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In Vivo Studies on the Incorporation of Microinjected Acidic Proteins of the Large Ribosomal Subunit from *Escherichia coli* and *Artemia salina* into Oocyte Ribosomes from *Xenopus laevis*†

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ABSTRACT: Tritium-labeled acidic proteins from the large ribosomal subunit of *Artemia salina* or *Escherichia coli* were microinjected into the cytoplasm of stage IV/V oocytes from *Xenopus laevis*. eL12 from the large ribosomal subunit of *A. salina* but not L7/L12 or L7/L12–L10 from *E. coli* is specifically incorporated into 60S ribosomal subunits of oocytes. This incorporation is not significantly inhibited by actinomycin D. Incorporation of eL12 into the 60S subunits occurs in enucleated oocytes, suggesting that active ribosomal ribonucleic acid synthesis and ribosome assembly as well are not prerequired for this reaction. Apparently the incorporation proceeds via an exchange reaction between a free cytoplasmic pool of eL12 and ribosomal eL12.

It is well established that procaryotic and eucaryotic ribosomes and their associated factors differ in their biochemical and physical properties, while the mechanism of protein synthesis is essentially similar in both classes [for a review, see Brimacombe et al. (1978)]. Structural differences may explain the observation that, in general, factors and ribosomes from the two classes are not freely interchangeable. There are only a few exceptions to the class specificity: bacterial type of elongation factor Tu can interact with 80S eucaryotic ribosomes (Krisko et al., 1969; Richter & Lipmann, 1970). Hybrid ribosomes consisting of *Escherichia coli* 50S and eucaryotic 40S subunits but not of 30S and 60S subunits show limited function in protein synthesis (Klein & Ochoa, 1972; Richter, 1973; Boublik et al., 1979). Functional interchangeability has also been found between the two acidic ribosomal proteins L7/L12 from *E. coli* and eL7/eL12 from yeast (Richter & Möller, 1974; Wool & Stöffler, 1974) or *Artemia salina* (Möller et al., 1975). Sequence (Amons et al., 1978) and immunological analyses (Wool & Stöffler, 1974; Stöffler et al., 1974; Howard et al., 1976; Leader & Coia, 1978) imply that eL7 and eL12 are the eucaryotic counterparts of *E. coli* proteins L7 and L12. So far little is known about formation of hybrid ribosomes under physiological conditions. Therefore, we used the oocyte system from *Xenopus laevis* as an in vivo model system for studying the incorporation of microinjected ribosomal proteins into ribosomes. As it turned out, this system may also be a promising model for investigating the ribosomal assembly process in eucaryotes. In the present communication we studied the in vivo incorporation of microinjected radioactively labeled acidic proteins L7/L12 from *E. coli* and eL12 from *A. salina* into ribosomes from oocytes of *X. laevis*.

Materials and Methods

Female frogs of *X. laevis* were obtained from South African Snake Farm (Fish Hoek, South Africa). Sodium boro-[^{3}H]hydride (sp act. 5–20 Ci/mmol) was purchased from Amersham-Buchler. Ribosomal proteins eL12 from *A. salina* (Möller et al., 1975) and L7/L12 from *E. coli* (Möller et al., 1972) were kindly provided by W. Möller, Leiden, and L7/L12–L10 from *E. coli* were provided by J. Dijk, Berlin (Dijk et al., 1977). Ribosomal proteins were labeled by the reductive methylation method using tritium-labeled sodium borohydride. Lyophilized proteins were dissolved in (0.5–1.0 mg/mL) and dialyzed against 20 mM Hepes buffer, pH 8.5, containing 10 mM $MgCl_2$, 20 mM KCl, and 6 mM 2-mercaptoethanol and methylated in the presence of 4 mM formaldehyde and 10 mCi of sodium boro[^{3}H]hydride at the time intervals indicated in published procedures (Means & Feeney, 1968; Kleinert & Richter, 1975). Aliquots were precipitated with 10% trichloroacetic acid, collected on filters (0.45-μm pore size, Sartorius), and analyzed in a Packard sample oxidizer and liquid scintillation counter to calculate the specificity of the labeled proteins. On the average 250–300 cpm/ng of protein

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was obtained. Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Sterilized buffers and solutions were utilized throughout.

