# Nucleus-associated polyribosomes are engaged in the replication- and transcription-dependent histone synthesis in early fish embryos

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## 1. INTRODUCTION

In most cases, histone synthesis is coupled to nuclear DNA replication [1–6]. The mechanism of this coupling, although repeatedly discussed [5,6,12], remains elusive. On the one hand, the mechanism seemed to involve a remote control since histones are believed to be synthesized in free polyribosomes [6–8]. On the other hand, the search for diffusible factors to mediate this control (i.e., to govern translation or survival of histone mRNAs depending on the state of the replication system) has been unsuccessful [5,12].

We have reported [13,14] that in early embryos of a teleostean, loach (*Misgurnus fossilis*), a large fraction of the total of cell polyribosomes, accounting for the major part of early protein synthesis, is associated with the cell nuclei.

This paper describes some experiments which show that the nucleus-associated polyribosomes ('n-polysomes') are the site of histone synthesis in blastula- and gastrula-stage loach embryos, and that the integrity of these polysomes is dependent on DNA and RNA synthesis.

On the basis of these data and [13,14], a model for the nuclear envelope-associated system of the chromatin biogenesis is proposed, where DNA replication and histone mRNA formation and translation are spacially coupled.

## 2. MATERIALS AND METHODS

The blastula or early gastrula developing eggs were used and the embryos per se (i.e., the blastoderms) were separated from the yolk by trypsin treatment [15].

Blastoderms were incubated in a double-strength Holtfreter medium in the presence of labeled precursors, as defined in the figure legends. To enhance incorporation of [$^3$H]uridine into RNA, the blastoderms were pre-incubated for 15 min in the presence of D-glucosamine (5 mM) which causes a decrease in the size of the UTP pool [14]. Higher concentrations of glucosamine (0.2 M) were used to inhibit RNA synthesis.

After washing away the excess labeled medium, the blastoderms were homogenized in the 'homogenizing medium' (KCl 0.3 M, $MgCl_2$ 5 mM, heparin 100 $\mu$g/ml, sucrose 0.32 M, Tris–HCl 50 mM, pH 7.4) to which diethylpyrocarbonate (DEPC, Sigma) was added up to 0.1% immediately prior to homogenization.

The homogenate was applied to 1.8 M sucrose underlayered with a cushion which contained 50% Ficoll 400 (Pharmacia) and 2.4 M sucrose, the two dense media being prepared on a high salt mixture (KCl 0.3 M, $MgCl_2$ 5 mM, Tris–HCl 50 mM, pH 7.4).

After 90 min spinning at 70000 × $g$, the nuclear

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pellet obtained was resuspended and stirred for 10 min in the medium containing: NaCl 100 mM, KCl 25 mM, $MgCl_2$ 1.5 mM, $CaCl_2$ 1.0 mM, Tris–HCl 10 mM (pH 7.4). After spinning down the nuclei (800 × *g*, 10 min), the supernatant contained n-polysomes.

Free and membrane-bound polyribosomes were separated as in [16] from the post-mitochondrial supernatant obtained by spinning the homogenate for 20 min at 16000 × *g*.

The nucleus-associated, free and membrane-bound polyribosomes were purified by pelleting through 1.5 M sucrose after resuspension in the homogenizing medium to which Tween-40 and sodium deoxycholate were added up to 0.5% each.

DNA, RNA and protein synthesis in vivo were measured as in [13,14]. For the in vitro translation, n-polysomes were obtained as described except that DEPC was pre-decomposed by heating the homogenizing medium, and dextran sulfate was added (50 μg/ml).

The 250 μl reaction medium contained: KCl 75 mM, $MgCl_2$ 5 mM, ATP 2 mM, GTP 1 mM, dithiothreitol 4 mM, creatine phosphate 10 mM, Tris–HCl 25 mM (pH 7.6), creatine phosphokinase 40 μg/ml, polyvinyl sulfate 30 μg/ml, polyribosomes 5 mg RNA/ml, cell sap from the same embryos, 4 mg protein/ml, non-labeled amino acids 20 μM each, [$^3$H]arginine and/or [$^3$H]lysine (16 and 18 Ci/mmol; 50 and 20 μCi/ml). The reaction was carried out at 20°C and stopped by adding $H_2SO_4$ to 0.4 N and sodium bisulfite. Products of translation extracted with $H_2SO_4$ were electrophoresed as in [17]. The gels were sliced, the slices dissolved in $H_2O_2$ and counted.

