Accumulation, spatial distribution and partial characterization of poly(A)$^+$RNA in the developing oocytes of *Xenopus laevis*

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**SUMMARY**

*In situ* hybridization using [$^3$H]poly(U) was applied to developing oocytes of *Xenopus laevis*, which had been fixed in Bouin's solution. Tissue sections were pretreated with DNase I, annealed with [$^3$H]poly(U) and post-treated with RNase A and TCA. After the autoradiographical processing, silver grains over the oocyte were counted. As a result of the control experiments which included RNase A, RNase T$_2$, DNase I and Pronase E hydrolysis and Cordycepin (3'-deoxyadenosine) incubation before *in situ* hybridization, it was concluded that the poly(U)-binding activity detected upon the oocytes was due to the possible presence of poly(A)$^+$RNAs. Spatial distribution of the poly(U)-binding sites changed during the development of the oocytes; in a small oocyte before the pachytene stage, silver grains developed over the nucleus, while in a larger oocyte after the diploteine the grains were concentrated over the cytoplasm. After yolk platelets were deposited in the cytoplasm, two types of poly(U)-binding activities were noted; a bound-type activity which was firmly associated with the cytoplasm, so that the positions of the silver grains were not influenced by fixation, and an unbound type which did not bind so firmly to the cytoplasm and was therefore easily influenced by inflow of fixative. The bound-type activity persisted in the cytoplasm throughout the oogenesis, but the unbound type appeared only after the vitellogenesis, especially in the yolky cytoplasm. The total poly(U)-binding activity per oocyte increased continuously with the growth of the oocyte.

**INTRODUCTION**

Since Spirin (1966, 1969) proposed that embryonic development depends on the utilization of masked maternal information stored in 'informosomes', a body of experiments has supported the existence of masked maternal messenger in the oocytes and eggs of many species of animals (reviewed in Davidson, 1976). Extensive analysis using biochemical techniques demonstrates that the maternal messenger stored in oocytes includes messenger RNA (mRNA) molecules (Slater, Gillespie & Slater, 1973; Lovett & Goldstein, 1977; Ruderman & Pardue, 1977; Jäckle, 1979). Although a molecular basis for the utilization of the masked mRNA is now becoming known in some species (Kawmeyer,

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Jenkins & Raff, 1978; Jenkins, Kawmeyer, Young & Raff, 1978), there is little information concerning the synthesis, storage and especially the masking mechanism of maternal information.

Many eukaryotic mRNA molecules contain a polyadenylic acid [poly(A)] sequence at their 3'-terminus (Brawerman, 1974). The kinetics of the poly(A)-containing RNA [poly(A)+RNA] have been intensively investigated in *Xenopus* oocytes and eggs (Rosbash & Ford, 1974; Darnbrough & Ford, 1976; Levenson & Marcu, 1976; Cabada, Darnbrough, Ford & Turner, 1977; Ford, Mathieson & Rosbash, 1977; Ruderman & Pardue, 1977; Miller, 1978; Dolecki & Smith, 1979; Sagata, Shiokawa & Yamana, 1980). Biochemical analysis indicates that even the previtellogenic (stage 1, after Dumont, 1972) oocytes contain almost the same amount of poly(A)+RNA as is stored in the full-grown (stage 6) oocytes (Rosbash & Ford, 1974). Moreover, a substantial number of poly(A)+RNA molecules which have been synthesized in the previtellogenic oocyte survive for lengths of time commensurate with the length of oogenesis (1–2 years) (Ford et al., 1977). Since the poly(A)+RNA stored in oocytes is able to direct protein synthesis in the cell free system (Darnbrough & Ford, 1976; Levenson & Marcu, 1976; Cabada et al., 1977; Ruderman & Pardue, 1977), these must be the presumptive mRNA molecules which are utilized during the early development after fertilization. On the other hand, the lampbrush-chromosome-stage oocyte, which has meiotic diplotene chromosomes, is believed to show a high transcriptional activity (Gall & Callan, 1962), but no substantial increase of the poly(A)+RNA is observed during this stage of oogenesis (Rosbash & Ford, 1974; Dolecki & Smith, 1979). In this respect, it is important to study the synthesis, accumulation and possible degradation of the poly(A)+RNA molecules during the oogenesis.

