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# RNase Resistant RNA in the Egg of *Xenopus laevis* I. RNA Extraction and *in Vitro* Labeling[1,2,3]

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# *Xenopus laevis*난에 존재하는 RNA분해효소에 저항성인 RNA I. RNA 추출 및 *in Vitro* Labeling

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## 적 요

RNA 분해효소에 저항하는 RNA 분자들이 양서류의 난에 존재하는지의 여부를 조사하기 전에 필요한 몇가지 예비실험을 하기 위하여 *Xenopus laevis*의 난에서 RNA를 추출 하였다.

Sephadex G-100 column chromatography는 세개의 peak을 항상 보여주고 있다. 첫째 peak에 포함되어있는 고분자량의 RNA만 $^{3}$H-dimethyl sulfate를 사용하여 시험관내에서 label하여 tRNA로부터의 base paired oligonucleotide의 참여를 배제하였다. 이 방법으로 아주 높은 specific activity를 얻을 수 있었으며 또한 부착된 methyl group은 대단히 안정성을 보였다.

## INTRODUCTION

It is a well-known fact that infection with a wide variety of small RNA containing bacterial, plant, and animal viruses leads to the formation of RNase resistant, double stranded-RNA (ds-RNA) which is referred to as a "replicative form" of viral RNA (Levinton, 1965). However, Colby and Duesberg (1969) have reported the finding of DNA virus specific ds-RNA in chick cells infected with vaccinia

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virus. The presence of ds-RNA in cells infected with a DNA virus is not restricted to eukaryotic cells since virus specific ds-RNA is also found in *E. coli* infected with $T_4$ phage (Jurale *et al.*, 1970) and bacteriophage MS 2 (Lago *et al.*, 1972).

Quite interestingly, ds-RNA can be detected in uninfected animal cells such as cultured human lymphoid cells, and a portion of this ds-RNA has been reported to be synthesized in the presence of actinomycin D (Stern and Friedman, 1971). Evidence for the presence of double stranded regions in heterogeneous nuclear RNA (Hn-RNA) from HeLa cells has been reported independently by Ryskov *et al.* (1972) and by Jelinek and Darnell (1972). According to them, double stranded regions are transcribed from reiterated sites in the genome, and are formed by intramolecular base pairing. Kronenberg and Humphreys (1972) purified and characterized ds-RNA from hatching blastulae of the sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus pictus*. On the bases of its being limited to the nucleus, and of its kinetic behavior as a rapidly turning-over nuclear RNA, they proposed that cellular ds-RNA is transcribed in concert with mRNA sequences as a part of Hn-RNA and that the complementary regions represent a portion of the transcript which is degraded in the nucleus in conjunction with the processing of mRNA and its transport to the cytoplasm. However, some informational RNA molecules determining amino acid sequences in proteins such as RNAs of bacteriophage $R_{17}$ (Sanger, 1971), globin mRNA (Williamson *et al.*, 1971) contain double stranded regions. White Ⅲ *et al.* (1972) suggested that the possible double stranded regions in mRNA of human cytochrome C could evolve through selections based on their greater stability in the cytoplasm. Riley (1973) has also predicted the presence of double stranded regions in mRNAs for human immunoglobulin K light chains and discussed the significance of double strandedness in relation to the evolution of the genetic code and suggested a possible mechanism whereby they could help to direct the sites of mutation.

Therefore, ds-RNA which had previously been thought to occur in nature only in the replicative cycle of RNA viruses and in a few RNA virus genomes appears now to be present widely in eukaryotic cells. In addition, ds-RNA is involved in several biological processes in animal cells. For example, it induces the production of interferon (Harel and Montagnier, 1971), can cause tumor regression (Cordell-Stewart and Taylor, 1971), inhibits protein synthesis both in cells infected with polio-virus (Hunt and Ehrenfeld, 1971) as well as in cell-free extract from reticulocytes (Darnbrough *et al.*, 1972), determines the cytoplasmically inherited killer-character of yeast (Vodkin and Fink, 1973).