## 3. RESULTS

### 3.1. *The size and labeling of n-polysomes*

Fig.1 shows that n-polysomes purified from the nuclear pellet (a) had a small average size (2–5 ribosomes) compared to the membrane-bound polyribosomes (b), both classes of polyribosomes revealing a significant extent of loading with ribosomes and [$^3$H]uridine incorporation into their mRNAs. As shown in [15,18] 18 S and 28 S rRNAs are not labeled at these early stages of loach embryogenesis.

In contrast, polyribosomes that were bound

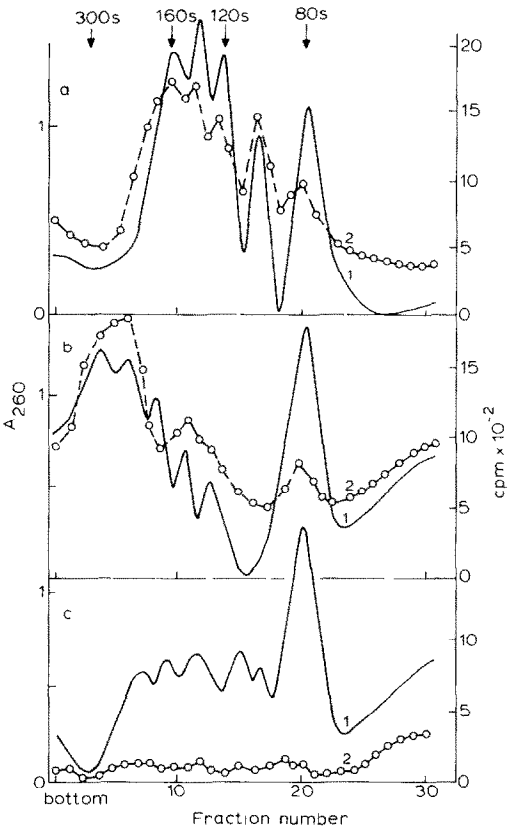


Fig.1. Sucrose gradient (17–50%) sedimentation of purified nucleus-associated (a), membrane-bound (b), and free (c) polyribosomes from the early gastrula-stage embryos incubated 60 min with 100 μCi/ml [$^3$H]uridine (24 Ci/mmol). Nucleus-associated polyribosomes were isolated from the Ficoll–sucrose-purified nuclei as described in the text. Free and membrane-bound polyribosomes were purified from the post-mitochondrial supernatant: (1) $A_{260}$; (2) radioactivity.

neither to nuclei nor to membranes of endoplasmic reticulum and defined here operationally as free polyribosomes (fig.1c), revealed a low level of loading with ribosomes and little, if any, labeling of mRNA.

Nucleus-associated, free and membrane-bound polyribosomes make 50%, 45% and 5% of the total of cell polyribosomes, respectively, in gastrula-stage embryos, while their rates of [$^{14}$C]lysine incorporation (per unit ribosomal material) relate as 1.0:0.52:1.3 [13,14]. Because of their low translational activity and insignificant labeling of mRNAs, free polyribosomes were not studied further.

### 3.2. *In vitro translation products of n-polysomes*

The previous in vivo labeling data suggest that n-polysomes synthesize histones [13,14]. However, since the nuclei are heavily labeled in their histones (>50% of total incorporation of [$^{14}$C]lysine upon a 15 min pulse) [13,14], the in vivo data could be misleading because of possible contamination of n-polysomes by nuclear leakage during their isolation. For the same reason, the reported histone mRNA-like characteristics (the absence of binding to poly(U)–Sepharose and sedimentation at 7–9 S) of the in vivo labeled RNA isolated from n-polysomes [13,14] could not prove their histone-making function.