Recently, a technique has been developed to demonstrate the cytological localization of poly(A)+RNAs by means of *in situ* hybridization using a [3H]-poly(U) probe (Lamb & Laird, 1976). Capco & Jeffery (1978) have succeeded in detecting a spatial distribution of the poly(A)+RNA in insect oocytes on paraffin-embedded histological sections. *In situ* hybridization with [3H]poly(U) has the merit of demonstrating accurate cytological localization of poly(A)+RNAs which are synthesized and in part degraded during oogenetic processes. In the present investigation, *in situ* hybridization using [3H]poly(U) was applied to *Xenopus* developing oocytes; the consequent accumulation and cytological localization of poly(A)+RNA is here described.

**MATERIALS AND METHODS**

**Animals**

African clawed toads, *Xenopus laevis*, were used: they were reared routinely and reproduced in our laboratory. Ovarian pieces were obtained from immature toads 1–6 months after metamorphosis.
In situ hybridization

In situ hybridization with [\(^3\)H]poly(U) was carried out according to the method developed by Capco & Jeffery (1978) with some modifications.

Ovarian pieces were fixed in Bouin's solution, dehydrated, embedded in paraffin and sectioned serially at a thickness of 6 \(\mu\)m. Hydrated slides were rinsed twice in DNase buffer (100 mM Tris-HCl, 3 mM MgCl\(_2\), pH 7.6) and then treated with 50 \(\mu\)g/ml of DNase (DNase I, grade III, Sigma) dissolved in DNase buffer for 1 h at 37 °C. After rinsing with DNase buffer, the slides were immersed in a hybridization buffer (10 mM Tris-HCl, 200 mM NaCl, 5 mM MgCl\(_2\), pH 7.6). Annealing was performed by applying 60 \(\mu\)l aliquots of 2.5 \(\mu\)Ci/ml of [\(^3\)H]poly(U) (17-61 Ci/m mole; 41–147 nucleotides in length, The Radiochemical Centre, Amersham) dissolved in hybridization buffer to each slide, sealing with a clean cover slip (24 x 50 mm), and incubating the slide for 3-5 h at 55 °C in a moist chamber. Following the incubation, the cover slip was carefully removed and the slide was rinsed twice with the hybridization buffer at 20 °C.

In order to remove the unhybridized [\(^3\)H]poly(U) from the tissue, the slide was treated with pancreatic RNase. All slides were rinsed with RNase buffer (50 mM Tris-HCl, 100 mM KCl, 1 mM MgCl\(_2\), pH 7.6) and then treated with 50 \(\mu\)g/ml of RNase A dissolved in the RNase buffer for 30 min at 37 °C. Finally, the slides were rinsed once in the RNase buffer and twice in distilled water; they were then immersed in ice-chilled 5 % trichloroacetic acid (TCA) for 15 min.

Autoradiography and grain count

Autoradiography was performed by coating the slides with Sakura NR II liquid emulsion (Konishiroku, Tokyo). After exposure in the dark at 4 °C (14 days), the slides were developed with D-19 developer for 2.5 min at 20 °C. The sections were stained through the emulsion with Delafield's haematoxylin and eosin after development.

The grain density was determined by counting the number of grains over a unit cytoplasmic square (100 \(\mu\)m\(^2\)). Silver grains over five given unit squares from a previtellogenic oocyte or ten given unit squares from a postvitellogenic oocyte were counted for one determination of the grain density. An average grain density was calculated from the grain densities of 20 similar oocytes.

