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Xenopus laevis oocyte Gα subunits mRNAs

Detection and quantitation during oogenesis and early embryogenesis by competitive reverse PCR

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The expression of mRNAs coding for different *Xenopus laevis* oocyte Gα subunits was analyzed by the PCR technique. Using the nucleotide sequences of five previously cloned cDNAs for oocyte Gα subunits [FEBS Lett. 244, 188–192, 1989; FEBS Lett. 268, 27–31, 1990] and the highly sensitive reverse PCR reaction we found that Gαo, Gαi-1, Gαi-3 and Gαs species are present in oocyte stage VI, Gαo mRNA being the most abundant transcript. Gαo mRNA was further quantitated through oogenesis, unfertilized eggs and early embryogenesis stages by a competitive PCR reaction using an 'in vitro' deleted Gαo mRNA as the internal standard. Using this approach we found that *Xenopus* Gαo mRNA levels were constant during oogenesis and unfertilized eggs at a concentration of 3.5 pg of mRNA/stage (5×10^5 molecules) and diminish gradually during early embryogenesis, reaching a level of 0.3 pg in the gastrula stage. These findings show that oocyte Gαo, and perhaps the rest of the α subunits, are expressed as maternal mRNAs and could play an important role in signal transduction at the beginning of oocyte cell differentiation.

G-protein; α subunit; Signal transduction; Oocyte; *Xenopus laevis*; PCR

1. INTRODUCTION

The first embryologic studies on amphibian oocytes, especially in *Xenopus laevis*, were mainly concentrated on the analysis of morphologic changes that take place during growth, maturation, fertilization and embryogenesis of the oocyte [1–3]. Later *Xenopus laevis* oocytes were used to study the mechanisms that govern cell division and gene expression [4–9]. From these studies it was found that maturation and fertilization can be initiated by the action of steroid hormones (progesterone), muscarinic ligands (acetylcholine), insulin and growth factors [10–15], and that the initial responses of oocytes to these molecules are modulated by different signal transduction systems [14].

Today it is known that *Xenopus laevis* oocytes contain at least two well-characterized signal transduction systems regulated by G-proteins: adenylyl cyclase and phospholipase C [16–26]. Both enzymes are present in the plasma membrane of the oocyte and are sensitive to different ligands.

Adenylyl cyclase can be inhibited by progesterone by an unusual mechanism that is responsible for the induction of the meiotic maturation process [16,17]. The inhibitory effect of progesterone is GTP-dependent, requires the participation of a G-protein, but this inhibition is not sensitive to the action of *Pertussis* toxin, which generally dampens the effect of Gi-mediated inhibition [18–21]. Oocyte adenylyl cyclase can also be activated by different Gs stimulators such as Gpp(NH)p, aluminium fluoride and *Cholera* toxin [16] and all these effects can be inhibited by progesterone through a mechanism in which the steroid hormone could be interfering with the activation of Gs [20,22]. It is of interest to point out that the inhibitory effect of progesterone only takes place in oocytes stage III [24].

Acetylcholine potentiates the action of progesterone, accelerating the maturation process. The muscarinic ligand increases the cytosolic levels of inositol 1,4,5-trisphosphate (IP_3) through the activation of phospholipase C by a not well characterized G-protein [26]. IP_3 causes release of cytosolic Ca^{2+} from endogenous stores and finally Ca^{2+} causes the opening of Cl^- channels and membrane depolarization. Recently we have cloned an oocyte muscarinic receptor that could be mediating this response (Olate et al., manuscript in preparation).

All these findings suggested the presence of different G-proteins in *Xenopus* oocytes and recently we have confirmed this by the cloning of five different cDNA encoding for Gαo, Gαi-1, Gαi-3 and two different Gαs, from a stage-VI cDNA oocyte library [27,28]. We have expressed these proteins in different systems and char-

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acterized some properties of these recombinant Gα subunits (J. Olate, unpublished results).