To test directly the proposed histone-making function of n-polysomes, we isolated them and allowed to translate their endogenous templates in a homologous cell-free system where the n-polysomes incorporated amino acids linearly for at least 1 h in a cycloheximide-sensitive way.

Fig.2 shows that acid-soluble polypeptides labeled in the cell-free system with n-polysomes had the same electrophoretic distribution as the in vivo-labeled histones isolated from the purified nuclei, which proves the histone-making function of nucleus-associated polyribosomes.

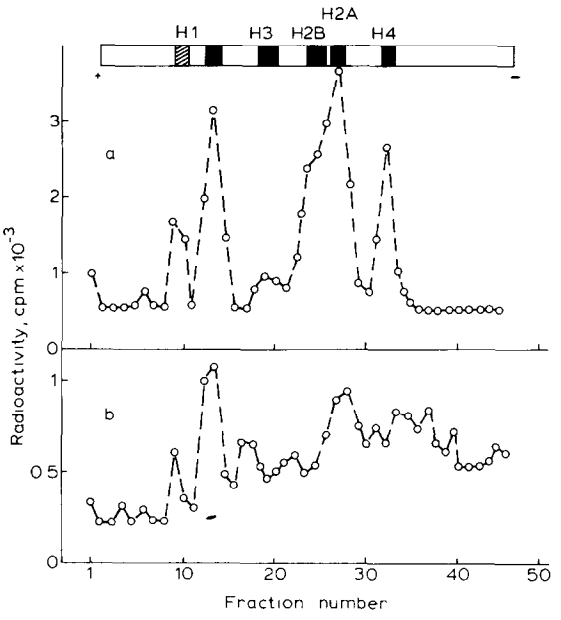


Fig.2. Electrophoresis of labeled in vivo (a) and in vitro (b) early gastrula protein products extracted with 0.4 N $H_2SO_4$. Blastoderms have been incubated for 15 min with [$^3$H]lysine and [$^3$H]arginine and histones were separated from Triton X-100-treated nuclei. Nucleus-associated polyribosomes were obtained as described in the text and translated in the cell-free system.

### 3.3. *Effect of inhibition of RNA synthesis on n-polysomes*

Depletion of the UTP pool with high doses of D-glucosamine is successfully used to inhibit RNA synthesis [14]. We used glucosamine treatment in combination with subsequent loading with cold uridine (200 mM and 2 mM, respectively) to follow the time-course of changes in various polyribosome classes under pulse–chase conditions.

Fig.3 shows that [$^3$H]RNA was rapidly chased from the nuclei to level down to 60% of the original value, while a more prolonged and profound decrease in [$^{14}$C]lysine incorporation into chromatin was observed.

From the 3 polyribosome classes of the embryos, only the n-polysome class was affected by inhibition of RNA synthesis under given conditions. The amount of ribosomes (measured as UV-absorbing material) that were found in polyribosomes purified from a constant number of blastoderms taken at different times of the experiment decreased drastically, the same as the amount of [$^3$H]uridine-labeled RNA and [$^{14}$C]lysine-labeled protein.

We interpret this result as the dissociation of n-polysomes upon inhibition of RNA synthesis. In fact, if n-polysomes were merely detached from their perinuclear compartment, they should be recovered with their accompanying labeled RNA and protein in the free polyribosome class, which was not the case (fig.3). Transition of n-polysomes into the membrane-bound class, which could be suggested by the increase in RNA labeling of the latter during the chase experiment (fig.3), is unlikely since the total amount of ribosomes and incorporation of [$^{14}$C]protein in the membrane-bound class remained virtually constant. The observed accumulation of [$^3$H]RNAs in membrane-bound polyribosomes is consistent with the report that these mRNAs, in contrast to mRNAs in n-polysomes, are polyadenylated and metabolically stable [13,14]. Thus, integrity of n-polysomes depends on nuclear RNA synthesis.