Since Xenopus oocytes increase in volume extensively during oogenesis, the grain density per unit cytoplasmic area does not represent the actual [\(^3\)H]-poly(U)-binding activity per oocyte. The total labelling per oocyte was therefore calculated from the volume of the oocytes at different diameters by treating the oocyte as a series of 6 \(\mu\)m-thick discs. The volume of the germinal vesicle was deducted from the volume of oocyte because the silver grains upon it were practically zero. The total grains per oocyte was estimated from the following
formula, in which $r_1$ is the radius of the oocyte and $r_2$ that of the germinal vesicle:

$$\frac{4/3\pi(r_1^3 - r_2^3)}{6 \mu\text{m (thickness)} \times 100 \mu\text{m}^2 \text{ (unit square)}} \times \text{grain density}$$

**Control experiments**

In order to check the specificity of $[^3\text{H}]\text{poly(U)}$ binding to tissue sections, several slides were pretreated with RNase and/or proteinase before *in situ* hybridization. The enzymes used were 50 $\mu$g/ml of RNase A, 2 units/ml of RNase T2 (Sigma) and 50 or 1 $\mu$g/ml of Pronase E (Tokyokasei, Tokyo).

Ovarian pieces, which contained about 100 oocytes, were cultured *in vitro*. The culture medium was diluted Leibovitz L-15 (which was a mixture of L-15, distilled water and foetal calf serum, in the ratio of 5:4:1, respectively) containing Cordycepin (3'-deoxyadenosine). After a short period of cultivation, the ovarian pieces were processed for *in situ* hybridization as described above.

**RESULTS**

**In situ hybridization with a $[^3\text{H}]\text{poly(U)}$ probe**

The first experimental step was to examine the effect of increasing concentrations of the $[^3\text{H}]\text{poly(U)}$ on the grain density over sections of the developing oocytes. Sixty $\mu$l of $[^3\text{H}]\text{poly(U)}$ at various concentrations were applied to similar sections. Figure 1 shows the poly(U)-binding activities on the cytoplasm of stage-1 oocytes (200 $\mu$m in diameter) at different concentrations of $[^3\text{H}]\text{poly(U)}$. After applying higher concentrations (above 0.8 $\mu$Ci/ml) a significant increase of the grain density was observed, but no substantial increase was observed above 2.5 $\mu$Ci/ml. Therefore, the saturation of the poly(U)-binding sites on histological sections seemed to be attained at a concentration between 1.0-2.5 $\mu$Ci/ml of $[^3\text{H}]\text{poly(U)}$. Subsequent experiments were carried out using $[^3\text{H}]\text{poly(U)}$ at the concentration of 2.5 $\mu$Ci/ml in order to assure an excess poly(U) hybridization.

The next experimental step was to establish the specificity of $[^3\text{H}]\text{poly(U)}$ binding to tissue sections. The grain density over the tissues which had been exposed to specific enzymes prior to annealing was examined. Table 1 is a summary of the effects of enzymatic digestions on the grain density upon the cytoplasm of stage-1 oocytes. RNase A was dissolved in two different buffer solutions according to Capco & Jeffery (1978), who stated that the poly(A) was protected from RNase hydrolysis in the presence of 0.1 M KCl, but not in the presence of 0.01 M KCl. However, both sections which had been pretreated with RNase dissolved in either low or high K$^+$ concentration buffers were labelled equally, and no substantial reduction of grains was detected even after a 24 h digestion period (Fig. 2b). On the other hand, RNase T$_2$ had a strong effect on the poly(U)-binding activity. No grains developed on the sections after the RNase T$_2$ digestion (Fig. 2c).
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Fig. 1. The grain density on the cytoplasm of *Xenopus laevis* oocytes at 200 μm in diameter after *in situ* hybridization with increasing concentrations of $[^3]$H]poly(U). Grain density ± SD.