Microinjection of Ribosomal Proteins into Oocytes. Oocytes from *X. laevis* were prepared as reported by Gurdon et al. (1971) modified according to D. Rungger (Geneva, personal communication). Fully grown female frogs were induced to ovulate by hormone treatment, and 2–4 weeks later a piece of ovary was excised and kept in modified Barth's medium (Gurdon, 1968). Oocytes of the late stage IV and early stage V (Dumont, 1972) were manually defolliculated with watchmaker forceps. A 40–60-nL amount of tritiated ribosomal proteins (10–40 fmol/nL) was injected into the cytoplasm of oocytes by calibrated micropipets with a tip diameter of 20–40 μm. Injected oocytes were transferred into modified Barth's medium containing 50 units/mL of both penicillin and streptomycin and incubated at room temperature as indicated in the captions to the figures and the footnotes to the tables. After incubation, oocytes were washed 3 times in modified Barth's medium and twice in cold buffer A (35 mM Tris-HCl, pH 7.5, 70 mM KCl, 9 mM $MgCl_2$, 0.1 mM NaEDTA,[1] and 2 mM dithiothreitol) containing 250 mM sucrose. When not immediately processed, microinjected oocytes were stored in liquid nitrogen.

Enucleated oocytes were prepared according to published procedures (Ford & Gurdon, 1977). Oocytes were left for 15 min in diluted (1:2) modified Barth's medium. Germinal vesicle was released by a small incision made at the animal pole of the oocyte. In order to allow healing of the incision, we incubated the enucleated oocyte at room temperature for 1–2 h in 100 mM potassium phosphate buffer, pH 7.1, with 2 mM $MgCl_2$ followed by an incubation (2 h) in modified Barth's medium.

Isolation of Ribosomes and Ribosomal Subunits. Batches of 20–40 microinjected oocytes were homogenized in a total volume of 4 mL of buffer A by using a glass douncer homogenizer. To minimize losses of ribosomes, we added 20–40 A_{260} units of carrier ribosomes prepared from *X. laevis* ovaries or *E. coli*. After centrifugation for 15 min at 15000 rpm (SM24 rotor) in a Sorvall centrifuge RC2B, both Triton X-100 and sodium deoxycholate were added to a final concentration of 1% (w/v). The supernatant fraction (4 mL) was layered on top of a 1.2-mL sucrose cushion (50% w/v sucrose in buffer A) and centrifuged at 45000 rpm for 14 h in a Beckman 50-Ti rotor. The ribosomal pellet was suspended in 0.5–1 mL of buffer B (10 mM Tris-HCl, pH 7.5, 500 mM KCl, 10 mM $MgCl_2$, and 2 mM dithiothreitol) and subjected to linear sucrose density gradient centrifugation (15–30% w/v sucrose in buffer B) at 20000 rpm for 14 h at 4 °C using a Beckman SW27 rotor. Fractions of 2 mL were collected, and 2 mL of 10% ice-cold trichloroacetic acid was added to each fraction. The precipitate was collected on Sartorius membrane filters (pore size 0.45 μm). The filters were processed in a Packard sample oxidizer; ^3H and/or ^{14}C radioactivity was counted in a liquid scintillation counter. Radioactivity was corrected for losses which occurred during ribosome preparation. The correction was calculated from the recovery of carrier ribosomes added to the batch of oocytes at the beginning of the preparation procedure (Hallberg & Smith, 1975).

