medium, although to label whole embryos $^{14}CO_2$ has to be used [24,28]. Shiokawa and Yamana [28] compared the overall RNA synthetic pattern in ^{3}H-uridine-labled dissociated cells and $^{14}CO_2$-labeled whole embryos, and concluded that as long as the overall profile of RNA-labeling pattern, which mainly reflects rRNA synthetic activity, is concerned, developmental change in the pattern of RNA synthesis is the same between the dissociated cell system and the whole embryos. Shiokawa and Yamana [28] found that per embryo activity of rRNA synthesis in dissociated blastula increases in the dissociated cell system just like that in the developing whole embryos, as embryos develop from the blastula stage to the neurula stage.

4. MBT IS ALSO CHARACTERIZED BY THE FIRST STAGE WHEN MATERNALLY PRESET PROGRAM OF APOPTOSIS IS EXECUTED AS A FAIL-SAFE MECHANISM OF DEVELOPMENT

MBT is characterized not only as the stage when G1 phase first appear in the cell cycle, but also the stage when maternally-preset program of apoptosis is executed. The execution of apoptosis in *Xenopus* embryos in the very early development could be summarized as in **Figure 2**. As shown in **Figure 2**, it appears that embryonic

cells check themselves to see if they are capable of continuing further development at MBT, and if some cells find themselves physiologically aberrant, they are removed into the blastocoel and disappear from the embryo by executing the apoptotic program [33]. In the cellular activities to be checked here are included the level of the methyl donor S-adenosylmethionine (SAM), DNA structure, DNA replication, DNA methylation, RNA transcription, and translation, as shown in the experiments which utilized SAMDC mRNA [33-35], γ-ray [36-38], cycloheximide [36-39], and 5-azadeoxycytidine (5-Aza-CdR) [40] as stimuli for execution of the apoptosis. From the experiments to trace cell lineage, we reached the conclusion that this apoptosis is to check and eliminate damaged cells shortly after MBT, and constitutes a surveillance or a "fail-safe" mechanism to save the rest of the embryo for normal development [33,35,41,42]. Since

Newmeyer et al. [43] showed that nuclear events typical of apoptosis can be reproduced in the cell-free extract of Xenopus eggs, the apoptosis mechanism seems to be a maternally-preset device in Xenopus unfertilized eggs.

5. EXTENSIVE MRNA CAP METHYLATION TAKES PLACE, BUT SYNTHESIS OF RRNA IS TOTALLY ABSENT IN THE PRE-MBT STAGE AS REVEALED BY THE ANALYSIS OF RRNA-SPECIFIC 2'-O-METHYLATION IN XENOPUS EARLY EMBRYOS

Ribosomal RNA synthesis was first considered to be initiated at the gastrula stage during Xenopus embryogenesis [26]. However, evidence that rRNA synthesis does not occur in pre-gastrular stages was not completely
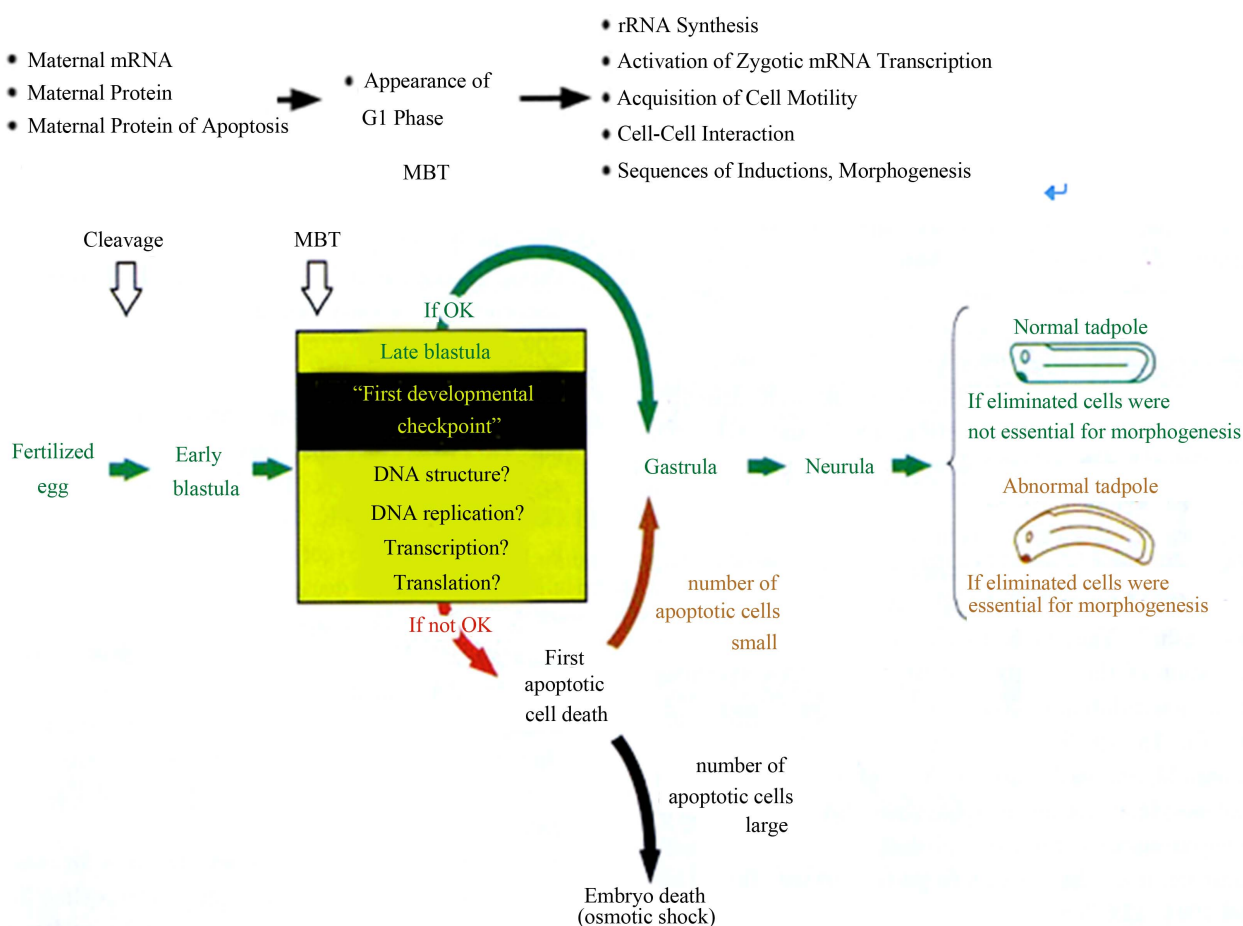