It is certain that unfertilized amphibian and sea urchin eggs store quite stable maternal mRNAs for post-fertilization development. Evidence for the presence of these stable mRNAs is that the sea urchin eggs treated with actinomycin D are

capable of protein synthesis without any *de novo* RNA synthesis and of consequent cleaving and forming blastulae (Gross and Cousinou, 1964; Raff *et al.*, 1972). The fact that the activated non-nucleated egg fragment of sea urchin (Harvey, 1940) or frog (Briggs *et al.*, 1951) and eggs with inactivated or destroyed nuclei (Dalq and Simmon, 1932) can cleave also supports its presence. About 60% of these stable mRNAs can still be detected by hybridization in cleaving *Xenopus* embryos (Crippa *et al.*, 1967) and about half of their sequences have been lost by early blastula stage (Crippa and Gross, 1969). Although there is some evidence indicating their presence in a "masked" form, probably as informosome-like ribonucleoprotein particles (Spirin, 1966), the nature of stored mRNA is still obscure.

Unfortunately, it is impossible to define the stabilization mechanism of stored mRNA with our present level of knowledge. The overall purpose of the experiments in this series is to explore whether double stranded cellular RNA reported by many workers exists in the amphibian eggs, and if so, to study the relationship between its presence and the stored mRNA that might provide some clue concerning the physical structure of the stable maternal mRNA and its stabilization mechanism. *Xenopus laevis* was chosen because it has many advantages as a developmental system (see Discussion). The first paper in this series describes the extraction of total RNA from the eggs, chromatographic isolation of high molecular weight RNA, and the efficacy of *in vitro* labeling.

## MATERIALS AND METHODS

1. *Xenopus* Eggs:

Ovulation was induced by injecting 300~500 IU of chorionic gonadotrophin (Sigma) into the dorsal lymph sac of a gravid female. Eggs were collected in De-Boer's solution (0.11 M NaCl, 0.0013 M KCl, 0.00044 M $CaCl_2$) and dejellied in 5% cysteine-HCl solution containing 0.1 M Tris-HCl (pH 7.8, Fisher Scientific Co.) for 3 to 5 minutes. They were then washed quickly with 5 changes of dechlorinated tap water according to Gussek and Hendrick (1971). Eggs were immediately used for RNA extraction or stored at −40°C in homogenizing buffer (0.1 M sodium acetate containing 4 $\mu$g/m*l* of polyvinyl sulfate, pH 5.0).

2. RNA Extraction:

Total RNA was extracted by the cold sodium dodecyl sulfate-phenol method of Brown and Littna (1964). Dejellied eggs were homogenized in 5 to 10 volumes of homogenizing buffer. Ten percent sodium dodecyl sulfate solution was added to a final concentration of 1% and the suspension was shaken with an equal volume of buffer-saturated phenol at 4°C for 10 minutes. The aqueous phase was collected after centrifugation at 3,000 G for 10 minutes at 4°C until there was no interphase. The interphase and phenol phase were further extracted with 0.5 volume of

homogenizing buffer. A 2 M NaCl solution was added to a final concentration of 0.1 M NaCl and the pooled aqueous phase was precipitated with 2 volumes of ethanol. The RNA was collected after centrifugation and dried by an air stream. Dried RNA was dissolved in TSE buffer (0.1 M NaCl, 0.001 M EDTA, 0.05 M Tris, pH 6.9) and precipitated with 2 volumes of ethanol. The above process was repeated until the ratios of light absorbancy at 260 nm and 270 nm, and of 260 nm and 280 nm reached to 1.2 and 2.0, respectively. Lowry tests were routinely performed to check for protein contamination (Lowry *et al.*, 1951). RNA was further extracted with a mixture of chloroform and buffer-saturated phenol (volume/volume, 1 : 1) until the Lowry test indicated negative values. The purified RNA was then precipitated with ethanol.

3. Sephadex G-100 Column Chromatography:

Gel was swollen in TSE buffer for 3 days according to the instructions of the manufacturer (Pharmacia Fine Chemicals), and the swollen gel was packed in a column of 1.5×79 cm. After equilibration with the same buffer at 4°C, total RNA extracted from the *Xenopus* eggs was placed on the column and fractions of 6.5 m*l* were collected at a flow rate of 0.1 m*l*/minute. Only high molecular weight RNA fractions which were excluded from the gel were pooled and precipitated with ethanol for *in vitro* labeling.

4. *In Vitro* Labeling:

Since extremely low specific activity was obtained by *in vivo* labeling (injection of 1 m*Ci* $^{3}$H-uridine per animal, see Results), an *in vitro* labeling method was adopted which had been successfully used in cases where low metabolic activity prevented labeling of RNA *in vivo* during its synthesis (Smith *et al.*, 1967; Hynes and Gross, 1972).