Table 1. Effects of specific enzyme treatments on $[^3]$H]poly(U)-binding activity on Xenopus oocyte cytoplasm

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Condition</th>
<th>Grain density ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase I (50 μg/ml)</td>
<td></td>
<td>37.3 ± 4.1</td>
</tr>
<tr>
<td>DNase-RNase A (50 μg/ml)</td>
<td>100 mM KCl, 24 h</td>
<td>28.2 ± 3.8</td>
</tr>
<tr>
<td>DNase-RNase A (50 μg/ml)</td>
<td>10 mM KCl, 24 h</td>
<td>21.6 ± 3.1</td>
</tr>
<tr>
<td>DNase-RNase T2 (2 μg/ml)</td>
<td>50 mM KCl, 24 h</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>DNase-Pronase E (50 μg/ml)</td>
<td>50 mM KCl, 10 min</td>
<td>6.2 ± 2.2*</td>
</tr>
<tr>
<td>DNase-Pronase E (1 μg/ml)</td>
<td>50 mM KCl, 10 min</td>
<td>33.2 ± 4.0</td>
</tr>
<tr>
<td>DNase-Buffer</td>
<td>100 mM KCl, 24 h</td>
<td>29.5 ± 4.3</td>
</tr>
</tbody>
</table>

* The stainability to haematoxylin and eosin of tissues was greatly reduced.

Mild Pronase E digestion (1 μg/ml for 10 min at 37 °C) did not alter the labelling patterns. Even after very strong Pronase E digestion (50 μg/ml) which removed a substantial amount of material from the sections, so that the stainability of sections to haematoxylin and eosin was significantly reduced, a few grains were still detected over the tissues.
Post-transcriptional polyadenylation in the oocyte

In order to examine the effects of Cordycepin on the poly(U)-binding activity and on the uptake of [3H]uridine into acid-insoluble fractions, ovarian pieces were cultured in vitro with or without Cordycepin. Cordycepin is reported to be a specific inhibitor of the post-transcriptional adenylation of nuclear RNAs.
Table 2. Effect of Cordycepin on $[^3H]$poly(U)-binding activity and $[^3H]$uridine uptake

<table>
<thead>
<tr>
<th>Dose of Cordycepin (µg/ml)</th>
<th>Duration in vitro (h)</th>
<th>Grain density ± sd</th>
<th>$[^3H]$poly(U) binding*</th>
<th>$[^3H]$uridine uptake†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>33.1 ± 4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>34.6 ± 5.0</td>
<td></td>
<td>258 ± 32</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>36.3 ± 4.5</td>
<td>35.0 ± 4.1</td>
<td>241 ± 40</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>24.3 ± 5.6</td>
<td>30.3 ± 5.8</td>
<td>226 ± 35</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>24.6 ± 4.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Grain density on 100 µm² cytoplasmic area of 200 µm oocyte.
† Grain density on the germinal vesicle.

(Darnell, Philipson, Wall & Adesnik, 1971; Jelinek et al. 1973). Table 2 shows the results of the poly(U)-binding activity of the sections and the uptake of $[^3H]$uridine after the Cordycepin incubation. Without Cordycepin, grain density resulting from the in situ hybridization was not significantly reduced even after 48 h in vitro. In contrast to this, the poly(U)-binding activities decreased in accordance with the duration of incubation and with the concentrations of Cordycepin used. Since the $[^3H]$uridine uptake into acid-insoluble fractions in the germinal vesicle did not alter following the Cordycepin incubation, the reduction of the poly(U)-binding activity cannot be due to the reduced rate of the nuclear RNA synthesis.