Results and Discussion

Microinjection of Ribosomal Proteins L7/L12 and of the Complex L7/L12–L10 from E. coli. We have shown previously that active hybrid ribosomes can be formed in vitro with 60S core particles from yeast or *A. salina* and *E. coli* L7/L12 (Richter & Möller, 1974; Möller et al., 1975). In order to study whether similar hybrid ribosomes could be obtained in vivo, we microinjected tritiated acidic proteins from the large ribosomal subunit of *E. coli* or *A. salina* into the cytoplasm of stage IV/V oocytes of *X. laevis*, known to be actively engaged in the assembly of ribosomes (Scheer, 1973; Hallberg & Smith, 1975). For analysis ribosomal subunits were isolated by sucrose density gradient centrifugation as described under Materials and Methods. Preliminary experiments suggested that microinjected [^3H]L7/L12 from *E. coli* were not incorporated into oocyte ribosomes (D. Richter and J. Allende, unpublished experiments). Since in the in vitro ribosomal system from *E. coli* L7/L12 are incorporated into the large ribosomal subunit only in the presence of L10 (Schrier et al., 1973; Highland & Howard, 1975), our microinjection experiments were modified by using a protein complex consisting of L7/L12 and L10 (Dijk et al., 1977). In this complex the molar ratio of L7/L12 and L10 was approximately 4:1. Even when injected in the presence of L10, incorporation of L7/L12 into oocyte 60S subunits was not observed (Figure 1). As judged from the radioactivity found in the top fractions of the gradient, a small but significant amount of *E. coli* L7/L12–L10 remained attached to oocyte ribosomes although, prior to gradient centrifugation, these ribosomes had been treated with detergents and centrifuged through a 50% sucrose cushion containing 70 mM KCl. Apparently [^3H]L7/L12–L10 are loosely bound to oocyte ribosomes and were washed off only when ribosomes were subjected to high salt treatment during sucrose density gradient centrifugation. That microinjected L7/L12–L10 from *E. coli* may have a weak affinity to oocyte ribosomes is also supported by competition experiments where approximately identical concentrations of unlabeled proteins L7/L12–L10 from *E. coli*

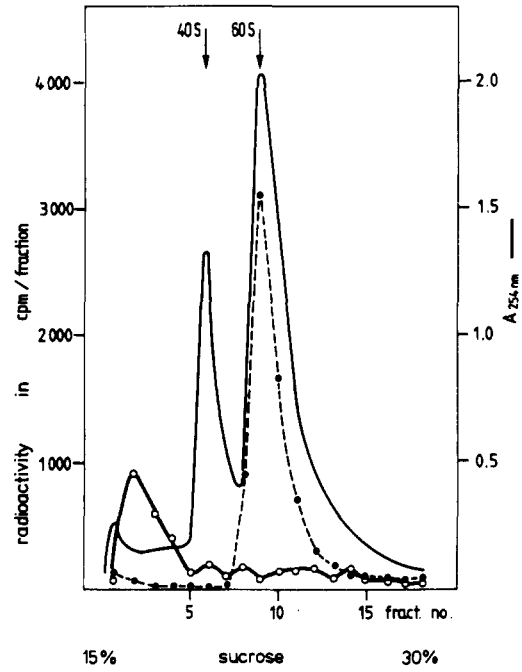

FIGURE 1: Sucrose density gradient centrifugation of ribosomes isolated from oocytes microinjected with [^3H]eL12 (*A. salina*) (●) or [^3H]L7/L12–L10 (*E. coli*) (○). Oocytes were injected with 50–60 nL of either [^3H]eL12 or [^3H]L7/L12–L10 and incubated at room temperature for 16 h in Barth's medium. 20 oocytes were homogenized and the ribosomes prepared according to Materials and Methods. 13 A_{260} units of ribosomes (26 A_{260} units/mL) were subjected to sucrose density gradient centrifugation (15–30% w/v sucrose in buffer B) using a Beckman SW27 rotor.

[1] Abbreviation used: EDTA, (ethylenedinitrilo)tetraacetic acid.