Different groups have shown that during early embryonic development inductive interactions between tissues depend on signal transduction processes. For example, protein kinase C and cAMP signal transductions pathways are involved in mediating neural induction [29–32]. G-proteins are also participating in the early development of higher organisms and complex changes in the temporal and spatial expression patterns of Gα subunits occur during mouse and *Drosophila* development [33,34]. Using antibodies against the Gαo protein, it was shown that the protein was expressed in the embryos of amphibia *Pleurodeles waltl* [35]. In *Xenopus laevis* oocytes, fertilization is a *Pertussis* toxin-insensitive event [36] and important additional clues about G-protein function in early development come from studies in the *Drosophila* fruitfly [37]. Therefore it would not be surprising if G-proteins are playing important roles in embryogenesis.

In this report we expand our research studying the expression of the Gα genes during *Xenopus* oocyte oogenesis, oocyte maturation and early embryogenesis, because differential expression of these mRNAs could lead the oocyte to respond to different ligands through the stages already mentioned in different ways.

2. MATERIALS AND METHODS

2.1. *Radiochemicals and biochemicals*

[α-^{32}P]dATP and [α-^{32}P]dCTP (3,000 Ci/mmol) were from Amersham. Restriction enzymes were from Boehringer Mannheim, Bethesda Research Laboratories (BRL) and Promega Corporation. AMV reverse transcriptase and Taq Polymerase were from BRL. RQ1 DNase, RNasin and pGEM-3 vector were from Promega. Calf intestinal phosphatase (CIP) and T4 DNA ligase were from Stratagene. Other general reagents were from Sigma, Difco and Fluka companies.

2.2. *Construction of the pGEM-3/GαoΔ129 vector*

The cDNA coding for *Xenopus* oocyte Gαo [27] was subcloned in the vector pGEM-3 and the recombinant plasmid, containing the Gαo cDNA in the right orientation with respect to the T7 RNA promoter, was digested with *Acc*I to eliminate a 3′ terminal fragment containing an undesired *Xba*I site. The purified linear form of the plasmid was religated and further digested with *Xba*I and *Hpa*I to create a deletion of 129 base pair fragment within the 3′ non-coding region. The resultant purified linear form of the plasmid was subjected to 3′ end filling in with Klenow fragment and religated to finally give the pGEM3/GαoΔ129 vector. This vector was used as template to produce the deleted form of the Gαo mRNA by an in vitro transcription reaction and used during the quantitation analysis.

2.3. *Primers used in the reverse PCR reaction*

Primers were all 21- and 22-mer, contained a G+C content between 50 and 60%, lacked 3′ complementarity and amplify 3′ non-coding regions of Gα mRNAs, except for the two Gαs clones. The following pair of primers were used to amplify each mRNA: Gαo, 5′-ACACTGCATGTGCCAACACGC-3′ and 5′-ATCCACCTTCCAGCAGGGTAC-′; Gαi-1, 5′-GATTCTTGATGAACAGCGGACC-3′ and 5′-CAGAATAAGGCAGCATGCATGC-3′; Gαi-3, 5′-ATAAGCCACGACTGGTTGGCG-3′ and 5′-AGACAGGAAAGCATGCAGAGC-3′; Gαs(12B2), 5′-ACACCAACAGACTCCAGGAAG-3′ and 5′-TCTGTGTCCACTGCACATGTG-3′; Gαs(6A1), 5′-TCTGCTGCTCGGTGCTGGAGA-3′ and 5′-GCTGGTACTCGTTGGATCGCT-3′. Underlined oligos correspond to the antisense primers used in the synthesis of cDNA. Fig. 1 shows in detail the regions to be amplified by each pair of primers for the different oocyte Gα mRNAs.

2.4. *Isolation of different oocyte and embryo stages*

Adult *Xenopus laevis* females were anesthetized by hypothermia and the ovary was removed surgically. Defolliculated oocytes were obtained from collagenase-treated ovaries as described [18] and separated into the different stages under microscopy. Embryos from various stages were harvested using morphologic criteria as described by Nieuwkoop and Faber [1] and frozen at −80°C until used.

2.5. *Obtention of total RNA*

Total RNA was extracted from defolliculated oocytes and embryos by using the method described by Sambrook et al. [38]. RNAs were stored at −80°C in 100% ethanol until their use.

