Post-transcriptional regulation of ornithine decarboxylase in *Xenopus laevis* oocytes

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Summary

The level at which ornithine decarboxylase expression is regulated in growing oocytes has been investigated. Immunoprecipitation of the *in vivo* labelled proteins showed that ornithine decarboxylase accumulated less rapidly in stage IV oocytes than in previtellogenic stage I+II oocytes. Quantitative Northern analysis showed that ornithine decarboxylase mRNA is abundant in oocytes (about 8 x 10⁸ transcripts/cell) and this number does not significantly change during oogenesis. Polysome analysis showed that this mRNA is present in polysomes in stage I+II oocytes but has passed into puromycin-insensitive mRNP particles by stage IV of oogenesis. Therefore, during the growth phase of oogenesis, ornithine decarboxylase expression is regulated at a translational level. These results are discussed relative to the temporal expression of ornithine decarboxylase and of other proteins whose expression also decreases during oogenesis. In order to perform these experiments, the cDNA (XLODC1) corresponding to *Xenopus laevis* ornithine decarboxylase mRNA was cloned and sequenced.

Key words: *Xenopus laevis*, mRNA, post-transcriptional regulation, ornithine decarboxylase.

Introduction

During oogenesis in *Xenopus laevis*, large amounts of mRNAs and proteins are accumulated which, after fertilization of the mature oocyte or egg, allow the embryo to perform the first twelve mitotic cell cycles in the absence of gene transcription (Newport and Kirschner, 1982). However, before an oocyte can be fertilised it must undergo a hormonally stimulated maturation process (reviewed in Smith, 1989). The competence to undergo this maturation process, which corresponds to a release of the oocytes from arrest in meiotic prophase I, is only acquired by the fully grown oocyte. Growing oocytes, although also blocked in prophase I are not competent to undergo hormonally stimulated maturation (reviewed in Masui and Clark, 1979; Maller, 1985; Taylor and Smith, 1987). It is necessary to differentiate here hormonally stimulated maturation from germinal vesicle breakdown (GVBD) caused by transfer of maturation-promoting factor (MPF). The progesterone-induced maturation of stage VI oocytes requires the synthesis of c-mos protein (Sagata *et al.* 1988) and the injection of synthetic c-mos mRNA into these oocytes activates MPF (Sagata *et al.* 1989). Although GVBD can be induced in stage IV oocytes by MPF transfer (Hanocq-Quertier *et al.* 1976) or injection of cyclin mRNA (Johnson and Smith, 1990), these oocytes do not contain c-mos protein (Sagata *et al.* 1988) and the injection of c-mos mRNA apparently does not activate MPF (Johnson and Smith, 1990). Therefore, the acquisition by stage VI oocytes of competence to undergo hormonally stimulated maturation must correspond to specific modifications that occur during oogenesis, modifications that possibly involve both the accumulation of certain gene products and the reduction of others. In the former case, these may be enzymes or cofactors that must attain a threshold level in the oocyte before the cascade triggered by hormonal stimulation can become active. The second case would concern inhibitory gene products whose presence or activity (enzymes) must decrease, before the cascade of events associated with maturation can become operative.

A number of different gene products have been identified whose amount per oocyte either increases or decreases during the growth phase of oogenesis. For the proteins that increase in amount during oogenesis, apart from the components of maturation-promoting...
factor, which exists in an inactive form in immature oocytes (Gerhart et al. 1984), it is not known whether their accumulation is required for acquisition of hormonally stimulated maturation competence. A test in this case would be, for instance, that the microinjection of the protein renders the growing oocyte responsive to progesterone.

The identified proteins whose amount or activity decreases during the growth phase of oogenesis are the transcription factor TFIIB (Shastry et al. 1984); poly(A) RNA-binding proteins (Richter and Smith, 1983) and ornithine decarboxylase (Osborne et al. 1989). Two of these proteins could play a role in acquisition of maturation competence. As a class, poly(A) RNA-binding proteins inhibited the translation of reconstituted mRNPs (Richter and Smith, 1984) and the 56×10^3 M_r poly(A) RNA-binding protein has been shown to associate with specific mRNAs (Crawford and Richter, 1987). Therefore, the decrease in the titre of these proteins could be important for the acquisition of maturation competence, by allowing the translation, towards the end of oogenesis, of previously masked (untranslated) mRNAs (Crawford and Richter, 1987).

