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Histone mRNA in Xenopus laevis ovaries: identification of the H4 messenger

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ABSTRACT

RNA extracted from ovaries of adult *Xenopus laevis* and introduced into a wheat germ cell-free system, directs the synthesis of polypeptides co-electrophoresing with all five histones. By fractionation of total ovary RNA through Sephadex G-200 and polyacrylamide gel, RNA was isolated that is translated into histone 4 (H4) only. In addition, this RNA labelled *in vitro* with $[^{125}I]$ hybridizes to a restriction fragment of cloned sea urchin DNA known to contain the H4 gene.

INTRODUCTION

The histone genes represent an interesting model system for the analysis of gene regulation in development and differentiation[1,2]. The isolation and amplification of these genes by molecular cloning has led to an advanced knowledge of their molecular structure in the case of sea urchins[3-7]. The reiteration of the histone genes in *Xenopus laevis* (20 to 50)[8] is much lower than in sea urchins (400 to 1000)[1]. The search for the histone genes from this amphibian was undertaken for two main reasons: first, *Xenopus* has been shown to be extremely useful as an experimental system for the study of developmental regulation mechanism, and secondly, its histones and their synthesis are well documented[9-11].

Different approaches have been envisaged to obtain histone mRNA of high specific activity as a probe for the detection of the histone genes from *Xenopus*. The use of radioactively labelled RNA obtained after injection of *Xenopus* embryos enabled Jacob *et al.*[8] to establish the reiteration frequency of the histone genes in this species. We have attempted to isolate *in vivo*-labelled histone mRNA from *Xenopus* culture cells; however, the specific activity as well as the purity of the messengers obtained in this way were not suitable for further use in the detection of the histone genes (Destrée, unpublished results). Since by their experiments Adamson and Woodland[12] have anticipated the existence of considerable amounts of histone mRNA in *Xenopus* oocytes, we have undertaken

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the isolation of unlabelled histone mRNA from ovaries of adult *Xenopus* in order to label it *in vitro* to a high specific activity.

We show by *in vitro* translation that RNA extracted from ovaries of adult *Xenopus* directs the synthesis of polypeptides co-electrophoresing with all five marker histones.Furthermore, one of the histone messengers directing the synthesis of histone 4 (H4) only, was isolated and was shown to hybridize to a DNA fragment, obtained by restriction of cloned sea urchin histone DNA, known to contain the H4 gene[7].

MATERIALS AND METHODS

Chemicals. *E. coli* tRNA (Sigma) was purified by two rounds of phenol extraction. Poly(U) was from Miles Laboratories, and radioactively labelled compounds from The Radiochemical Center, Amersham.

Extraction of RNA. Total RNA was extracted from ovaries of adult *Xenopus laevis* essentially according to Parish and Kirby[13]. Approximately 10 g of ovary were homogenized at room temperature for 2 min at a speed setting of 2 in a Sorvall Omnimix homogenizer in 250 ml of 1% triiso-naphtalene sulphonate, 6% 2-butanol, 6% sodium 4-aminosalicylate and 1% NaCl. The pH of the homogenate was then adjusted to about 7 with 15 ml of 1 M Tris-HCl, pH 8.0; 250 ml of phenol/*m*-cresol/hydroxychinoline (PCH) in ratios of 100/14/0.1 were then added and homogenization continued for 2 min at a speed setting of 5. The homogenate was centrifuged for 10 min at 12 000 x *g* at room temperature. The aqueous phases were re-extracted for 10 min with an equal volume of PCH. After centrifugation, the combined aqueous phases were brought to 0.45 M NaCl and re-extracted with 0.5 vol. of PCH. The combined aqueous phases obtained by centrifugation were re-extracted with a 1 to 1 mixture of PCH and chloroform. The RNA in the final aqueous phases was precipitated overnight at -20°C with 2.5 vol. of 95% ethanol and recovered by centrifugation.