2.6. *In vitro synthesis of GαoΔ129 mRNA*

Vector pGEM-3/GαoΔ129 was digested with *Hin*dIII and 10 μg of the linear DNA template subjected to an in vitro transcription reaction that contained 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 5 mM NaCl, 10 mM DTT, 100 μg/ml of BSA, 500 μM of each rNTPs, 500 U/ml of RNasin and 500 U/ml of T7 RNA polymerase. The mixture was incubated 1 h at 37°C and the remanent DNA plasmid template was digested by the addition of 10 units of DNase Q for 15 min at 37°C. mRNA was extracted, precipitated and finally stored at −80°C in 100% ethanol at a concentration of 1 μg/ml.

2.7. *Reverse PCR reaction*

(a) First step. The synthesis of the cDNA was done in a volume of 20 μl containing variable amounts of total RNA (see figure legends) from different oocyte and embryogenesis stages, 500 μM of each cold dNTPs, 1 μM of the antisense primer, 1 × PCR buffer (50 mM Tris-HCl pH 8.2, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.001% gelatin), 1 U of RNasin and 0.2 U of reverse transcriptase. The mixture was incubated 1 h at 37°C and then the reaction was stopped by incubation at 100°C during 3 min.

(b) Second step. The PCR reaction was done in a volume of 100 μl containing 20 μl of the reverse transcription reaction (first step) and 80 μl of a PCR master mix (1 × PCR buffer, 200 μM of each dNTPs, 5 μCi of [α-^{32}P]dCTP and [α-^{32}P]dATP, 0.2 μM of antisense and sense primers and 0.2 U Taq polymerase) and 50 μl of mineral oil. Samples were amplified by 25 cycles at 93°C for 30 s, 72°C for 1 min, and 55°C for 30 s. Aliquots of 10 μl of each reaction were subjected to electrophoresis on 2% Nusieve–1% Agarose gels. For autoradiography gels were dried and exposed for different periods of time with Kodak X-Omat AR films.

2.8. *Quantitative reverse PCR*

Quantitation was done according to Gilliland et al. [39] with some modifications. Normally, 2 μg total RNA from different oocyte and embryo stages, were mixed with increasing quantities of the deleted GαoΔ129 internal standard mRNA and both species of RNA were amplified as described previously. The quantitation was done through a densitometric analysis of autoradiographic films and the intensities of the bands were graphically plotted to obtain the respective calibration curves [39].

3. RESULTS

3.1. *Detection of Gα mRNAs in stage VI oocytes by the reverse PCR reaction*

Fig. 2 shows the PCR products obtained when 5 μg of total RNA from stage VI oocytes were amplified with