Ornithine decarboxylase (ODC) is the first enzyme in the polyamine biosynthetic pathway and it is one of the rate-limiting enzymes (Heby, 1981; Pegg, 1986). The decrease in this enzyme activity during oogenesis is accompanied by a reduction in the intracellular polyamine concentration (Osborne et al. 1989). Artificially increasing the concentration of either spermidine or spermine in the stage VI oocytes inhibited their hormonally stimulated maturation, suggesting that the reduction in ODC activity and the associated decrease in polyamine concentration are required for the acquisition by the fully grown oocyte of their competence to undergo hormonally stimulated maturation (Osborne et al. 1989).

The identification of other gene products, which are involved in maturation competence and whose activity must decrease during oogenesis, would be helped by a knowledge of the regulatory controls that are common to both ODC and the 56×10^3 M_r poly(A) RNA-binding protein. Therefore, we have analysed and reported here the level at which ODC expression is regulated during the early growth phase of oogenesis. Comparison of these results with those previously reported for the 56×10^3 M_r poly(A) RNA-binding protein show similarities and differences in their mode of regulation.

Materials and methods

Biological material

Ovaries were removed from anaesthetized Xenopus laevis females and the oocytes isolated by treatment with dispase and collagenase successively (Belle et al. 1986). The oocytes were sorted manually into the different oogenesis stages (Dumont, 1972). When required, the oocytes were incubated in buffered saline (88 mM NaCl, 0.33 mM Ca(NO_3)_2, 1 mM KCl, 0.41 mM CaCl_2, 0.82 mM MgSO_4, 10 mM Hepes pH 7.6) with puromycin (150 μg ml⁻¹) for 2 h at 20°C, or with [³⁵S]methionine (50 μCi ml⁻¹) for 15 h at 18°C. Protein synthesis, during the second hour of puromycin treatment, was inhibited by at least 70% as measured by [³⁵S]methionine incorporation into total acid-insoluble protein.

Protein extraction and analysis

Cytosol extracts were prepared as previously described (Osborne et al. 1989). Immunoprecipitation of in vivo (50 μCi) and in vitro (reticulocyte lysate diluted 60-fold, final volume 500 μl) [³⁵S]methionine-labelled proteins, in 30 mM Tris–HCl pH 7.4, 60 mM KCl and 1% Nonidet NP 40, was achieved by successive incubations (15 h, 4°C) with normal rabbit serum and anti-mouse ODC serum (Persson, 1982). The immunoprecipitates were collected by incubation with Pansorbin beads (Calbiochem) as described by the supplier. Proteins were analysed by electrophoresis in 12% polyacrylamide gels in the presence of SDS (Laemmli, 1970), treated for fluorography if required, dried and autoradiographed at —70°C.

cDNA library screening and DNA sequencing

A cDNA corresponding to Xenopus ODC mRNA was isolated from an Xenopus laevis unfertilized egg lambda gt10 cDNA library (Paris and Philippe, 1990) using a [α-³²P]CTP-labelled RNA probe (Melton et al. 1984), complementary to the 940bp 3’ HindIII fragment of mouse ODC (Gupta and Coffino, 1985). Prehybridisation and hybridisation were performed at 60°C in 30% or 40% formamide with 5× Denhardt solution (1×Denhardt’s solution is 0.02% bovine serum albumin, 0.02% Ficoll and 0.02% polyvinylpyrrolidone). 5×SSPE (1×SSPE is 10 mM NaPO₄, pH 7.0; 180 mM NaCl; 1 mM EDTA), 0.1% SDS and 50 μg ml⁻¹ tRNA. The filters were washed at low stringency, 1×SSC (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate; pH 7.4), 0.1% SDS at 50°C for 20 min. Positive clones were plaque purified using a [³²P]-labelled cDNA probe corresponding to the same fragment of mouse ODC. Hybridization was performed at 42°C in the same medium as above except that 20% formamide was used. The temperature of the 1×SSC wash was also reduced to 42°C.