Figure 2. Changes which take place during Xenopus early embryonic development. This model shows how early development proceeds by indicating occurrence of apoptotic checkpoint at the midblastula stage or the stage of MBT. Fertilized eggs cleave rapidly until the early blastula stage, and when they reached MBT stage, the "first developmental checkpoint" comes when G_1 phase first appears. We assume that this check mechanism determines cell-autonomously if each embryonic cell may continue or may not continue development. If there is a cell which is not good for continued development, the cell is eliminated by execution of the maternal program of apoptosis. If the number of apoptotic cells was large, the whole embryo stops development and dies. However, if the number of apoptotic cells was small, such cells are confined within the blastocoel and disappear due to apoptosis, permitting the rest of the embryo continues on development. From Shiokawa [42].

conclusive, because RNA fractionation methods routinely used in previous experiments were sucrose density gradient centrifugation and agarose or polyacrylamide gel electrophoresis, and these methods did not completely separate rRNA from heterogeneous mRNA-like RNA, which is synthesized very actively especially in embryos during blastula stages [10,11].

18S and 28S rRNA molecules contain a relatively large number of 2'-O-methyl groups (90 per 40S pre-rRNA), and almost all of these 2'-O-methyl groups are conserved during pre-rRNA processing and in the matured rRNAs. Most eukaryotic mRNAs, on the other hand, contain no 2'-O-methyl group but the one in the cap terminus position. Therefore, rRNA and mRNA can be separately quantitated if nucleotides having 2'-O-methylation were determined in nuclease digests of these RNAs. Since rRNA precursor is methylated immediately after its transcription, the detection of rRNA-specific 2'-O-methylation is a sensitive method to detect rRNA gene products shortly after its transcription. Thus, the methods to detect rRNA-specific 2'-O-methylation is the methods to detect rRNA synthesis without being affected by the presence of inevitably-contaminating mRNA in the rRNA samples.

We labeled *Xenopus* embryonic cells with (methyl-^{3}H) methionine at the morula, blastula, gastrula and neurula stages, and purified ^{3}H-labeled high-molecular-weight RNA from each embryos on sucrose density gradient centrifugation. We digested the RNAs pooled from the high-molecular-weight RNA regions with RNases A and T$_2$, and analyzed the nucleotide digests on DEAE-Sephadex columns. **Figure 3** shows a set of results of such experiments. Judging from the sucrose gradient profiles (inset in each figure) rRNA synthesis indicated by the presence of distinct 18S and 28S rRNA radioactivity peaks occurred only at post-gastrula stages. In the profiles of morula (definitely, this stage is the pre-MBT stage) and blastula RNAs, however, radioactivity was widely distributed in the high-molecular-weight RNA region, and one can not discriminate if this is the labeling of rRNA or mRNA. We found here that about 80% of the radioactivity was resistant to alkali hydrolysis, which implies that most of the label in the high-molecular-weight RNA region at these early developmental stages (morula and blastula stages) was due to methylation of DNA which was fractionated also in this region. Incorporation of the label into 4S RNA, which is another RNA with ample 2'-O-methylation, occurred at all the stages examined (from morula to neurula stages). The DEAE-Sephadex column chromatographic profiles of morula cell RNA labeled for 3 hr showed methylation peaks at regions of charge value-2 (mononucleotide due to base methylation) and charge value-5 (mRNA cap), but there was no appreciable amount of 2'-O-methylation in the region of charge value-3 (rRNA-specific dinucleotides;

NpmNp) or charge value-4 (rRNA-specific trinucleotides; NmpNmpNp) nucleotides. This shows that morula cells do not synthesize rRNA at all, although they synthesize a considerably large amount of capped mRNA. In RNA from early blastula cells which were labeled for 4 hrs until the end of late blastula stage, the largest component obtained in the DEAE column chromatography was the charge value-3 compound (rRNA-specific dinucleotides). Here, the charge value-4 compound (specific to 2'-O-methylation in 28S rRNA) was also detected though in a much smaller amount. These results show that rRNA synthesis occurs already in embryos at the blastula stage. The relative amount of the charge value-5 (mRNA cap) component showing cap methylation in the blastula stage was smaller than in morula stage. At the gastrula stage, the relative amounts of charge value-3 and charge value-4 substances, both indicating rRNA synthesis, increased greatly and the relative amount of the cap methylation became much smaller. The methylation profile of neurula cells was essentially the same as that of gastrula cells.

6. RRNA SYNTHESIS IS ACTIVATED IN THE LATTER HALF PERIOD OF BLASTULA STAGE AS DEMONSTRATED BY ANALYSIS OF 2'-O-METHYLATION

Since we found that embryos starts to synthesize rRNA during the 4 hr from early blastula to late blastula stage, we divided this period into two 2 hr-periods, and studied rRNA synthesis by detecting 2'-O-methylation in each period. The results obtained showed that embryos contained only a trace amount of rRNA-specific dinucleotides in the former 2 hrs of the blastula stage but contained a very large amount of rRNA-specific dinucleotides in the latter 2 hrs, indicating that rRNA synthesis starts in the latter half of the MBT stage (**Figure 4**). These results are consistent with the timing of the expression of exogenously-injected ribosomal RNA genes [44]. By contrast, the extent of cap formation and base-methylation were not greatly different in the former and the latter half periods of blastula stage, indicating that only rRNA synthesis is activated during the latter 2 hr period of the blastula stage. From these results we concluded that rRNA synthesis starts during the midblastula to late blastula stage [10,11].

7. ONCE STARTED IN THE LATTER HALF PERIOD OF THE MBT STAGE, THE RATE OF RRNA SYNTHESIS PER CELL IS CONSTANT THROUGHOUT LATER STAGES