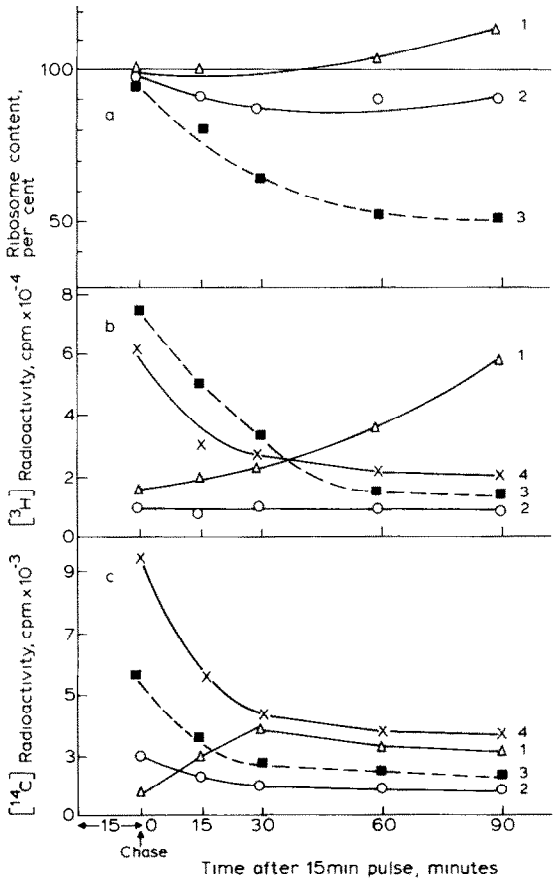


Fig.3. Effect of glucosamine treatment on content of polyribosomes (a), [$^3$H]uridine incorporation (b) and [$^{14}$C]lysine incorporation (c) into polyribosome classes of loach blastoderms under pulse-chase conditions. Blastoderms have been incubated for 15 min with [$^3$H]uridine/[$^{14}$C]lysine, washed and treated with glucosamine and cold uridine to inhibit RNA synthesis: (1) membrane-bound; (2) free and (3) nucleus-associated polyribosomes; (4) chromatin.

### 3.4. *Effect of inhibitors of DNA synthesis on n-polysomes*

In most animal cells, inhibitors of DNA replication, hydroxyurea and cytosine arabinoside, cause inhibition of histone synthesis through dissociation of histone polyribosomes and decay of histone mRNAs [1–5,11,12].

Fig.4 shows that when hydroxyurea was added to the incubation medium together with labeled thymidine, the relative incorporation of thymidine in the nuclear DNA of blastoderms, as compared

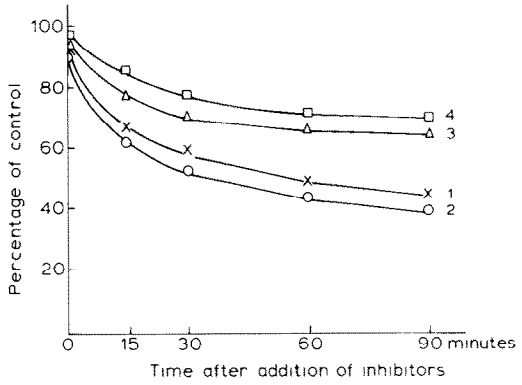


Fig.4. Effect of hydroxyurea (5 mM) on incorporation of [$^{14}$C]thymidine into chromosomal DNA (1) and the quantity of nucleus-associated polyribosomes (2), and protection of the polyribosomes by simultaneous inhibition of protein synthesis with 10 $\mu$g cycloheximide/ml (3). (4) Effect of cycloheximide alone on the quantity of nucleus-associated polyribosomes. The incorporation of [$^{14}$C]thymidine and the quantity of polyribosomes are expressed as a percentage of controls.

to control embryos, decreased as a function of time. The amount of n-polysomes in treated embryos decreased in a remarkably similar way (cf. curves 1 and 2). Cytosine arabinoside, too, caused a decrease in the amount of n-polysomes together with a decrease in the amount of [$^3$H]uridine-labeled RNA and [$^{14}$C]lysine-labeled protein of the nuclei (table 1). Other polyribosome classes did not respond to a comparable extent. Furthermore, it follows from table 1 that n-polysomes were