The cDNA fragments isolated from the lambda clones and restriction fragments were subcloned into Bluescript™ plasmids. Single-stranded DNA was prepared and sequenced by the dyeoxy chain termination method (Sanger et al. 1977).

RNA preparation and Northern analysis

RNA was prepared using the LiCl/urea procedure (Auffray and Rougeon, 1980). RNA concentrations were evaluated from the A₂₆₀nm and controlled by gel analysis. RNA samples were fractionated by electrophoresis in 1.2% denaturing agarose gels containing 2.2M formaldehyde and transferred onto nylon membranes (Hybond, Amersham). Hybridization to [³²P]-labelled Xenopus cDNA probe was carried out in 50% formamide, 1% SDS, 10×Denhardt solution, 10% dextran sulphate, 0.1% PPI, 1 M NaCl and 0.05 M Tris–HCl pH 7.5 at 42°C for 15 h. The filters were then washed twice in 2×SSC, 0.5% SDS at 65°C for 30 min and twice in 0.1×SSC at room temperature for 30 min and autoradiographed at —70°C.

Polysome analysis

Polysonal and non-polysomal RNA were separated essentially as described by Dworkin et al. (1985). 50 isolated stage I+II or stage IV oocytes, and isolated oocytes preincubated with puromycin, were homogenised with a Dounce homogeniser (tight-fitting piston) in 2 ml HKM buffer (Hepes 20 mM, pH 7.4; KCl 300 mM; MgCl₂ 10 mM; Nonidet NP40 0.5% and polyvinyl sulfate 20 μg ml⁻¹) and centrifuged
Mt or normal rabbit serum followed by monospecific anti-

the molecular weight markers are indicated on the left as
to equal amounts of total labelled protein. The position of
immunoprecipitates loaded in each lane correspond
autoradiographed as described in the Methods. The volume
analysed by electrophoresis in 12 % acrylamide gels and
mouse ODC serum (anti-ODC). The proteins were
immunoprecipitated with either normal rabbit serum (NRS)
(the above ODC activity changes during this
extracted proteins were immunoprecipitated with
polyacrylamide gels and autoradiographed (Fig. 1).

Results

Protein synthesis

We have previously shown that ODC activity decreases
during the early growth phase (stage II to IV) (Osborne
et al. 1989). Therefore, in order to analyse the
regulation of ODC in oocytes, we determined initially
whether the reduction in ODC activity during this
period of oogenesis was associated with a change in the
accumulation of ODC protein. Batches of stage I+II
and stage IV oocytes were incubated overnight in
[35S]methionine-supplemented medium and the
extracted proteins were immunoprecipitated with
monospecific anti-mouse ODC serum. The proteins
precipitated by this antibody or with non-immune
serum were analysed by electrophoresis in SDS-
polyacrylamide gels and autoradiographed (Fig. 1).

Cloning and sequencing of Xenopus ODC cDNA

In order to analyse Xenopus ODC mRNA, the
corresponding cDNA was isolated from a Xenopus egg
cDNA library as described in Materials and methods.
The isolated clones contained inserts of either 1.6 kb or
1.9 kb, which cross-hybridized in Southern analysis and
which hybridized to a 2.4 kb mRNA in Northern
analyses of total egg RNA. The longest of these cDNAs
(XLODC1) was subcloned in Bluescript plasmid and
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(XLODC1) was subcloned in Bluescript and sequenced.
Fig. 2A indicates the partial restriction analysis and the
sequencing strategy. The sequence of the
1.9 kb cDNA is shown in Fig. 2B.