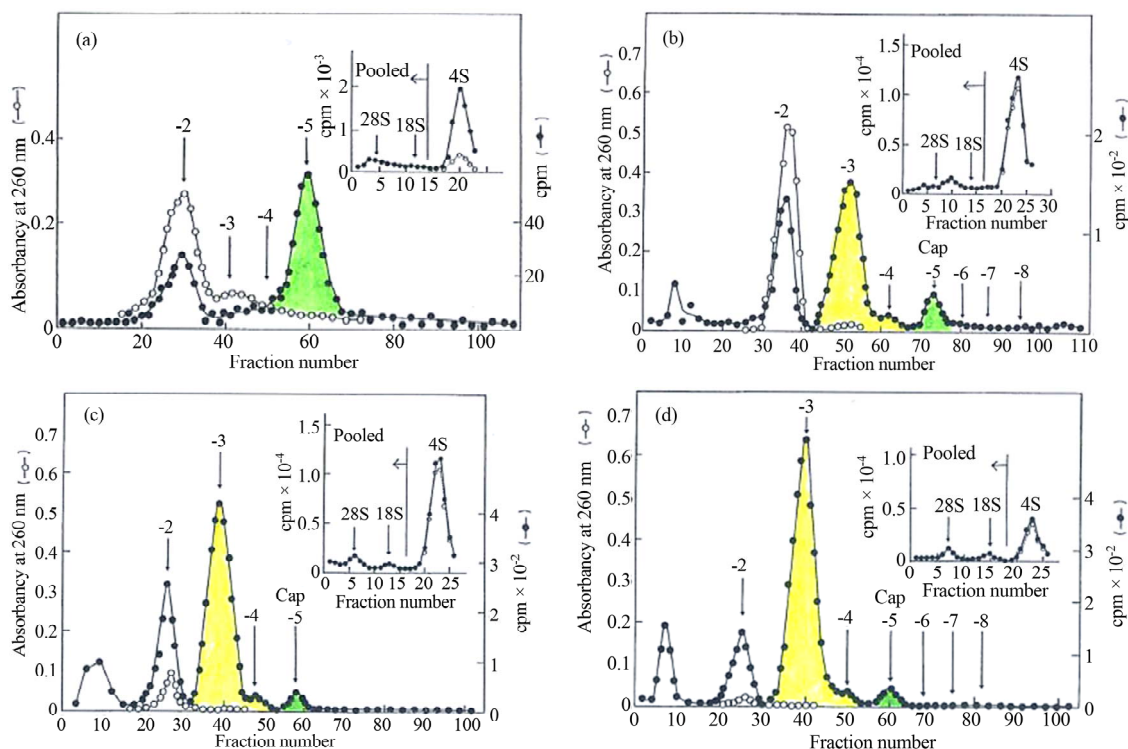From the amounts of incorporation of the (methyl-^{3}H)-group into rRNA-specific dinucleotides (NmpNp) and

Figure 3. Analysis of 2'-O-methylation in *Xenopus* early embryonic cells. DEAE-Sephadex A25 column chromatographic profiles were obtained for nuclease digests of (methyl-^{3}H)methionine-labeled high-molecular weight RNAs, which had been purified by sucrose density gradient centrifugation (insets). Embryos were dissociated and their cells were labeled with (methyl-^{3}H)methionine. Numbers indicated are positions of marker oligonucleotides of the indicated charge values. The number of embryos used for each labeling experiment was 3000 morulae (a); 300 early blastulae (b); 50 gastrulae (c) or 20 neurulae (d). The use of the changing number of embryos at different stage was in order to obtain RNA from 10^6 cells at all the stages. In the case of the experiment in (a), one labeling experiment was carried out using cells from 300 morulae and RNA extracted was subjected to sucrose density gradient centrifugation, and high-molecular-weight RNA was pooled. This was repeated 10 times, and 10 high-molecular-weight RNA preparations were pooled, and subjected to digestion with RNase A and T$_2$, and analyzed on DEAE Sephadex column at a time. Yellow peak is charge value-3 rRNA-specific dinucleotides ($N^m_pN_p$). The −4 charge value component is also for rRNA-specific methylated trinucleotides ($N^m_pN^m_pN_p$). Green peak (−5 component) is for methylated type I cap structure (m7G$_{ppp}$N^m_pN$_p$). Abscissa, Fraction number. From Shiokawa [42].

mRNA-specific cap, and the specific radioactivity of S-adenosylmnethionine which was determined by separate column chromatographic analyses, the rates of syntheses of 18S rRNA and 28S rRNA and capped mRNA were estimated, assuming that the specific radioactivity of the methyl-^{3}H in S-adenosylmethionine is the same as the specific activity of methyl-^{3}H in the methyl group contained in 2'-O-methylated nucleotides. The rate of rRNA synthesis per embryo was about 1 ng/embryo/hr at the blastula stage when rRNA synthesis starts in the latter 2 hr of the MBT stage, and this continued to increase greatly from the late blastula stage to the neurula stage. When the rate per cell of rRNA synthesis was calculated, it is undetectable (less than 0.02 pg/cell/hr) during the morula stage, 0.02 pg/cell/hr in the former 2 hr-period of the MBT stage, and about 0.12 pg/cell/hr in the latter 2 hr-period of the MBT stage, and was constantly about 0.2 pg/cell /hr in the following 15 hrs of development

(from gastrula stage to early tailbud stage). Since the appearance of nucleoli can be correlated with the start of rRNA synthesis, we corrected the rate of rRNA synthesis for the number of cells with definitive nucleoli. The approximate percentage of nucleolated cells increased sharply during the blastulas stage: it is about 50% in the late blastula stage and in later stages most cells have nucleoli. Thus, the rate of rRNA synthesis was about 0.2 pg/nucleolated cell/hr, and this did not change greatly in the later stages. It appears, therefore, that once rRNA synthesis starts, its rate per cell does not change greatly throughout development (**Figure 5**).

8. WEAK BASES SUCH AS AMMONIUM SALTS AND AMINES SELECTIVELY INHIBIT RRNA SYNTHESIS IN XENOPUS LAEVIS NEURULA CELLS

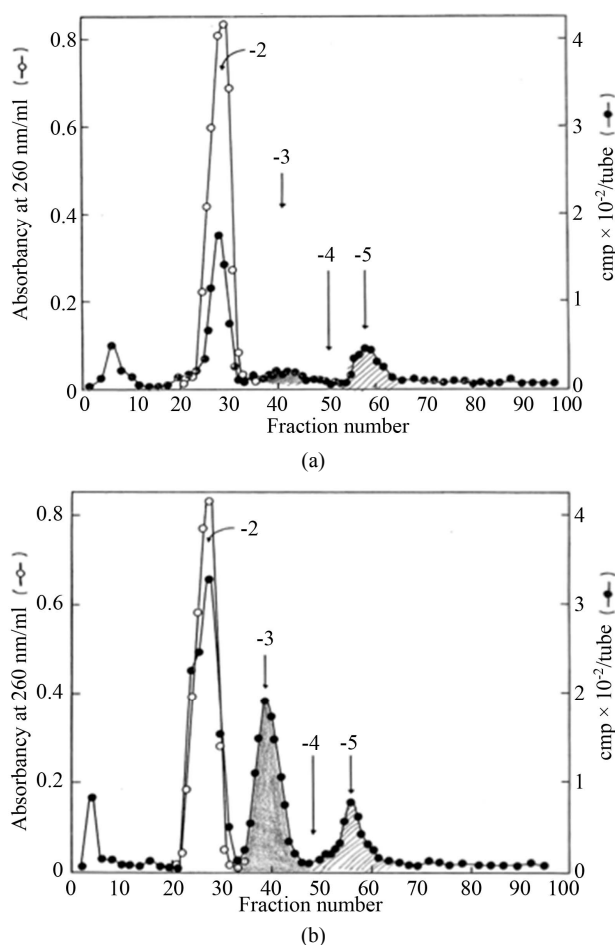As for the mechanism of the developmental control of