Gel filtration of RNA through Sephadex G-200. Total ovary RNA (not more than 20 mg) was dissolved in 20 ml of G-200 buffer (10 mM sodium acetate, pH 5.0, 0.1 M NaCl, 0.1 mM EDTA, 0.01 mM sodium azide and 0.1% of diethylpyrocarbonate) and applied onto a Sephadex G-200 column (5 x 90 cm) at a flow rate of 40 ml/h. Eight ml fractions were collected and the A_{260} determined. For the characterization of the RNA in a wheat germ cell-free system, the RNA from two consecutive fractions was precipitated with 2.5 vol. of 95% ethanol in the presence of *E. coli* tRNA (15 µg/ml). When the

RNA was to be further purified on polyacrylamide gels, appropriate fractions were pooled and precipitated with 2.5 vol. of 95% ethanol in the presence of *E. coli* tRNA where necessary.

Polyacrylamide gel electrophoresis of RNA. The RNA samples collected by centrifugation were dissolved in electrophoresis buffer[14] containing 0.2% sodium dodecyl sulphate (SDS) and 10% glycerol. RNA (100 to 200 μg) was layered onto cylindrical 4% polyacrylamide gels (0.7 x 11 cm) overlaid with a 2.4% polyacrylamide spacer gel (1 cm long). Electrophoresis was carried out for 4 h at room temperature at 7 mA/gel. The gels were soaked overnight in 500 ml of distilled water at 4°C. After scanning at 260 nm, they were frozen on dry ice and cut into 0.5 mm slices. Four consecutive slices were combined, 2 ml of 0.6 M lithium acetate, pH 5.8, containing 0.2% SDS were added[3] and the RNA was eluted by shaking at room temperature for at least 24 h. The RNA in the eluates was precipitated at -20°C with 2.5 vol. of 95% ethanol in the presence of carrier RNA: 10 μg/ml of *E. coli* tRNA when it was to be analyzed in the wheat germ cell-free system, or 10 μg/ml of poly(U) when it was to be iodinated.

Translation of mRNA *in vitro*. The RNA pellets collected by centrifugation after fractionation by G-200 filtration or polyacrylamide gel electrophoresis were washed three times with cold 95% ethanol, dried under reduced pressure and dissolved in 50 μl of distilled water. A wheat germ cell-free system was prepared and preincubated essentially as described by Roberts and Paterson[15]. Parameters of the cell-free system were optimized for the translation of total RNA from *Xenopus* ovary. The reaction mixtures (25 μl) contained 10 μl of preincubated wheat germ S-30, 20 mM Hepes, pH 7.6, 3 mM Mg^{++}, 88 mM K^{+}, 2 mM DTT, 1 mM ATP, 25 μM GTP, 8 mM creatine phosphate, 250 μM spermidine, 25 μM of each of the unlabelled amino acids except lysine, 5 μM $[^{3}H]$ lysine (28 Ci/mmol) and where indicated 10 μl of RNA solution. After incubation for 90 min at 23°C, 3 μl aliquots of each reaction mixture were assayed for hot TCA precipitable radioactivity essentially as described by Roberts and Paterson[15].

Analysis of the *in vitro* translation products by SDS-polyacrylamide gel electrophoresis. To 3 μl aliquots of the translation mixtures, 3 μl of a solution containing 200 mM DTT, 100 mM Tris-HCl, pH 6.8, 2% SDS, 40% glycerol and 0.05% bromophenol blue were added and the samples heated for 2 min at 90°C. After cooling, the samples were applied into the wells of a 0.1% SDS - 12.6% polyacrylamide slab gel (14 x 16 x 0.1 cm) prepared

according to Laemmli[16]. Electrophoresis was performed for 4.5 h at 12 mA. The radioactivity in the gel was detected by the fluorographic procedure of Bonner and Laskey[17].

Tritium-labelled marker histones were isolated from *Xenopus* kidney culture cells as follows. Cells in roller culture were labelled with 3 μCi/ml of [^{3}H]lysine (18 Ci/mmol) for 7 days and lyzed in 10 mM Tris-HCl, pH 7.4, 10 mM KCl and 1.5 mM $MgCl_2$. Nuclei and histones therefrom were prepared as described previously[9].

Iodination of RNA. The RNA containing messenger activity for H4 was precipitated with poly(U) after elution from the 4% polyacrylamide gel (see above), recovered by centrifugation and redissolved in 25 μl of distilled water.