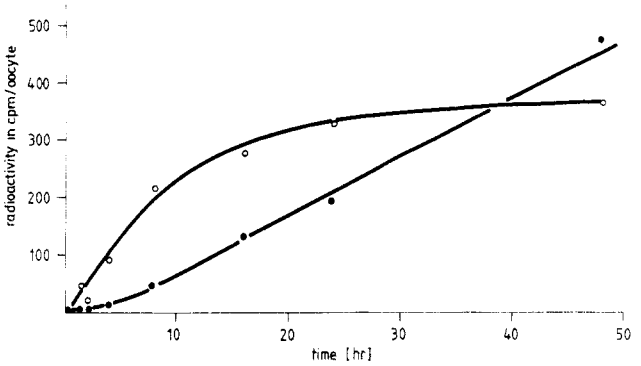

FIGURE 2: Incorporation of [^{3}H]eL12 and [^{14}C]uridine into ribosomes of oocytes. [^{3}H]eL12 (40–60 nL) and [^{14}C]uridine (100–150 nCi) were coinjected into oocytes; at the indicated times, 20 oocytes were removed and the ribosomes analyzed for radioactivity by sucrose density gradient centrifugation as outlined under Materials and Methods. A blank of 80 cpm obtained from nonincubated oocytes (microinjected as indicated above and kept for 15 min in buffer A at 4 °C) was subtracted. This blank results from in vitro binding of [^{3}H]eL12 to carrier ribosomes from *X. laevis* ovaries added at the beginning of the subunit isolation procedure (see Materials and Methods). The blank was significantly reduced when *E. coli* ribosomes were used as carrier. Incorporation of [^{14}C]uridine into rRNA (●) and of [^{3}H]eL12 (○) into 60S ribosomal subunits.

were coinjected with [^{3}H]eL12 from *A. salina*. Under these conditions incorporation of [^{3}H]eL12 was diminished to ~80% of the control value (unpublished data).

Microinjection of A. salina eL12. As shown in Figure 1, eL12 from *A. salina* is incorporated into ribosomes and, as expected, is present in 60S but not in 40S ribosomal subunits. After 20 h of incubation, 0.1–0.4 pmol of eL12 was incorporated into 60S subunits per oocyte. On the basis of earlier calculations (Scheer, 1973), 1.2 pmol of ribosomes is present in stage IV/V oocyte, suggesting that maximally every third ribosome carries an injected [^{3}H]eL12. For several reasons this number may be a minimal estimate, primarily affected by the existence of a free cytoplasmic pool of eL7/eL12 which has been found in *A. salina* yeast (van Agthoven et al., 1978; Zinker & Warner, 1976) and *E. coli* (Gupta & Singh, 1972; Subramanian, 1974; Ulbrich & Nierhaus, 1975; Ramagopal, 1976) and should exist in oocytes as well. In addition to pool effects, microinjected [^{3}H]eL12 that is not associated with ribosomes is degraded during incubation and hence will also cause a lower number for the molar ratio (data not shown). The possibility that radioactivity found in the 60S ribosomal subunit may be due to metabolic interconversion of [^{3}H]eL12 during the course of the incubation can be excluded. Ribosomal subunits of 60S isolated from oocytes incubated for 14 h with microinjected [^{3}H]eL12 were analyzed by sodium dodecyl sulfate gel electrophoresis and found to contain only one significantly radioactive component which comigrated with authentic *A. salina* eL12.

Figure 2 shows that the incorporation of [^{3}H]eL12 into ribosomes was linear for the first 6 h followed by a reduced rate of incorporation. The lower incorporation rate after prolonged incubation may be explained either by the cytoplasmic pool of eL12 and/or by degradation of microinjected [^{3}H]eL12. In contrast, incorporation of coinjected [^{14}C]uridine into rRNA had a significant lag period and then was later linear. The experiment suggests that [^{3}H]eL12 was incorporated into the ribosome via an exchange reaction since the maximal rate of incorporation of eL12 was observed prior to the onset of rRNA synthesis. This assumption was supported by a number of different experiments. Table I shows that actinomycin D had little effect upon the incorporation of

Table I: Incorporation of [^{3}H]eL12 and [^{14}C]Uridine into Ribosomes in the Presence or Absence of Actinomycin D[a]

conditions	% [^{3}H]eL12	% [^{14}C]uridine
without actinomycin D	100	100
5 μg/mL actinomycin D	80	28.5
20 μg/mL actinomycin D	82	15.7

[a] Oocytes were preincubated for 5 h at room temperature in Barth's medium containing, where indicated, 5 or 20 μg/mL actinomycin D. The preincubated oocytes were microinjected with 40–60 nL of [^{3}H]eL12 and 100–150 nCi of [^{14}C]uridine, and incubation with or without actinomycin D was continued for an additional 16 h. Analysis of ^{3}H and ^{14}C activity was according to Figure 2. 100% of ^{3}H or ^{14}C radioactivity corresponds to 190 and 120 cpm/oocyte, respectively.