Figure 4. Comparison of RNA methylation pattern between the former half and the latter half of the blastula stage. (a) Blastula cells were labeled for 2 hrs with (methyl-^{3}H)methionine immediately after cell dissociation; (b) Blastula cells were first cultured for 2 hrs, then labeled as in (A) for 2 hrs. RNA was extracted and DEAE-Sephadex column chromatography was performed as in Figure 4 to find out 2'-O-methylatiom. Charge value-3 component which is specific for rRNA synthesis occurs only in the latter half of the blastula stage, whereas the labeling of charge value-5 substance, which is mRNA cap-specific methylation, occurred equally in both (a) and (b). From Shiokawa *et al*. [10].

rRNA gene expression, the classical nuclear transplantation experiment by Gurdon and Brown [26] suggested that a cytoplasmic factor is involved, because a nucleus from later stage embryos transplanted into the unfertilized egg ceased rRNA synthesis during cleavage and restored it at and after the gastrula stage. Shiokawa and Yamana [45] prepared dissociated blastula and neurula cells and cultured these cells together or separately and obtained results which suggested that *Xenopus* blastula cells may release some factor that inhibits rRNA synthesis in neurula cells [45]. Since the boiled culture medium (conditioned medium) from the blastula cell culture was effective as the unheated culture medium, we thought
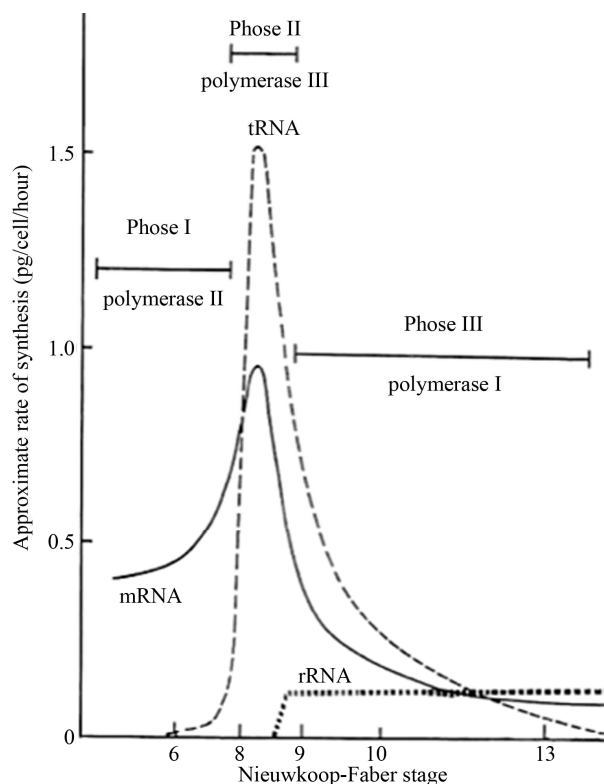

Figure 5. Rates of syntheses of three major RNA species during *Xenopus* development. Early stages were divided into three periods with respect to RNA synthetic activity. Phases I-III are characterized by active synthesis of mRNA, tRNA and then rRNA by DNA-dependent RNA polymerases II, III, and I, respectively. From Shiokawa *et al.* [13].

that the active substance that inhibits rRNA synthesis in neurula cells is a low-molecular-weight substance. The experiments utilizing the conditioned medium, however, were not of high reproducibility [45,46]. Therefore, we homogenized early embryos with 0.5 N perchloric acid (PCA) and after neutralizing it with KOH, applied it onto a small charcoal column, and eluted acid-soluble materials from the charcoal column with ammonia-alcohol (0.2 N ammonia-50% ethanol). The white powder obtained by lyophilization of the ammonia-alcohol eluate selectively inhibited rRNA synthesis in neurula cells [45,46]. After a long lasting efforts for purification of the active substance, we finally reached the conclusion that the active substance in the charcoal eluate was ammonium perchlorate that was formed artifactually during the charcoal column chromatography [47]. In these results, however, the inhibition of rRNA synthesis induced by the ammonium perchlorate was quite strong and in spite of the inhibition of rRNA synthesis 4S RNA synthesis was not affected appreciably. Therefore, we continued to study how ammonium salts affect rRNA synthesis in *Xenopus* neurula cells.

The concentration of ammonium perchlorate which

selectively inhibits rRNA synthesis turned out to be ca. 2.5 mM. We compared the effects of the same concentration (2.5 mM) of ammonium perchlorate and sodium perchlorate in *Xenopus* neurula cells, with the finding that only the former but not the latter inhibited rRNA synthesis selectively (**Figure 6**). This implied that the effective part of the ammonium perchlorate is not the perchlorate moiety, but the ammonium moiety. We cultured *Xenopus* neurula cells in the medium which contained 2.5 mM of ammonium chloride and ammonium sulfate, and found that both of these inhibit rRNA synthesis, with little inhibition on 4S RNA synthesis [47-49]. We tested ammonium dihydrogenphosphate, ammonium monohydrogenphosphate, and ammonium phosphate, using as references, potassium dihydrogenphosphate, potassium monohydrogenphosphate and potassium phosphate all at 2.5 mM. We found that only ammonium ion-releasing salts were effective in inhibiting rRNA synthesis, and the effectiveness was roughly proportional to the number of ammonium ions to be released. We also found that while ammonium acetate and ammonium aspartate selectively inhibited rRNA synthesis at 2.5 mM, again, their potassium salt were ineffective. We also found that ammonium salts of all the four major ribonuleotide monophosphates (5'-AMP, 5'-GMP, 5'UMP, and 5'-CMP) at 5 mM were effective in selectively inhibiting rRNA synthesis in neurula cells. We also tested the effects of amines such as monomethylaminehydroperchloride, dimethylaminehydroperchloride, and trimethylaminehydroperchloride, and found that all of these are active as selective inhibitors of rRNA synthesis at 2.5 mM. Similar positive results were obtained when three kinds of ethylamines were tested. From these results, we concluded
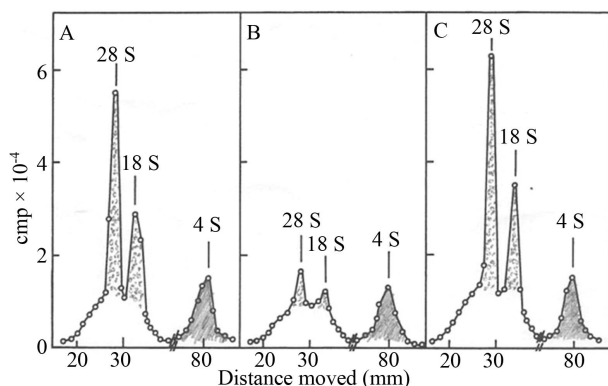
that weak bases such as ammonium ion and amines are similarly effective as selective inhibitors of rRNA synthesis in *Xenopus* neurula cells.