This insert (1970 nucleotides) contains an open
reading frame of 1380 nucleotides and a single
polyadenylation signal 10 nucleotides from the 3' end.
As no poly (A) track was found at the 3' end of the
insert, additional sequence may exist between the end
of the cDNA and the polyadenylation cleavage site.
The shorter cDNA (1.6 kb) also contained a single
polyadenylation signal but no poly (A) track (data not
shown). The deduced amino acid sequence of
Xenopus ODC (Fig. 2B) was compared with the mouse (Gupta
and Coffino, 1985; Kahana and Nathans, 1985) and
human (Hickok et al. 1987) ODC proteins. The
homology between the Xenopus protein and the human
and mouse proteins is 81 % and 80 %, respectively,

(6000g, 6 min) to eliminate cellular debris and nuclei. The
appropriate controls showed that neither ODC mRNA nor
the mRNAs corresponding to several Eg cDNAs (Paris and
Philippe, 1990) were present in this pellet. 1.8 ml of the clear
supernatant was deposited over a 10 ml sucrose gradient
(15 %-30 % w/w) in HMK buffer and centrifuged for 5½ h at
41000 revs min-1 in a Beckman SW 41 rotor. The 10S–80S
supernatant fraction, and the pelleted RNA, resuspended in
10 mM sodium acetate, pH 5.5; 100 mM NaCl, 2 mM EDTA and
0.4 % SDS, were extracted once with 1 vol phenol/
chloroform (1:2), once with 1 vol chloroform and then
precipitated with 2 vol ethanol (–20°C). The same procedure
was followed when the Mg2+ in the homogenisation and
gradient buffers was replaced by 20 mM EDTA.

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**Fig. 2.** Sequencing strategy and nucleotide sequence of *Xenopus laevis* ODC. (A) The restriction sites shown are those used for obtaining the nucleotide sequence, the EcoRI sites correspond to the linkers added during the construction of the cDNA library and used for subcloning (HindIIIand EcoRI; see Fig. 1). The construction of the cDNA library and sequence reading of each fragment. Bg, Bg/II; F, EcoRV; FokI; H, HindIII; X, XhoI.

(B) Nucleotide sequence of ODC transcript, and the deduced amino acid sequence. The polyadenylation signal is underlined. Numbering starts at the first nucleotide of the insert. (C) Comparison of the deduced *Xenopus* and the murine and human ODC protein sequences. Alignment of the *Xenopus*, mouse (Gupta and Coffino, 1985; Kahana and Nathans, 1985) and human (Hickok et al. 1987) ODC protein sequences was done manually. The numbering on the right corresponds to the *Xenopus* sequence. Only amino acids that are not identical are shown for the mouse and human sequences and the conservative replacements (S-T; A-D; E-R; K-L; I-V; and Y-F) are denoted by an asterisk. *Xenopus* ODC contains an insertion (K) and two deletions (EQ) at amino acid positions 313 and 432-433 respectively.
spread over almost the entire sequence (Fig. 2C). Confirmation that the mRNA, transcribed from this cloned cDNA, codes for a protein with ornithine decarboxylase activity has been obtained by transfection of ODC-deficient mutant C57.7 Chinese hamster ovary cells (Steiglich and Scheffler, 1982). These cells, when transfected with a plasmid containing the coding region of XLODC1 under the control of the SV40 early promoter, expressed ODC activity and grew in the absence of exogenous polyamines whereas the parent cells did not (Osborne et al. 1990).

Analysis of ODC mRNA in oocytes

To evaluate the changes in ODC mRNA during oogenesis, RNA was extracted (as described in Materials and methods) from oocytes at different stages. Northern analysis using a 32P-labelled XLODC1 cDNA probe (Fig. 3A) showed that, relative to total RNA, ODC mRNA decreased during oogenesis. Densitometer quantification (Fig. 3B) of autoradiograms, similar to that shown in Fig. 3A, determined that this decrease in ODC mRNA (relative to total RNA) is what would be expected for a stable transcript that is being diluted during oogenesis due to the increase in total RNA (Davidson, 1986). Between stages II and VI, the total RNA extracted per oocyte increased 11-fold, which is close to the published value of 13.7 (Taylor and Smith, 1985), whilst the amount of ODC mRNA per μg of total RNA decreased about 12-fold. Similar comparable values were obtained for the other stages of oogenesis. The number of ODC transcripts per oocyte was also determined by comparing the autoradiogram signals from fixed amounts of total RNA extracted from oocytes, with those from known amounts of RNA transcribed in vitro from XLODC1 (Fig. 3C). Densitometer quantification of the autoradiogram signals showed that one oocyte equivalent of total RNA contains 1±0.4 ng of ODC mRNA (about 8×10⁸ transcripts). ODC mRNA, therefore, is an abundant mRNA in oocytes and the amount of this transcript per oocyte does not significantly change during oogenesis.