Iodination was performed essentially as described by Getz *et al.*[18]. Reaction mixtures for iodination contained 1 to 5 μg of RNA plus poly(U), 2 mCi of carrier-free Na[^{125}I] (100 mCi/ml), 62.5 μM KI, 23 mM $TlCl_3$ and 0.04 M sodium acetate, pH 5.0, in a total volume of 30 μl. The reaction was carried out at 50°C for 30 min. Unstable bound [^{125}I] was dissociated by adding 3 μl of 0.1 M Na_2SO_3 and 30 μl of 0.5 M NH_4HCO_3, pH 8.0, and incubating at room temperature for 5 min. The labelled RNA was separated from free [^{125}I] by filtration through Sephadex G-50 (medium) in a Pasteur pipette and washed with 10 mM Tris-HCl, pH 7.5. Five-drop fractions were collected and the [^{125}I]-RNA-containing fractions monitored and pooled. The RNA was further purified by vigorous shaking for 2 min with an equal volume of 2-methoxyethanol and of 2.5 M potassium phosphate, pH 8.0. After centrifugation for 10 min at 4 000 x *g*, the upper phase was collected. To it was added *E. coli* tRNA (50 μg), 0.5 vol. of 1% cetyltrimethylammonium bromide and an equal volume of 0.2 M sodium acetate, pH 5.6. The mixture was kept at -20°C overnight , the RNA recovered by centrifugation and washed with 70% ethanol, 0.1 M sodium acetate, pH 5.6. The RNA was dissolved in 100 μl of distilled water; its specific activity was about 22×10^{6} cpm/μg.

Hybridization of mRNA to restriction fragments of cloned sea urchin histone DNA. Restriction fragments obtained by excision of histone genes of *Psammechinus miliaris* from λh22 DNA and transferred to nitrocellulose filters[19] were kindly supplied by W. Schaffner.

Hybridization was with either polysomal [^{3}H]-labelled RNA from

P. miliaris (a kind gift of K. Gross) or $[^{125}I]$-labelled mRNA from *Xenopus*. In the case of homologous hybridization, the incubation and subsequent processing of the nitrocellulose strips was essentially as described by Schaffner *et al.*[4], except that 2 x SSC (SSC = 0.015 M trisodium citrate, 0.15 M NaCl, pH 7.0) was used instead of 4 x SSC, that 100 µg/ml of proteinase K were included, and that the reaction was for 15 h at 65°C. For heterologous hybridization, the incubation mixture contained 4 x SSC, 0.2% SDS, 1 mg/ml of *E. coli* tRNA, 100 µg/ml of proteinase K, 1 mg/ml of unlabelled poly(U) and 1 to 2 x 10^6 cpm/ml of the $[^{125}I]$-mRNA; the nitrocellulose strips were incubated for 15 h at 60°C and washed with 2 x SSC without RNase treatment.

The strips were impregnated with 7% PPO in diethylether[20] and exposed to a flash-activated X-ray film[21] to locate the regions of hybridization. Scans of the resulting fluorograms are presented.

RESULTS AND DISCUSSION

Fractionation of total ovary RNA by Sephadex G-200. When total RNA from *Xenopus* ovary is added to a wheat germ cell-free system, using $[^{3}H]$-lysine as a precursor, the most abundant polypeptides synthesized co-electrophorese with the five histone markers of *Xenopus* (results not shown). Similar observations have been made by Ruderman and Pardue (personal communication).

In order to separate the mRNAs from the bulk of the RNA, filtration through Sephadex G-200 was performed. In these conditions, total ovary RNA separates into three A_{260} peaks (Fig. 1). The excluded material represents mainly 28S and 18S RNA, that make up about 95% of the total RNA in *Xenopus* ovaries[22]; the other two A_{260} peaks correspond to 5S and 4S respectively.