9. WEAK BASES ALSO SUPPRESS DEVELOPMENTAL ACTIVATION OF RRNA SYNTHESIS IN XENOPUS BLASTULA CELLS

Weak bases inhibit rRNA gene expression in *Xenopus* neurula cells in which rRNA synthesis is fully activated. We cultured blastula cells for 5, 10, 15 hrs in the medium which contained 2.5 mM of ammonium salts. When control blastula cells were labeled with ^{3}H-guanosine for 5 hrs a small amount of incorporation was detected in 28S and 18S rRNA with a large amount of incorporation into 4S RNA and DNA (**Figures 7(A)-(C)**). Ammonium chloride, but not potassium chloride, inhibited rRNA synthesis but not 4S RNA and DNA syntheses. When we first cultured blastula cells in the ammonium chloride-containing medium for 5 hrs, and then labeled them for 5 hrs in the continued presence of the ammonium chloride, much clearer inhibition of rRNA synthesis was obtained. This is an indication of the inhibition of post-MBT activation of rRNA synthesis (**Figures 7(D)-(F)**). Essentially the same results were obtained when we cultured blastula cells for 10 hrs in the presence of ammonium chloride and then labeled them for 5 hrs (**Figures 7(G)-(I)**). Importantly, syntheses of 4S RNA (tRNA) and heterogeneous nuclear RNA, and in addition, DNA were not interfered greatly under these conditions. Using 10% polyacrylamide gel, we separated small-molecular-weight RNAs in the RNA preparations obtained and confirmed that ammonium chloride did not inhibit the labeling of 4S RNA, 5S RNA, and U_1, U_2, and U_5 small nuclear RNAs.

When we cultured blastula cells for 5 hrs in the ammonium ion-containing medium and after this transferred them into the normal medium which contained ^{3}H-guanosine. We found here that rRNA synthesis can be restored to a large extent, indicating that the effects of ammninium salts are reversible. By HPLC analysis of the acid-soluble faction of the embryonic cells, we confirmed here that ammonia was incorporated into neurula cells within 1 hr. We also confirmed that ammonium salts at ca. 3 mM did not inhibit protein synthesis, cell division, and cellular reaggregating activity at least for the first 10 hrs of incubation, although after treatment for 10 hrs, cell reaggregation was found to be significantly inhibited. These data indicates that ammonium salt actually selectively inhibits not only the initiation of rRNA synthesis at MBT but also subsequent activation of rRNA synthesis in post-MBT stages.
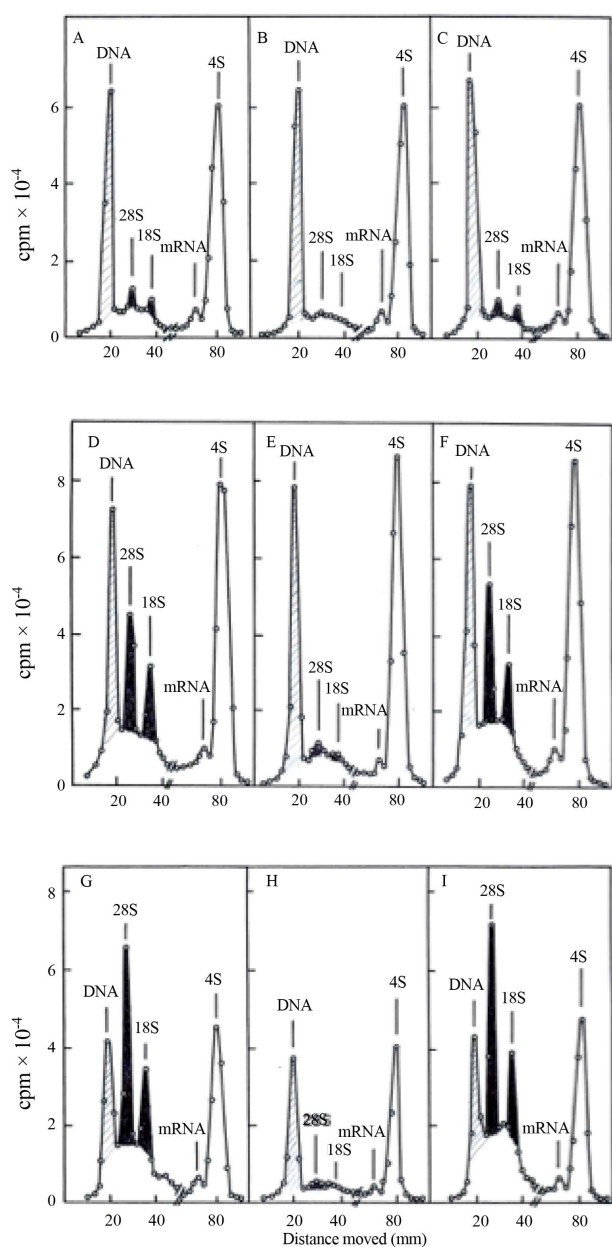

Figure 6. Effects of ammonium perchlorate and sodiumn perchlorate on RNA synthetic pattern of neurula cells.Neurula cells were labeled for 3 hrs with 20 μCi of ^{3}H-uridine for 3 hrs in the presence of 2.5 mM of ammonium perchlorate (B) or sodium perchlorate (C); RNAs were extracted and electrophoresed on 0.5% agarose-2.4% polyacrylamide gels; (A) Control neurula cells. Dotted peaks are for 28S and 18S rRNAs, and black peaks are for 4S RNA (tRNA). From Shiokawa *et al*. [47].

Figure 7. Inhibition of the initiation and activation of rRNA gene expression by ammonium chloride but not by potassium chloride in *Xenopus* blastula cells. Blastula cells were treated with 2.5 mM of either ammonium chloride ((B), (E), (H)) or potassium chloride ((C), (F), (I)) and labeled with 3H-guanosine. ((A)-(C)) Blastula cells were labeled for 5 hrs. ((D)-(F)) Cells were cultured for 5 hrs in the presence of ammonium chloride (E) or potassium chloride (F), and then labeled for 5 hrs with ^{3}H-guanosine in the continued presence of the salts. ((G)-(I)) Cells were cultured for 10 hrs in the presence of the salts and then labeled for 5 hrs in the continued presence of the salts. RNAs were extracted and electrophoresed on 0.5% agarose-2.4% polyacrylamide gels. In this profile the radioactivity appeared also in DNA. ((A), (D), (G)) Control cells. Black peaks are for 28S and 18S rRNA, and white peaks are for 4S RNA, and shaded peaks are for DNA. From Shiokawa *et al*. [48].