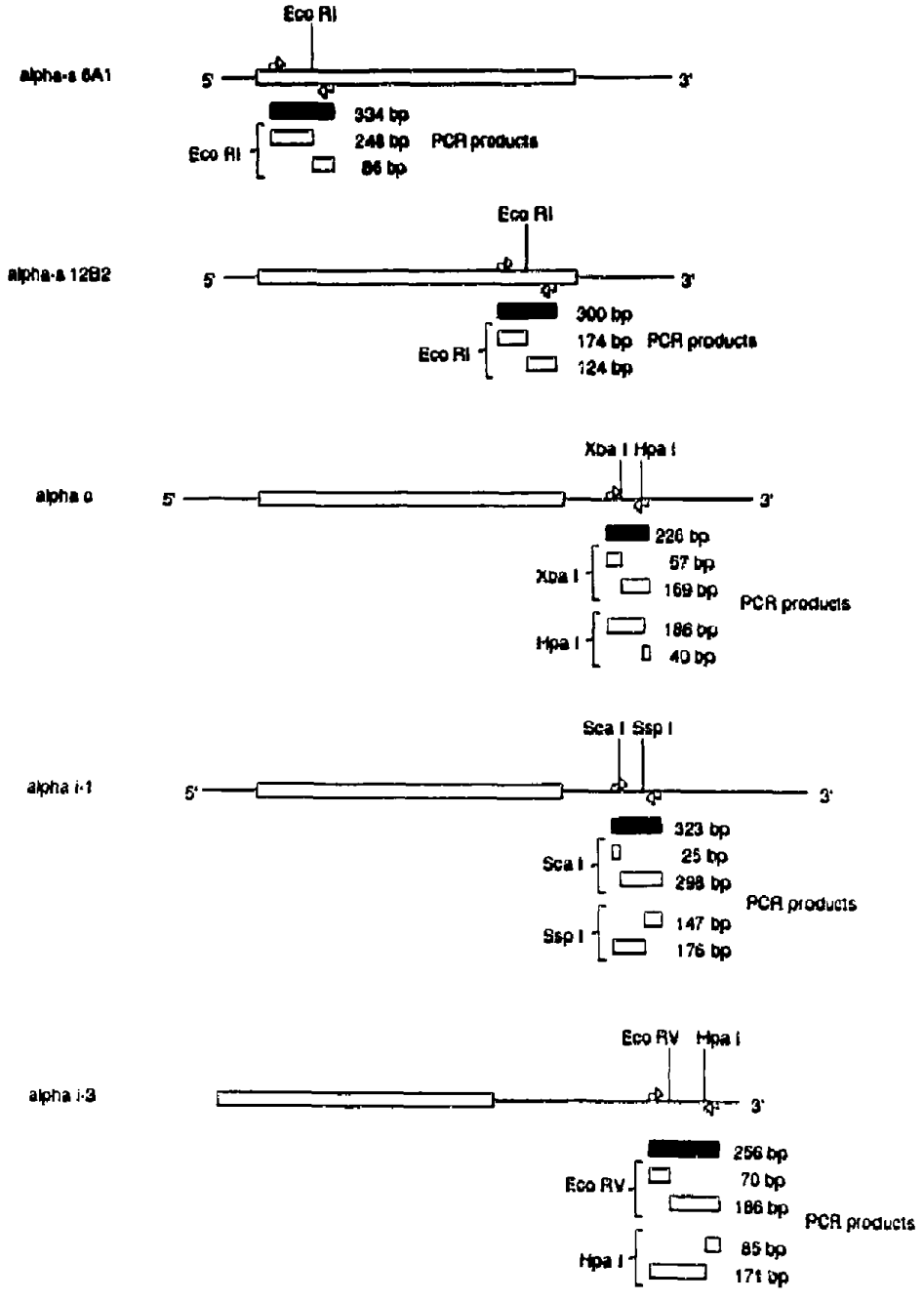

Fig. 1. Structure of *Xenopus* oocyte mRNAs and DNA regions to be amplified by the reverse PCR reaction. Coding regions are represented by empty large boxes. 5'- and 3'-non-coding regions are represented by thin lines. Regions amplified are flanked and delimited by a pair of arrows which represent the sense (⟩) and antisense (⟨) primers. The sizes for the main PCR products and restriction fragments are indicated below each amplified region.

the respective pair of primers for each Gα subunit and analyzed by ethidium bromide staining (part A) and autoradiography analysis (part B). All five Gα transcripts could be detected and the size of the PCR products correspond to the predicted sizes shown in Fig. 1, indicating their presence in mature oocytes. Another important observation from these results is the fact that the quantity is different for each of them, Gαo being the most abundant species and Gαs(6A1) the less abundant species.

In order to demonstrate that each PCR product corresponds specifically to each amplified Gα mRNA, the labelled PCR products were digested with restriction enzymes that cut once within each fragment and the digestion products analyzed through agarose gels. Fig. 3 shows that each PCR product contains the expected restriction site and the digestion products the expected sizes shown in Fig. 1, confirming the identity of the amplified mRNAs. Both Gαs and Gαo PCR products were in some degree resistant to the action of *Eco*RI and