Table II: Subcellular Distribution of Microinjected [^{3}H]eL12 in Oocytes from *X. laevis*[a]

subcellular fractions assayed	^{3}H radioact (%)
expt 1	
total oocyte	100
cytoplasm	95.2
nuclei	4.8
washed nuclei	0.7
expt 2	
oocyte	100
enucleated oocyte	120

[a] Oocytes were microinjected and incubated for 21 h in experiment 1 and for 12 h in experiment 2 at room temperature. The values for total oocyte, cytoplasm, and nuclei were based on trichloroacetic acid precipitated material which was processed as described under Materials and Methods. Nuclei were manually released by transferring oocytes into 10% trichloroacetic acid (R. Bravo, personal communication). Washed nuclei or enucleated oocytes were prepared by the enucleation procedure described under Materials and Methods. The released nuclei were washed in 10 mM Tris-HCl buffer, pH 7.1, 1 mM $MgCl_2$, 250 mM sucrose, and 0.1% Triton X-100 and analyzed for ^{3}H radioactivity; in experiment 1 100% corresponds to 305 cpm/oocyte and in experiment 2 to 260 cpm/oocyte. A blank of 80 cpm resulting from in vitro binding of [^{3}H]eL12 to added carrier ribosomes was subtracted.

[^{3}H]eL12, yet inhibited rRNA synthesis significantly. Table II (expt 1) shows that when labeled protein was microinjected into the cytoplasm of the oocyte, most of the label was found in the cytosol and little was found in the nucleus. The residual ^{3}H radioactivity found in nuclei most likely was due to unspecific contamination; less than 1% of the total radioactivity was present when adherent material of the nuclei was washed off. The contention that incorporation of [^{3}H]eL12 into 60S ribosomal subunits is not part of the assembly process in nucleoli is clearly strengthened by experiments with enucleated oocytes. Here the injected protein was incorporated to the same extent as in intact oocytes (Table II, expt 2).

In conclusion, we have shown that in vivo [^{3}H]eL12 from *A. salina* is incorporated into 60S ribosomal subunits from oocytes of *X. laevis*; the incorporation proceeds via an exchange reaction which is not dependent on the ribosome assembly process. This is also supported by experiments where microinjected eL12 was incorporated into 60S ribosomes of the stage VI oocytes known to have greatly reduced rRNA synthesis. Apparently, a pool of eL12 protein exists in the cytoplasm which can be exchanged with ribosomal-bound eL12. This finding coincides with recent experiments (van Agthoven et al., 1978) in *A. salina* which indicate that about 30% of eL12 is present in the ribosomal fraction and 70% in the supernatant fraction. The physiological basis of the exchange of certain ribosomal proteins such as eL12 in the

cytoplasm is presently under study. It would be of particular interest to determine whether 80S ribosomes and polysomes differ in their capability to exchange this protein.

The data in Table II (expt 1) suggest that eL12 cannot cross the nuclear membrane, which is considered as a barrier not easily penetrated by microinjected proteins (Gurdon, 1974). Proteins with a molecular weight of lower than 60000 should cross this membrane more quickly than larger ones. Lack of microinjected eL12 in the nucleus may imply that eL12 is one of the "late" ribosomal proteins that is not actively engaged in the ribosomal assembly process and therefore directly attached to the ribosome in the cytosol (Zinker & Warner, 1976). Alternatively, eL12 takes part in the assembly reaction in the nucleoli; however, entry of the protein into the nucleoli is facilitated only when it is synthesized on polysomes of the oocyte.

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