10. WEAK BASES INHIBIT RRNA TRANSCRIPTION, BUT NOT POST-TRANSCRIPTIONAL PROCESSING

Xenopus neurula cells were first inhibited for 2.5 hrs by 5 mM of ammonium chloride or monomethylamine hydroperchloride and then pulse-labeled for 2.5 hrs in the continued presence of the weak bases [47]. We obtained active labeling of heterogeneous nuclear RNA (hnRNA), and 40 S rRNA primary transcript, in addition to the labeling of 28S and 18S mature rRNAs and 4S RNA. Under these conditions, both of the weak bases inhibited labeling of 40S pre-rRNA, 28S rRNA, and 18S rRNA almost completely (**Figure 8**), suggesting that the inhibition of rRNA synthesis by these weak bases is at the transcription level.

We then selected conditions of partial inhibition of rRNA synthesis by these weak bases. When we labeled the control neurula cells for 1 hr (**Figure 9(A)**), we obtained 40S pre-rRNA (black peak), 30 S rRNA intermediate (shaded peak) and 18S rRNA (dotted peak), in addition to a large amount of heterogeneous mRNA-like RNA [50]. The appearance of 18S mature rRNA before 28S mature rRNA is due to the fact that the processing of the former is finished before that of the latter [51]. The identification of 30S component is based on slight re-
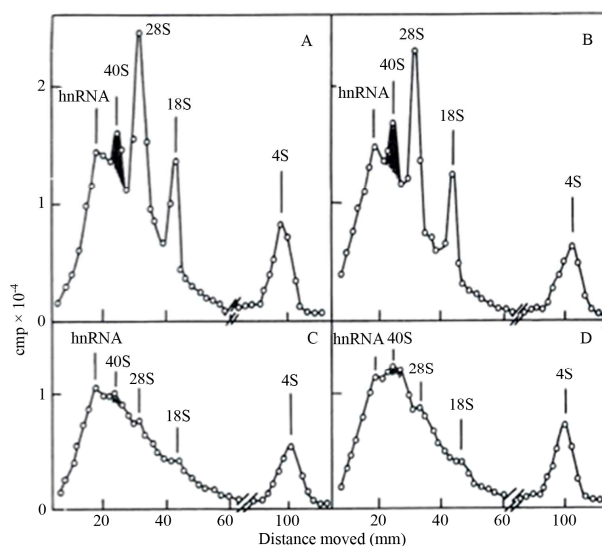

Figure 8. Effects of ammonium salts and amine on pulse-labeled RNA synthesis. *Xenopus* neurula cells were treated with 5 mM each of potassium chloride (B); ammonium chloride (C); or monomethylaminehydroperchloride (D) for 2.5 hrs and then pulse-labeled with 3H-uridine for 2.5 hrs in the continued presence of the potassium and ammonium salts. RNA was extracted and gel electrophoresed, and radioactivity determined using the sliced gels. Densitometric scanning of gels before slicing are omitted from the radioactivity profiles. Peaks of 40S rRNA precursor are marked by black colour. A, Control untreated cells. From Shiokawa *et al*. [47].
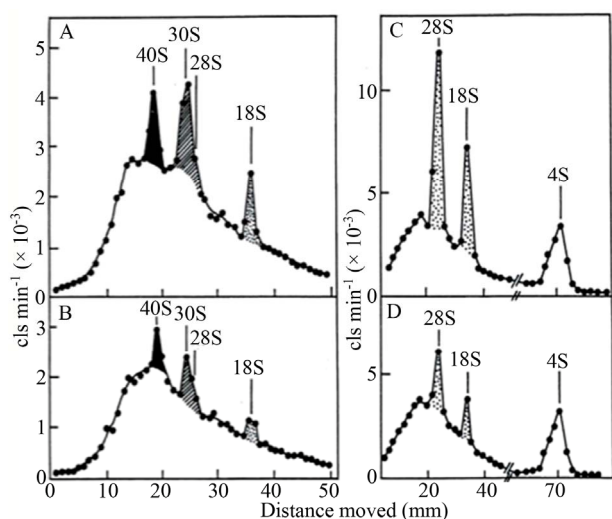
Figure 9. Effects of ammonium chloride on the labeling of 40S pre-rRNA, 30 S pre-rRNAs and 18S and 28S mature rRNAs. Neurula cells were labeled for 1 hr ((A), (B)) or for 3 hrs ((C), (D)) with ^{3}H-uridine in the presence of 2.6 mM ammonium chloride. RNAs were gel electrophoresed for 2 hrs ((A), (B)) or 1 hr ((C), (D)). Longer electrophoresis was for obtaining better resolution in the high-molecular-weight RNA region. Densitometric scanning of gels before slicing are omitted from the radioactivity profiles. Black peaks are for 40S pre-rRNA, shaded peaks are for 30S pre-rRNA, and dotted peaks are for 28S and 18S mature rRNAs. From Shiokawa *et al.* [50].

tardation of this peak from 28S optical density peak on the agarose-polyacrylamide gel. When neurula cells pre-treated for 2.5 hrs with 2.6 mM ammonium chloride (which was the conditions for partial inhibition of rRNA synthesis) were labeled with ^{3}H-uridine for 1 hr, the extent of the inhibition of labeling was 60%, 59%, and 70% for 40s pre-rRNA, 30s rRNA intermediates and 18S rRNA, respectively (**Figures 9(A)** and **(B)**). When the same cultures were labeled for 3 hrs, labeling of 18 S and 28 S mature rRNAs but not 40S and 30S pre-rRNA became predominant, and inhibition here was 72%, 69%, and 7% for 28S and 18S rRNAs and 4S RNA, respectively (**Figures 9(C)** and **(D)**). When we performed a parallel experiment using 1 mM trimethylammonium perchloride, the inhibition was 67%, 70%, 75%, 73% and 8%, for 40S pre-rRNA, 30S rRNA intermediate, 28S rRNA, 18S rRNA and 4S RNA, respectively. Thus, the extents of the inhibition of the labeling of 40S pre-rRNA, 30S intermediate rRNA, 18S and 28S mature rRNA were much the same (67% - 75%), and in spite of the large inhibition of the labelinbg of rRNA species, inhibition of the labeling of 4S RNA (tRNA) was only 8%. Therefore, we concluded that inhibition was at the level of the formation of 40S primary transcript and [50] probably not at the processing of 40S pre-rRNA.