Spatial distribution of the $[^3H]$poly(U)-binding sites

Cytological localization of the $[^3H]$poly(U)-binding sites seemed to change according to the developmental stages of oocytes. Even in very small oocytes during the earlier stages of the meiotic prophase (leptotene and zygotene), silver grains were already recognized over the nucleus, but not the cytoplasm (Fig. 3a, b). In the pachytene oocyte, silver grains were more concentrated over the nucleus (Fig. 3c) than during the former stages, but somewhat fewer over the extrachromosomal ‘cap’, which corresponded to an accumulation of the ribosomal DNA molecules (Ficq, 1970; Van Gansen & Schram, 1974). When the oocytes grew to the diplotene stage, the labelling pattern changed drastically; the silver grains over the nucleus diminished and appeared over the cytoplasm (Fig. 3d). This pattern of labelling – heavy silver grains over the cytoplasm and no or few grains over the germinal vesicle – was not altered thereafter. The cytoplasm was evenly labelled except for the mitochondrial mass or Balbiani body, which was a peculiar cytoplasm specific to the previtellogenic oocyte and composed of mitochondria (Dumont, 1972). The silver grains over the mitochondrial mass were somewhat fewer than over the other cytoplasmic region (Fig. 3e, f).
After vitellogenesis began in the oocyte cytoplasm, the labelling pattern was modified: there were relatively dense silver grains over the yolk-free cytoplasm and more scattered grains over the yolky peripheral cytoplasm (Fig. 3g, h). It is worth noting that the poly(U)-binding sites on the yolky cytoplasm seemed influenced by the inflow of the fixative. On the other hand, the silver grains over the yolk-free cytoplasm were firmly fixed regardless of the direction of fixative inflow. The direction of the fixative invasion into oocytes was assumed from the location of the detached site of the germinal vesicle from the cytoplasm (Fig. 4a), resulting from the different rate of shrinkage of the cytoplasm and the germinal vesicle caused by the fixative. The yolky cytoplasm at the 'distal' end (Fig. 4b) represented a much higher silver-grain density than that at the 'proximal' end (Fig. 4e) in terms of the direction of the fixative inflow.

After vitellogenesis, the presumptive animal–vegetal axis of oocyte was assumed on the histological sections according to the distribution of melanin granules and the size of yolk platelets; animal pole cytoplasm contained dense melanin granules and small yolk platelets while vegetal pole cytoplasm had few melanin and large yolk platelets. However, accurate spatial distribution of the poly(U)-binding sites along the animal-vegetal axis could not be determined, since the grain density over the yolky cytoplasm was influenced by the fixative invasion regardless of the animal–vegetal axis of the oocyte.

**Changes of poly(U)-binding activities during oogenesis**

Figure 5 shows changes of the grain density upon the cytoplasm of oocytes after *in situ* hybridization during oogenesis. The data from three different experiments using different ovaries were combined. During the early phase of oocyte growth (up to 200 μm in diameter) the average grain density over the cytoplasm rapidly increased in accordance with the growing diameter of the oocyte. The highest grain density was attained over the cytoplasm of oocytes of 200 μm diameter. Once the oocyte grew larger than 200 μm, the average grain density dropped and thereafter gradually decreased with increasing size of oocyte. Although the silver grains were uniformly distributed over the cytoplasm during the previtellogenic stage (35–300 μm in diameter), the distribution of grains showed a bimodal pattern after the vitellogenesis: relatively higher.
Fig. 4. Autoradiographs of developing oocytes of *Xenopus* after *in situ* hybridization with [3H]poly(U). (a) Low magnification photograph showing a vitellogenic oocyte. Each cytoplasmic region enclosed by squares is enlarged in Figs. 4b–e. Large arrow indicates a presumed direction of fixative inflow, judged from the location of detached site (*) of the germinal vesicle from the cytoplasm. The silver grains are much more concentrated over the yolked cytoplasm at the ‘distal’ end (b) than at the ‘proximal’ end (e), in terms of the direction of fixative inflow. The silver grain density is the same upon the yolk-free cytoplasm of ‘distal’ (c) and ‘proximal’ (d) regions. Haematoxylin and eosin stained. Scale bars: 100 μm (a), 20 μm (b–e).
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Fig. 5. The grain density on the cytoplasm (solid line) and the total silver grains per oocyte (broken line), determined in Xenopus developing oocytes after in situ hybridization with $[^3H]$poly(U). Closed circles, $\sigma$ represent grain density upon the yolk-free cytoplasm and open circles represent grain density upon the yolky cytoplasm after the vitellogenesis.

density over the yolk-free cytoplasm and lower density over the yolky peripheral cytoplasm. The grain densities over both yolk-free and yolky cytoplasms decreased gradually with the growth of the oocyte.