Separate experiments have shown that when total *in vivo* labelled *P. miliaris* histone mRNA is filtered through Sephadex G-200 together with total ovary RNA from *Xenopus*, the radioactivity appears in a region of the column corresponding to fractions 70 to 110 of Fig. 1 (results not shown). Therefore, these fractions were challenged in a wheat germ cell-free system: two major peaks of incorporation appeared (Fig. 1). Analysis of the products of translation in SDS-polyacrylamide gels indicates that virtually all the proteins synthesized *in vitro* co-electrophorese with *in vivo* labelled histones of *Xenopus* (Fig. 2). The relatively low incorporation of label in

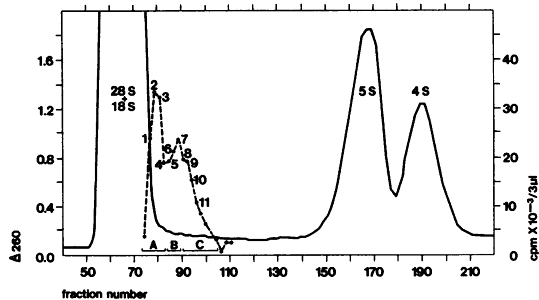

Fig. 1 : Fractionation on Sephadex G-200 of total RNA extracted from *Xenopus laevis* ovaries. Total ovary RNA was prepared and fractionated by filtration through Sephadex G-200 as described in Materials and Methods. The RNA from the pertinent fractions was tested for translation activity in the wheat germ cell-free system and the products made by the RNA contained in fractions 1 to 11 analyzed by SDS-polyacrylamide gel electrophoresis (see Fig. 2). A, B and C : RNA fractions pooled for further purification on polyacrylamide gels. ——— , A_{260}; •- - -• , incorporation of [^{3}H]lysine into hot TCA precipitable material.

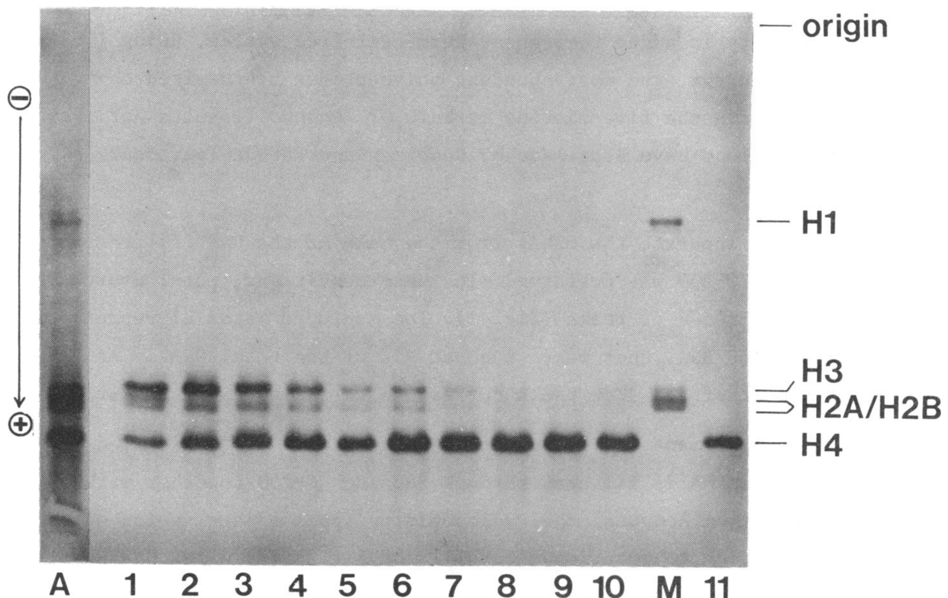

Fig. 2 : SDS-polyacrylamide gel analysis of the translation products from RNA fractions separated on Sephadex G-200. A and 1 to 11 : cell-free translation products from fractions A and 1 to 11 of Fig. 1 analyzed by SDS-polyacrylamide gel electrophoresis performed as indicated in Materials and Methods. M : [^{3}H]-*in vivo*-labelled marker histones (9 000 cpm) isolated from *Xenopus* kidney culture cells. Identification of histones was according to Destrée *et al.*[9]. Fluorographic exposure time after flash-activation was 3 days.

the tentative H1 might be due to poor translation of the corresponding messenger in the wheat germ system, or to post-translational degradation, as H1 is very susceptible to proteolytic breakdown[23]. These results demonstrate that the mRNAs for all five histones are stored in *Xenopus* ovaries.