Approximately 30 - 60 min is needed for the pulse-labeled 40S pre-rRNA to be processed completely into

18S and 28S rRNAs [50,51]. Therefore, we first pulse-labeled neurula cells for 35 min and chased the label in rRNA species for 2 hrs. Labeled RNAs in neurula cells under these conditions were 40S pre-rRNA and hetero-geneous mRNA-like RNA (**Figure 10(A)**). These neu-rula cells were administered with actinomycin D (10 µg/ml) as a transcription inhibitor, and treated either with ammonium chloride (5 mM) or with trimethylammonium perchloride (5 mM) for 2 hrs in the continued presence of actinomycin D. During the chase period of 2 hrs, the label in the 40S pre-rRNA in the control culture com-pletely disappeared and 18S and 28S mature rRNAs ap-peared (**Figure 10(B)**). Such changes in the labeling pro-file were observed also in the cultures treated with 10 mM ammonium chloride (**Figure 10(C)**) and trimethyl-ammonium perchloride (**Figure 10(D)**). These results show that weak bases used here inhibited rRNA tran-scription but did not inhibit the processing of 40S pre-rRNA into two mature rRNAs [50]. Also, these results exclude the possibility of aberrant processing or rapid wastage of the mature rRNAs.
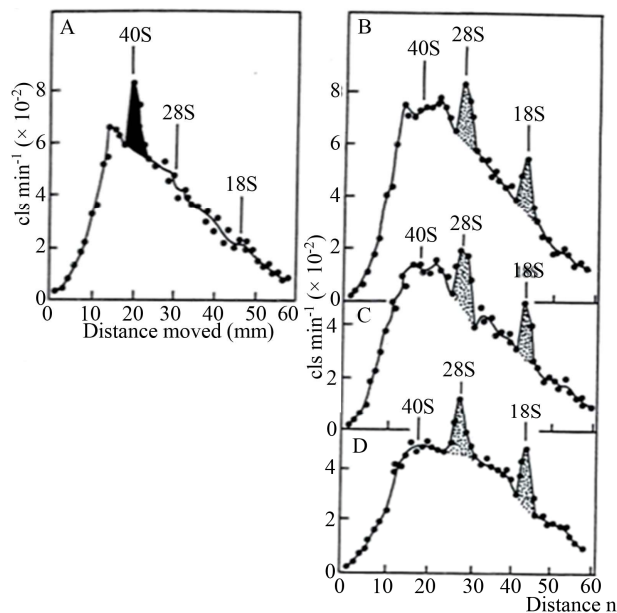

Figure 10. Effects of ammonium chloride on the processing of 40S pre-rRNA which was labeled before administration of am-monium chloride. Neurula cells were pulse-labeled with ^{3}H-uridine for 35 min. One culture was then immediately frozen as a zero-time control (A). Three cultures were then administrered with 10 µg/ml of actinomycin D and further cultured for 2 hrs in the presence of either 10 mM ammonium chloride (C) or 10 mM of trimethylammonium perchloride (D). Control cells were incubated for 2 hrs without being exposed to these weak bases (B). RNA were extracted and electrophoresed on agarose-poly-acrylamide gels. Densitometric scanning of gels before slicing are omitted from the radioactivity profiles. A black peak is 40S pre-rRNA and dotted peaks are 28S and 18S rRNAs. From Shi-okawa *et al.* [50].

11. POSSIBLE INVOLVEMENT OF PH CHANGE IN THE WEAK BASE-TREATED NEURULA CELLS

In *Xenopus* embryos, a slight increase of intracellular pH has various important effects [52]. Such pH changes have been implicated as a necessary step in oocyte maturation [53], fertilization [54], and commencement of cleavages [55]. We suspected that a slight elevation of intracellular pH might be involved in the inhibition of rRNA synthesis. Since it is known that pH-mediated changes are abolished when Na^+ was eliminated from the surrounding medium [53,56], we tested the effect of 3 mM ammonium chloride and 1.5 mM trimethylammonium perchloride on neurula cells in the medium in which all the Na^+ was replaced by choline ions. Results of the labeling experiment showed that approximately 80% of the inhibitory activity of both ammonium chloride (**Figure 11(B)**) and trimethylammonium perchloride (**Figure 11(C)**) completely disappeared when their effects were tested in the medium whose Na^+ had been replaced by choline ions (respectively, **Figures 11(E)** and **(F)**). Therefore, these weak bases seem to exert their rRNA synthesis-inhibiting effects via slight pH elevation within the neurula cells.

We prepared acid-soluble fractions of these weak base-treated neurula cells, and found that the level of ATP as well as other ribonucleotidetriphosphates remained unchanged. Therefore, neither ammonium chloride nor trimethylammonium perchloride disturbed the energy generating system in *Xenopus* neurula cells [50].

From these results, the mechanism by which weak bases inhibit rRNA synthesis in *Xenopus* embryonic cells can be summarized as in **Figure 12**.
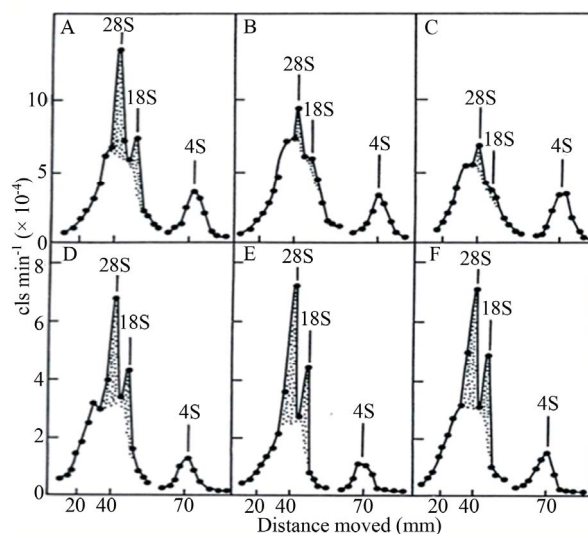