The broken line shown in Fig. 5 represents changes of total silver grains per oocyte during the oogenesis. The silver grains per oocyte increased almost linearly in accordance with growth of the oocytes, so far as these were examined.

DISCUSSION

The $[^3H]$poly(U)-binding activities on the developing oocytes of Xenopus laevis after in situ hybridization with DNase pretreatment and RNase-TCA post-treatment are equivalent to the activity which has been seen in insect oocytes (Capco & Jeffery, 1978, 1979). We conclude that the poly(U)-binding activity described in this paper is a result of the presence of poly(A)$^+$ RNAs in the oocyte. We offer the following reasons.

First, the poly(U)-binding activity is sensitive to RNase T$_2$ hydrolysis (which preferentially attacks the adenylic acid bonds), but it is insensitive to DNase I,
RNase A and Pronase E treatments (Table 1). Second, after Cordycepin incubation labelling after in situ hybridization with [³H]poly(U) decreases considerably (Table 2), but the uptake of [³H]uridine into acid-insoluble fractions is not altered. This agent acts at the level of post-transcriptional addition of poly(A) to mRNA (Darnell et al. 1971; Mendecki, Lee & Brawerman, 1972; Jelinik et al. 1973). Cytological localization of the poly(U)-binding activities changes significantly during development of the oocyte. Before entering the diplotene stage, the poly(U)-binding sites were concentrated in the nucleus, but after the diplotene were concentrated in the cytoplasm. The polyadenylation of presumptive mRNAs in the nucleus has been demonstrated in mammalian cells (Mendecki et al. 1972; Jelinik et al. 1973). In Xenopus, also, it has been reported that presumptive vitellogenin mRNA is polyadenylated in the nucleus just after transcription (Ryffel et al. 1980). When applying in situ hybridization using [³H]poly(U) onto Xenopus liver and kidney, almost all silver grains are concentrated over the nucleus (M. Wakahara, unpublished). Therefore, it seems that nuclear RNAs are polyadenylated in the nucleus of differentiating or differentiating cells. In Xenopus oocytes after diplotene, however, the silver grains become concentrated over the cytoplasm. This implies two possibilities. The first is that nuclear RNAs are polyadenylated in the germinal vesicle and released into the cytoplasm. However, the rapid release of poly(A)⁺RNA into the cytoplasm and rapid increase in the volume of the germinal vesicle, causes the poly(U)-binding activity upon the germinal vesicle to be hardly detectable. The second is that nuclear RNAs are instantly released into the cytoplasm without polyadenylation and then polyadenylated in the cytoplasm. Although the results of Cordycepin incubation (Table 2) demonstrate conclusively that the post-transcriptional adenylation of RNAs occurs in the oocyte, we could not determine the cytological location of the polyadenylation, in either nucleus or cytoplasm, after the diplotene stage.

Since the in situ hybridization procedure employed in this study did not allow us to assess the length of the poly(A) sequence, the presence of two size classes of poly(A) with different lengths, short and long poly(A), designated as poly(A)ₜ and poly(A)ₗ (Cabada et al. 1977), could not be confirmed. But, as a result of behaviour during fixation, two types of poly(U)-binding activities were demonstrated; one type is firmly bound to the cytoplasm, so that the silver grains are not influenced by fixative inflow, while the other may not be so firmly bound to the cytoplasm, and so is easily influenced by the fixative invasion. The former, bound-type poly(U)-binding activity, persists throughout oogenesis, and the latter, unbound type, appears after vitellogenesis. Since the appearance, accumulation and persistence of the bound and unbound types correspond to those of the poly(A)ₗ and poly(A)ₜ respectively, the bound-type activity may correspond to the poly(A)ₗ and unbound type to the poly(A)ₜ. Furthermore, it is possible to assume that the bound-type activity reflects the poly(A)⁺RNA associated with the polysomes and the unbound type represents
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A poly(A)+RNA in the form of mRNP particles which are presumed to represent a store of mRNAs (Rosbash & Ford, 1974). At present, however, no evidence is available to allow further characterization of these two types of poly(U)-binding activities.

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