While filtration of total ovary RNA through Sephadex G-200 has proved very useful for the isolation of the smallest histone messenger (H4), the other larger histone messengers may be better separated from the 28S and 18S RNA by Sepharose 6B or equivalent gel filtration (results not shown).

For further purification of the H4 messenger, the RNA from the various fractions of the G-200 column was pooled (A, B and C of Fig. 1) and fractionated on polyacrylamide gels.

Fractionation of histone mRNAs by polyacrylamide gel electrophoresis. The RNAs from the pooled fractions were separated on 4% polyacrylamide gels containing 0.2% SDS. The A_{260} scans of the gels carrying the RNA populations A, B and C of Fig. 1 are presented in Fig. 3. The shift in the molecular weights of the RNA populations, reflecting the separation by Sephadex G-200, is evident. Translation of the RNA from the 9S to 12S region[8] of the gels

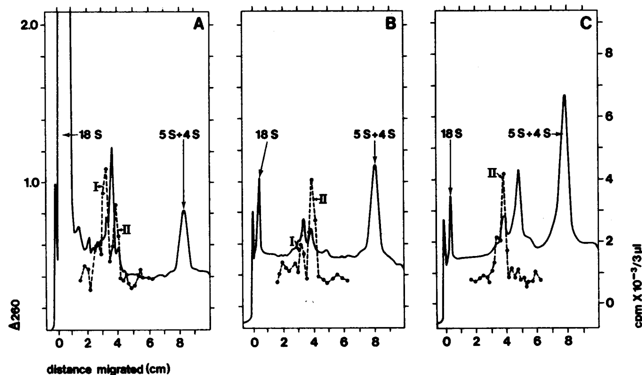

Fig. 3 : Separation of histone mRNA by polyacrylamide gel electrophoresis. The RNAs from the fractions pooled after Sephadex G-200 filtration (A, B and C of Fig. 1) were separated on cylindrical 4% polyacrylamide gels as described in Materials and Methods. A, B and C : RNA populations from pools A, B and C respectively of Fig. 1. I and II : translation products analyzed by SDS-polyacrylamide gel electrophoresis (see Fig. 4). ——— , A_{260}; ●- - -● , incorporation of [^{3}H]lysine into hot TCA precipitable material.

reveals two major peaks of activity (peaks I and II of Fig. 3) that do not exactly coincide with the optical density peaks. Most of this A_{260} absorbing material (which varies in amount from one preparation to another) represents ribosomal RNA breakdown products as determined by hybridization experiments (results not shown).

The products representing maximum activity in the translation system were analyzed on SDS-polyacrylamide gels (Fig. 4). From this analysis it appears that the protein of peak II in all three cases co-migrates with marker H4. The activity of peak I, on the other hand, corresponds to two proteins that co-migrate with H3 and H2A or H2B.

Because in these conditions, the RNA with messenger activity for H4 (peak II of Fig. 4) could be easily separated out from the other histone mRNAs, it was iodinated and used in hybridization experiments.