The high molecular weight RNA from the Sephadex column was dissolved in distilled water and a 2.5 M sodium acetate solution (pH 5.0) was added to make a 1 M solution with a RNA concentration of 100~200 O.D./m*l*. Aliquots of $^{3}$H-dimethyl sulfate (375~385 m*Ci*/mM, New England Nuclear) dissolved in ether were added. Following the elimination of ether by aeration, samples were kept for 20 hours with agitation at 4°C. The samples were then precipitated with 2 volumes of ethanol and dissolved in TSE buffer. Ethanol precipitation was repeated until there was no detectable amount of radioactivity in the supernatant after centrifugation. Three changes of dialysis against 500 m*l* of appropriate buffer were sometimes carried out following ethanol precipitation. To obtain high specific activity same procedures were done for the RNase treated RNA species.

5. Measurement of Radioactivity:

Aliquots of RNA samples were precipitated in 8~10% TCA solution with 2 dr-

ops of carrier RNA (yeast soluble RNA, 3 mg/ml) for more than one hour in an ice bath. RNA was collected on 0.45 $\mu$ Millipore filters and washed with an excess of cold 5% TCA. Dried filter papers were counted in 10 ml of toluene cocktail in a Beckman Liquid Scintillation Counter Model LS-233. The scintillation counting cocktail contained the followings; toluene (reagent grade) 1,000 ml, PPO 5 g, POPOP 0.1 g.

## RESULTS

1. Molecular Sieve Chromatography on Sephadex G-100 column:

Total RNA extracted from spawned, unfertilized eggs of *Xenopus laevis* was applied to a Sephadex G-100 column and was eluted as described in Materials and Methods. Fig. 1 shows that it is separated into three peaks. Recoveries were always near 100%. Since the purpose of this chromatography was the elimination of the possible contribution of base paired oligonucleotides from various RNA species, only high molecular weight RNA in the first peak was pooled and precipitated with 2 volumes of ethanol for further experiments.

2. *In Vitro* Labeling:

Although radioactive tracers are easily incorporated into macromolecules if precursor materials are injected into oocyte or growing embryos (Brown and Littna, 1964), very low specific activity was obtained by *in vivo* labeling. For example, injection of 1 mCi $^3$H-uridine into the dorsal lymph sac of gravid female gave only few hundred counts per O.D. Here, it was necessitated to do an *in vitro* labeling.

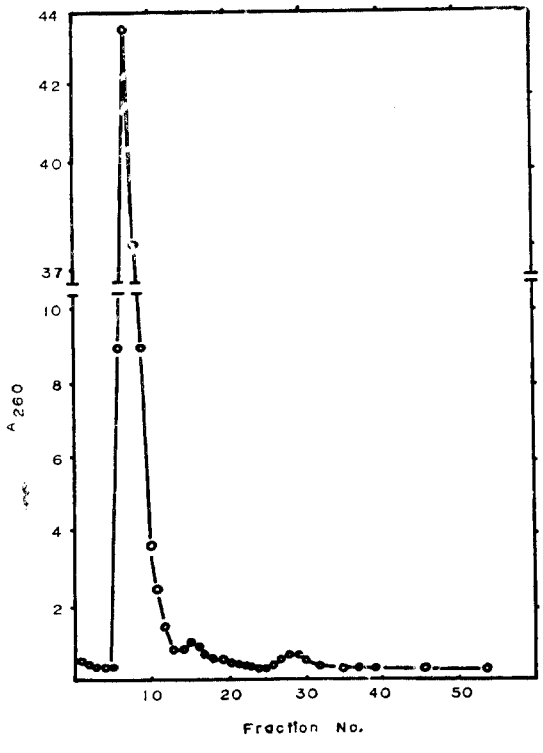


**Fig. 1.** Sephadex G-100 column chromatography of total RNA from *Xenopus* eggs.
500 O.D. units of RNA were dissolved in 3 ml of TSE buffer and placed on the column. RNA was fractionated with the same buffer and fractions of 6.5 ml were collected with a flow rate of 6 ml/hour. High molecular weight RNA (fractions 6-11) fractions were pooled and precipitated with 2 volumes of ethanol.
Column size: Height, 70 cm
Diameter, 1.5 cm

Total RNA from *Xenopus* eggs or high molecular weight RNA samples (sometimes after RNase treatment) were labeled with $^3$H-dimethyl sulfate in 1 M sodium-acetate solution (pH 5.0) for 20 hours at 4°C with agitation. The reacted solutions were repeatedly precipitated with 2 volumes of ethanol to eliminate unreacted radioactive dimethyl sulfate. Table 1 reveals that the unreacted dimethyl sulfate could be eliminated almost completely after 5 ethanol precipitations.