Polysome recruitment

Since the amount of ODC mRNA remains at a constant level during oogenesis, the decrease in ODC accumulation and in enzyme activity that occurs between stages I and IV of oogenesis can only be caused by a regulatory control at a translational or post-translational level. In order to evaluate the contribution of changes in ODC mRNA translation, total RNA from stage I+II and stage IV oocytes was separated into polysomal and non-polysomal fractions by centrifugation through sucrose gradients in the presence of magnesium as described in

![Fig. 3. Northern blot analysis and quantification of ODC mRNA present in oocytes at different stages. (A) 5 μg (lanes a) or 1 μg (lanes b) of total RNA from each stage of oogenesis were analysed by electrophoresis in formaldehyde-agarose gels and transferred to nylon membranes. These membranes were hybridized in stringent conditions with 32P-labelled XLODC1 cDNA and autoradiographed. (B) The relative amount of ODC transcripts per μg of total RNA, normalised to 1 for stage VI oocytes, was calculated from densitometer scans of several autoradiograms similar to that shown in (A) and exposed for different periods of time. (C) 5 μg of total RNA from stage IV and VI oocytes, in vitro transcriptions of XLODC1 (lanes 1–6 respectively 4 ng, 2 ng, 1 ng, 0.5 ng, 0.25 ng and 0.125 ng) supplemented with 5 μg of poly(A)⁺ RNA from eggs and 5 μg of poly(A)⁺ RNA from eggs as a control (lane 7), were subjected to Northern analysis as described in (A). Densitometry of the autoradiograms allowed the number of ODC transcripts per oocyte equivalent of total RNA to be calculated (see text).]
Materials and methods. Northern analysis of the ODC mRNA contained in these fractions (Fig. 4) showed that for the extracts from stage I+II oocytes this mRNA is present in both the fast-sedimenting polysomal and the non-polysomal fractions. For stage IV oocytes, however, ODC mRNA was only present in the fast-sedimenting polysomal fraction. Densitometer quantification of the autoradiograms confirmed that the total amount of ODC mRNA recovered from these magnesium-containing gradients (P+S for stage I+II; P for stage IV) did not vary by greater than 10%. Therefore, the lack of ODC mRNA in the non-polysomal fraction from stage IV oocytes is not due to a preferential loss of these mRNAs during the fractionation procedure.

*Xenopus* oocytes contain untranslated mRNP complexes which, in addition to certain phosphoproteins also include a ribosome and have a sedimentation coefficient of 80–120 S (Cummings and Sommerville, 1988). Therefore, these stored mRNAs may also be present in the polysomal fraction of sucrose gradients and confuse the interpretation of the results. To distinguish polysomal ODC mRNA from that in stored mRNPs, the effect of a prior incubation of the oocytes in the presence of puromycin on this fractionation was analysed. Puromycin treatment of oocytes causes mRNAs that are being translated to move from the polysomal fraction to the non-polysomal fraction of sucrose gradients, whereas the sedimentation of mRNAs in stored mRNP particles is unaffected (Cummings and Sommerville, 1988).

Northern analysis of the different fractions (Fig. 4) showed that the amount of ODC mRNA in the non-polysomal fractions from stage I+II oocytes increased after puromycin treatment, whereas, the distribution of ODC mRNA in stage IV extracts was unaffected by the prior treatment of the oocytes with this drug. Protein synthesis was inhibited by puromycin to an equal extent in stage I+II and stage IV oocytes (data not shown). That the puromycin treatment of the oocytes did not affect the total amount of ODC mRNA recovered from the different gradients was verified by densitometer quantification of the autoradiograms. These measurements also determined that the puromycin treatment caused a 35% decrease in the ODC mRNA present in the polysomal fraction from stage I+II oocytes. This decrease was accompanied by a quantitatively equivalent increase in the non-polysomal fraction from these cells. This corresponds to an exchange of about 20% of total ODC mRNA between the polysomal and non-polysomal fractions from stage I+II oocytes. As a control, oocytes (puromycin-treated and untreated) were disrupted in the presence of EDTA and the extracts centrifuged in EDTA-containing gradients. For these conditions and all the batches of oocytes, ODC mRNA was only found in the non-polysomal fraction.