Figure 11. Disappearance of the inhibitory effect on rRNA synthesis of ammonium chloride and trimethylammonium-perchloride in the medium in which Na^+ was replaced by choline ion. Neurula cells were treated for 2.5 hrs either in the normal medium ($0.1 \times$ Steinberg's solution) ((A)-(C)) or in the choline$^+$-containing Na^+-free medium ((D)-(F)) with 3 mM of ammonium chloride ((B), (E)) or 1.5 mM of trimethylammonium perchloride ((C), (F)). ((A), (D)) are untreated control neurula cells. All the cultures were administered with ^{3}H-udirine and cells were labeled for 3 hrs. Dotted peaks are 18S and 28S rRNAs. From Shiokawa *et al*. [50].
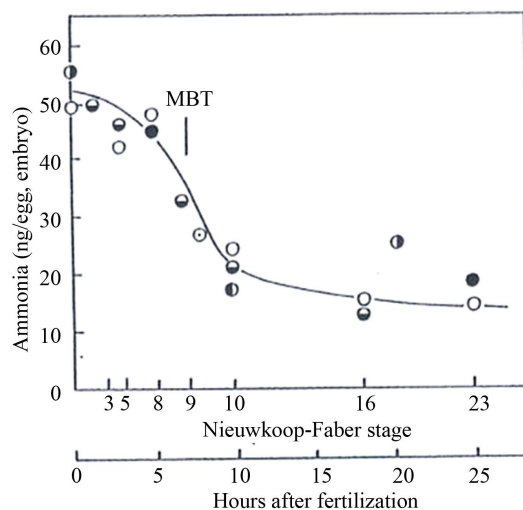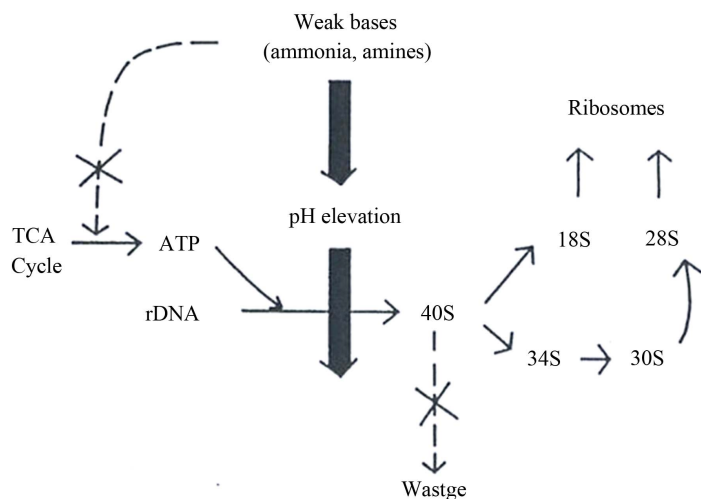

Figure 12. A working hypothesis that weak bases, most probably ammonium ion within the embryonic cells, may be involved in the regulation of rRNA transcription in *Xenopus* early embryos. Left: Weak bases do not inhibit TCA cycle and do not induce wastage of 40S pre-rRNA and 18S and 28S mature rRNAs. Also, processing of pre-rRNAs is not inhibited. Instead, weak bases inhibit transcription of rRNA genes (rDNA). This inhibition is probably mediated by intracellular slight pH elevation. From Shiokawa *et al*. [50]. Right: Developmental changes in the amount of ammonium ion within the embryo determined by amino acid analyzer using the PCA-soluble fraction of the embryo homogenates. The changing amount of the ammonium ion is expressed as ammonia (ng/egg or embryo). From Shiokawa *et al*. [48].

12. UPTAKE OF AMMONIUM ION AND AMINES IN *XENOPUS* NEURULA CELLS

Using amino acid analyzer, we analyzed ammonia and amines in acid-soluble fractions of *Xenopus* neurula cells which had been treated with 2.5 mM of either ammonium chloride, ammonium phosphates, or methylamines. In the amino acid profiles, it has been shown that the peak representing ammonia became quite large in the cells treated with ammonium salts. In cells treated with monomethylamine hydroperchloride, a large amount of monomethylamine was detected, with no increase in the amount of ammonia. However, amounts of other components such as ornithine and other amino acids in the neurula cells did not change appreciably in spite of the treatments with ammonium salts and amines. These results indicate that both ammonium ion and amines selectively inhibit rRNA synthesis after being uptaken into *Xenopus* neurula cells.

13. AMMONIA IS A CANDIDATE OF THE REGULATOR OF RRNA SYNTHESIS WHICH CONTROLS THE INITIATION AND ACTIVATION OF RRNA SYNTHESIS AT AND AFTER MBT

When we examined ninhydrin-positive materials in the acid-soluble fraction of *Xenopus* early embryos, there was a sizable amount of ammonia but not amines within the embryo. When we performed similar analyses at various stages of development, we found that the level of ammonia in an egg was ca. 55 ng/egg before fertilization and this value did not change greatly during cleavage, but decreased promptly to the level as low as ca. 20 ng/embryo. The occurrence of ca. 55 ng/egg of ammonia corresponds ca. 3 mM of ammonia as an intra-egg concentration, and 20 ng/embryo of ammonia corresponds ca. 1 mM as an intra-embryo concentration, since the volume of an egg is ca. 1 μl. The lowered level of ammonia was maintained throughout later stages until the muscular response stage (**Figure 12**).

We attempted to isolate ammonia (and amines if any) from cleavage stage embryos. Starting from 25,000 cleavage embryos, we isolated about 10 mg of residual materials after evaporating the hydrochloric acid solution that was used to capture the cellular volatile ammonia components. Mass spectrometric analysis revealed the presence of ammonium chloride but not of amines. Ten milligrams of ammonium chloride obtained here corresponds to ca. 4 mg of ammonia. We tested effects of the ammonium chloride isolated from cleavage stage embryos for its activity to inhibit rRNA synthesis in *Xenopus* neurula cells at 1.0 and 5.0 mM. The results showed that the synthesis of rRNA was inhibited by 60% (at 1 mM) and 90% (at 5 mM) with only a slight inhibition (less than 10%) in 4S RNA (tRNA) synthesis. These observations also suggest that ammonium ion is a candidate for the factors that regulate initiation and activation of rRNA synthesis in *Xenopus* embryogenesis.

14. CONCLUSION

It has been long believed that RNA synthesis never takes place from zygotic nuclei during pre-MBT stage or cleavage stage in early *Xenopus* embryogenesis. However, Shiokawa's old data [12] and Klein's new data [17] show that there is RNA synthesis in pre-MBT embryos. However, not all the RNA is synthesized during the pre-MBT stage. For example, rRNA is the RNA whose genes are totally silent during the pre-MBT stage, and transcription of rRNA genes is initiated from the latter half of the MBT stage. This developmental change in the initiation of rRNA genes coincides with the developmentally controlled appearance of nucleoli not before but after MBT. Weak bases were found to selectively inhibit rRNA synthesis in neurula cells and also inhibit the developmental activation of rRNA synthesis which takes place after MBT. Based on some evidence, weak bases such as ammonia could be a factor that constitutes the control mechanism of the developmental regulation of rRNA genes at the transcriptional level, but not at the step of the post-transcriptional processing level. Our data suggest that ammonia and amines exert their effect via slight elevation of intracellular pH in the early *Xenopus* embryos.

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