**Table 1.** Elimination of unreacted dimethyl sulfate by repeated ethanol precipitations.

| No. of ethanol precipitations | Radioactivity in supernatant* (cpm/0.1 m*l*) | |
|---|---|---|
| | Expt. I | Expt. II |
| 1st EtOH | 683,900 | # |
| 2nd EtOH | 21,650 | — |
| 3rd EtOH | 5,250 | — |
| 4th EtOH | 565 | 225 |
| 5th EtOH | 99 | 106 |
| 6th EtOH | —# | 72 |
| 7th EtOH | — | 50 |

Expt. I. Labeled 1,575 O.D. in 10.25 m*l* of 1 M Na-acetate with 5 m*Ci* of tritiated dimethyl sulfate.

Expt. II. Labeled 450 O.D. in 2 m*l* of Na-acetate with 5 m*Ci* of tritiated dimethyl sulfate.

* After centrifugation at 15,000 G for 10 minutes, 0.1 m*l* of supernatant at each step was put on Millipore filters and dried. Radioactivity was counted in a toluene cocktail. Back ground (30 cpm in both experiments) was subtracted.

\# Not measured.

**Table 2.** Efficacy of *in vitro* labeling of RNA with $^3$H-dimethyl sulfate.*

| RNA | Enzyme treatment | Total O.D. | Volume (m*l*) | O.D./m*l* | $^3$H-dimethyl sulfate (m*Ci*) | m*Ci*/O.D. | Specific# activity (cpm/O.D.) |
|---|---|---|---|---|---|---|---|
| Xenopus | no RNase | 536.5 | 2.87 | 1.87 | 1.8 | 1/298 | 23,300 |
| Xenopus | no RNase | 23.5 | 0.25 | 94 | 0.6 | 1/39.2 | 169,000 |
| Xenopus | RNase | 23.5 | 0.25 | 94 | 0.6 | 1/39.2 | 72,000 |
| Mycophage ds-RNA | | 23.5 | 0.25 | 94 | 0.6 | 1/39.2 | 53,700 |
| Xenopus | no RNase | 200.0 | 1.0 | 200 | 5.0 | 1/40 | 200,000 |
| Xenopus | RNase | 35.0 | 0.25 | 140 | 0.46 | 1/76 | 25,300 |
| Xenopus | no RNase | 1575.0 | 10.25 | 154 | 5.0 | 1/300 | 15,400 |
| Xenopus | no RNase | 450 | 2.0 | 225 | 5.0 | 1/90 | 55,505 |
| Sea urchin | no RNase+ | | | | | 1/3-1/5 | 1.5-2millions |

* Labelings were carried out as described in Materials and Methods.

\# Specific activities were counted after complete elimination of unreacted $^3$H-dimethyl sulfate.

\+ Recalculated from the data by Hynes and Gross (1972).

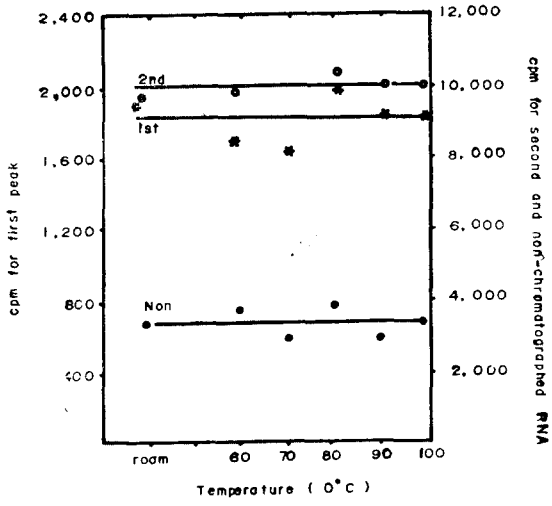


**Fig. 2.** Stability of $^{3}$H-methyl group on RNA molecule against heat treatment. All samples were dissolved in 1 m*l* 1 x SSC and then TCA precipitable radioactivities were measured after 30 minutes incubation at each temperature.
•-•-•-•-•- RNase treated RNA which has not been applied to cellulose column.
*-*-*-*-*- RNase treated RNA eluted in the first peak on cellulose column.
o-o-o-o-o- RNase treated RNA eluted in the second peak on celluose column.