**Discussion**

The results presented in this paper show that the previously described decrease in ODC enzyme activity, which occurs principally between stage II and IV of oogenesis, is at least partially due to a change in the polysome recruitment of this mRNA. Immunoprecipitation of *in vivo* labelled proteins showed that the accumulation of ODC relative to that of total protein is greatly reduced between stages I+II and stage IV of oogenesis. Quantitative Northern analysis showed that ODC mRNA is already abundant in stage I oocytes and does not change significantly in amount throughout oogenesis; each oocyte contains about 8×10^8 ODC transcripts. Further evidence that the expression of ODC was controlled at a translational level was obtained by polysome analysis of ODC mRNA extracted from stage I+II and stage IV oocytes. These experiments demonstrated that at least 20% of this mRNA is actively translated in stage I+II oocytes but that in stage IV oocytes it is only detected in puromycin-insensitive mRNP particles.

The results presented here show, therefore, that ODC expression is regulated at a translational level in growing oocytes; this mRNA is actively translated in stage I+II oocytes but in stage IV oocytes it has moved to a pool of untranslated mRNP particles. This leads to a decrease in the accumulation of ODC and, therefore, the enzyme activity, during the growth phase of oogenesis. At present we cannot exclude that a change in protein stability leading to a more rapid turnover of the protein also occurs.
During maturation, the recruitment and the release of certain mRNAs from polysomes is correlated with a change in their polyadenylation (Galili et al. 1988; Hyman and Wormington, 1988; McGrew et al. 1989). However, we have observed that the polyadenylation status of ODC mRNA does not change during oogenesis (F. Omillii and H. B. Osborne, unpublished results). This suggests that release from polysomes and targeting to mRNPs ‘storage’ particles are regulated differently.

Comparison of the level of regulation of ODC described here with that of the $56 \times 10^3 M_r$ poly(A) RNA-binding protein (Lorenz and Richter, 1985), which also decreases in amount during oogenesis (see Introduction), shows similarities but also a significant and interesting difference. The translation of both of these mRNAs decreases during oogenesis. However, the amount of ODC mRNA per oocyte is stable during oogenesis while that encoding the $56 \times 10^3 M_r$ poly(A) RNA-binding protein decreases. This difference can be correlated with the patterns of expression of these two gene products during oogenesis and early embryogenesis. The $56 \times 10^3 M_r$ RNA-binding protein is encoded by an oocyte-specific mRNA; this transcript was not detected in either embryos or somatic cells (Lorenz and Richter, 1985). Therefore conservation of the maternal mRNA is not required. The situation is different however, for ODC. Although this enzyme activity decreases during the early growth phase of oogenesis (Osborne et al. 1989), it increases during the maturation and/or ovulation of fully grown oocytes (Younglai et al. 1980; Sunkara et al. 1981). This maturation-associated increase in ODC activity does not require gene transcription (Younglai et al. 1980). In addition, ODC activity increases again after fertilisation (Osborne et al. 1990). Therefore, growing oocytes must retain a store of ODC mRNA to allow synthesis of this enzyme during maturation and early development, which proceed in the absence of transcription.

A question that is now pertinent to ask is what are the characteristics of ODC and/or the $56 \times 10^3 M_r$ poly(A) RNA-binding protein mRNAs that target them for decreased translation during oogenesis? The identification of sequence motifs responsible for this particular mode of translational control during the growth phase of oogenesis can be addressed by injecting chimeric mRNAs containing different parts of these Xenopus mRNAs into Xenopus oocytes cultured in vitro.

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