Table 1

Effect of cytosine arabinoside treatment (100 $\mu$g/ml, 45 min) of loach blastoderms on the amount of [$^3$H]uridine-labeled RNA content and [$^{14}$C]lysine incorporation into polyribosome classes (% of untreated control values)

| Subcellular fractions | UV-absorbing material | [$^3$H]RNA content | [$^{14}$C]lysine incorporation |
|---|---|---|---|
| Chromatin | – | 90 | 27 |
| Polyribosomes – | | | |
| Nucleus-associated | 35 | 58 | 40 |
| Free | 75 | – | 90 |
| Membrane-bound | 93 | 88 | 100 |

dissociated, rather than detached intact, from their perinuclear compartment, as a result of cytosine arabinoside treatment, since if the latter was the case, the $^{3}H$- and $^{14}C$-label accompanying n-polysomes would have appeared in the free polyribosome class, which was not observed.

Another feature of the replication-coupled histone synthesis consists in the fact that degradation of histone mRNAs in replication-blocked cells can be prevented by cycloheximide [5,11]. In our experiments, cycloheximide protected n-polysomes from dissociation in hydroxyurea-treated embryos (fig.4), according to cycloheximide action on histone-making polyribosomes in HeLa cells [5,11].

These results allow us to conclude that the integrity and function of n-polysomes depend on DNA synthesis, a feature characteristic of the replication-coupled histone synthesis in most animal cells.

## 4. DISCUSSION

These data show, in confirmation and extension of [13,14], that fractionation of particulate components of loach early embryo cells under conditions apparently preventing degradative enzymatic processes due to treatment with diethylpyrocarbonate at the homogenization step, allowed us to isolate a special class of polyribosomes which are pelleted with the nuclei and are termed nucleus-associated polyribosomes, or n-polysomes.

These n-polysomes differ in a number of features from two other polyribosome classes isolated from loach embryo cells (i.e., free polyribosomes and those bound to membranes of endoplasmic reticulum) and can be considered as a physically and functionally distinct subgroup of polyribosomes. If the embryos were pre-treated with inhibitors of DNA or RNA synthesis the same technique yielded nuclear pellets from which much less n-polysome could be recovered. This argues for the reliability of the procedure used, and for a functionally meaningful nature of association of these polyribosomes with the nuclei. No polyribosomes were pelleted with the nuclei if the homogenate contained 1% Triton X-100 [14].

The n-polysomes are shown here to synthesize histones in a homologous cell-free system, which is consistent with our previous in vivo data [13,14], and we consider these polyribosomes as the major, if not the sole, site of histone synthesis at the developmental stages studied (blastula and early gastrula).

Histone synthesis by n-polysomes is coupled to DNA replication: inhibition of DNA synthesis led to dissociation of the histone-making polyribosomes, the effect being also observed in HeLa cells [1,2,5,11] and sea urchin embryos [3,4]. However, in contrast to loach embryos, in sea urchin embryos and other kinds of animal cells, histone polyribosomes have been repeatedly reported to belong to the free polyribosome class [6–8]. Only in [19] was it reported that histone mRNAs were found associated with the nucleus [19].

The spacial proximity of histone-making polyribosomes to the nuclear envelope in loach embryo cells appears to be highly significant, especially given the possibility that at least part of DNA replication complexes are associated with the nuclear membranes, as reported for sea urchin embryos [20] and HeLa cells [21]. Such a spatial organization may provide a structural basis for the functional organization of the system involved in coordinated biogenesis of chromatin, where coupling of histone and DNA synthesis can be effected through short-range, as opposed to the diffusion-dependent, interactions at the level of the nuclear envelope. This proposal, detailed in [14], is compatible with other models proposed to explain the coupling [5,6,12]. It differs in that it envisages a minimal contribution of diffusion in the well-known, and still unexplained, extremely rapid transport of newly made histone mRNAs and histones [5,6,9,10] and dependence of histone template translation on antecedent DNA synthesis [5,6].

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