Table 2 shows that specific activities of non-RNase treated RNA depend largely on the ratio of radioactive dimethyl sulfate and RNA content in the reactive mixture. The specific activities were in the range of 45,000~70,000 cpm/O.D. when 1 m*Ci* of dimethyl sulfate was reacted with 100 O.D. of RNA. Fig. 2 indicates that the attached methyl groups are quite stable since there is no decrease in specific activity at each step of the experiment even after 5 times boiling. To check the efficacy of the methylation of ds-RNA by *in vitro* labeling, Mycophage ds-RNA was treated under the same condition. Mycophage is known to form a genuine ds-RNA naturally in its replication cycle. They were labeled as effectively as RNase treated RNA of *Xenopus* egg (Table 2).

## DISCUSSION

The wide spread occurence of ds-RNA is now an established fact. The presence of ds-RNA is extended to the region in Hn-RNA and some mRNA molecules of uninfected eukaryotic cells. The discovery of poly A segments in Hn-RNA and in cytoplasmic mRNA has stimulated further studies on the relationship between these RNAs with an apparent precursor-product relationship. The presence of ds-RNA and poly U region in Hn-RNA also triggered studies mainly on the post-transcriptional modification of Hn-RNA in which some sequences are selected to become cytoplasmic mRNA.

So far, there has been very little work reported on the state of stored mRNA. One purpose of the present studies is to determine whether ds-RNA exists in amphibian eggs, and it is the case, to examine the physical structure of stored m-RNA and the mechanism of its stabilization. *Xenopus laevis* provides an useful

experimental tool to achieve the objective of this study. The reasons are that the development is fully described from the morphological and anatomical standpoints (Nieuwkoop and Farber, 1956); large quantities of eggs are routinely obtainable (Hamburger, 1960); microsurgical manipulations are readily performed (*e.g.* micro-injection (Gordon and Malacinski, 1970), tissue grafting (Chung and Briggs, 1975), mechancial enucleation and nuclear transplantation (Gurdon, 1963); and valuable mutant genes are on hand (Brown and Gurdon, 1964).

Gel filtration on the Sephadex G-100 column was performed in order to isolate high molecular weight RNA which by itself can possibly provide base paired oligonucleotides. Results were always the same with 3 peaks. Stern and Friedman (1971) who isolated RNA from Burkitt lymphoma cells also obtained three peaks and charactrized them as high molecular weight RNA containing 28 S and 18 S rRNAs, 5 S rRNA and 4 S RNA, respectively. It is quite safe to conclude here that the first peak contains those 28 S and 18 S rRNAs as well as high molecular weight Hn-RNA.

Results in this study that the attached methyl groups are quite stable. It has been found that methylation occurs only at the nitrogen 7 position of guanine residues in RNA under this experimental condition. Since the genuine Mycophage ds-RNA was effectively labeled, all the above results indicate that RNA can be adequately labeled *in vitro*. Furthermore, the values of specific activity of RNA from *Xenopus* egg agree well with those of Hynes and Gross (1972) who did *in vitro* labeling with RNA from sea unchin embryo. The high range of specific activity is enough to carry out further experiments characterizing these RNA species such as RNase treatment, chromatographic studies on benzoylated DEAE-cellulose and cellulose column and thermal denaturation.

## SUMMARY

RNA was extracted from the eggs of *Xenopus laevis* to do preliminary experiments before testing the possibility that if RNase resistant RNA molecules exist in the amphibian egg. Chromatography on Sephadex G-100 column indicated 3 peaks consistently. Only high molecular weight RNA species eluted in the first peak were labeled *in vitro* using $^{3}$H-dimethyl sulfate to eliminate the possible contribution of base paired oligonucleotides from tRNA. By this method, high specific activity could be obtained and the attached methyl groups were quite stable.

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