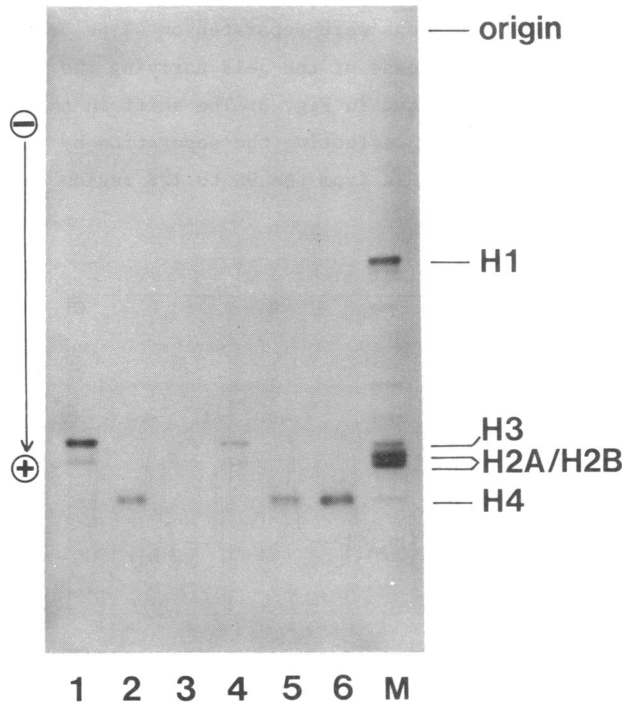

Fig. 4 : SDS-polyacrylamide gel analysis of the translation products of RNA fractions from the 4% acrylamide gels. The products of the cell-free translation were analyzed by electrophoresis in SDS-polyacrylamide slab gels as indicated in Materials and Methods. 1 and 2 : fractions I and II respectively of Fig. 3A; 3 : endogenous products of the cell-free system; 4 and 5 : fractions I and II respectively of Fig. 3B; 6 : fraction II of Fig. 3C; M : [^{3}H]-*in vivo*-labelled marker histones (9 000 cpm) from *Xenopus* kidney culture cells. Fluorographic exposure time after flash-activation was 3 days.

Hybridization of H4 mRNA. The $[^{125}I]$-labelled H4 mRNA was hybridized to the restriction fragments obtained by digestion of the recombinant λh22 DNA with endonuclease *Hin*dIII. From the results of this experiment (Fig. 5A and B) it is clear that the $[^{125}I]$ mRNA of *Xenopus* specifically hybridizes to the 6 kilobase pair (kb) fragment of *P. miliaris* DNA, as does also polysomal $[^{3}H]$-labelled RNA from *P. miliaris* that contains all five histone mRNAs[1,22].

To further verify that the $[^{125}I]$-labelled RNA indeed contains H4 mRNA, it was hybridized to the DNA restriction fragments obtained by cleavage of the isolated 6 kb unit with the endonuclease *Hpa* II. The cleavage sites of this enzyme within the 6 kb unit have been mapped by Smith and Birnstiel[7]. According to this map, four large fragments contain histone gene sequences, and this is illustrated in Fig. 5C by a hybridization experiment with polysomal $[^{3}H]$-labelled RNA from *P. miliaris*. The fragment of 1.6 kb contains the H4 and H2B DNA sequences, and indeed, this is the only fragment to which the $[^{125}I]$ mRNA hybridizes (Fig. 5D). That this hybridization is not due to the presence of H2B mRNA in the iodinated mRNA is clear from the results of the translation experiments (Fig. 4).

In conclusion, these results demonstrate that the mRNA coding for H4

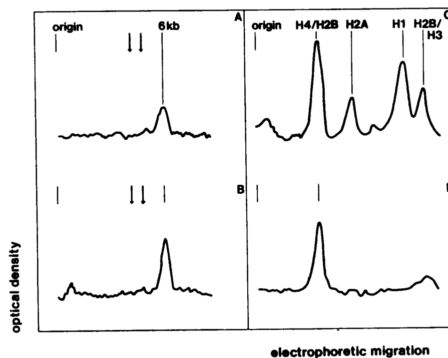

Fig. 5 : Hybridization of histones mRNAs to total or restricted histone DNA of *P. miliaris*. The conditions for hybridization were as specific under Materials and Methods. *Hin*dIII digests of λh22 DNA were hybridized to polysomal $[^{3}H]$-labelled RNA of *P. miliaris* (A), or to $[^{125}I]$-labelled mRNA of *Xenopus* (B); arrows refer to λ vector fragments as detected by ethidium bromide staining. *Hpa* II fragments obtained by digestion of the 6 kb unit (excised from the λh22 DNA with *Hin*dIII) were hybridized to polysomal ^{3}H - labelled RNA of *P. miliaris* (C), or to $[^{125}I]$-labelled of *Xenopus* (D).

can be separated relatively easily from the bulk of the messengers present in *Xenopus* ovaries. Experiments are now in progress to test the usefulness of this messenger as a probe for the detection of the histone genes of *Xenopus*.

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