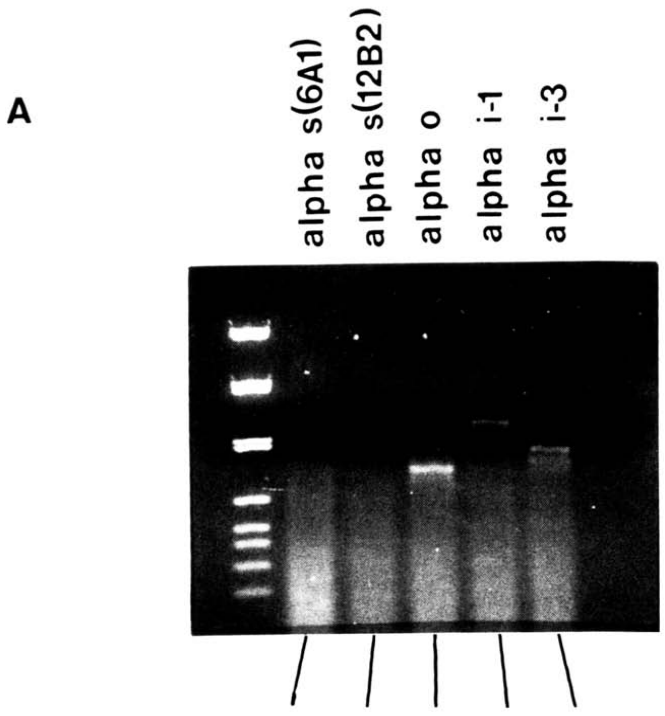

Fig. 2. PCR detection of Gα mRNAs. 5 μg total RNA from stage-VI oocytes were subjected to PCR amplification with the corresponding pair of primers. PCR products were analysed by agarose gels and ethidium bromide staining (part A) or autoradiography of labelled products (part B).

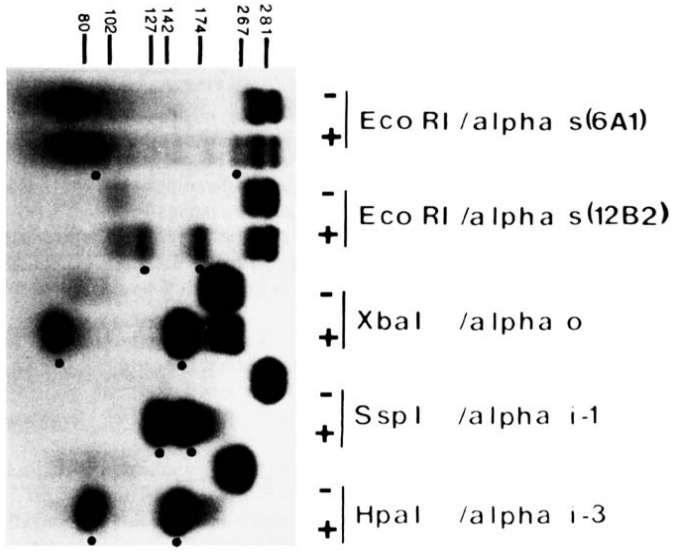

Fig. 3. Restriction enzyme digestion of Gα PCR products. 10 μl of the PCR reaction mixture was subjected to digestion with the indicated restriction enzymes and the digested products analyzed by agarose gels and autoradiography. Dots indicate the migration of each digested DNA fragment.

this can be due to the production of single-strand species by asymmetric amplification.

The sensitivity of the method was determined amplifying Gαo mRNA from decreasing amounts of total RNA. As shown in Fig. 4, Gαo mRNA can be detected when as little as 500 ng of total RNA is used (part A) and this level can be lowered to 6 ng when the analysis is done using labelled PCR products and subsequent autoradiography (part B). This represents an advantage over other RNA detection methods, like Northern and dot blot analysis, which normally need much more total RNA and much longer exposure times.

3.2. *Quantitation of Gαo mRNA through oogenesis and early embryo stages*

Based on the facts that Gαo has been proposed to participate in the transduction of muscarinic signals and initiate different events in the oocyte [40] and that it is the most abundant transcript in mature oocytes, we decided to quantitate Gαo transcripts in all oocyte stages (I–VI), eggs and earlier embryo steps.

We used the highly sensitive quantitative reverse PCR reaction in which a known amount of the deleted *Xenopus* Gαo mRNA is used as an internal standard that can be amplified equally well as the endogenous Gαo mRNA in the same tube with the same primers, but the PCR products of which could be distinguished by their different size. Fig. 5 shows the quantitation curves done for stage I to stage VI oocyte total RNA (part A) and the corresponding plots of the ratio of the signal intensities of the Gαoδ129 internal standard/*Xenopus* Gαo as a function of the amount of deleted internal standard mRNA (part B). We determined that *Xenopus* Gαo mRNA is present in all oocyte stages analyzed, at concentrations that varied between 9 and 1 attomol of Gαo transcript per 2 μg of total RNA. These values were adjusted and expressed as function of total RNA contents per oocyte stage [41]. Table I shows that this amount was constant through all oocyte stages and moves between 3.2 and 3.75 pg of Gαo mRNA/oocyte, corresponding to about 500,000 mRNA copies. Using the same approach we have done the quantitation during maturation and early embryogenesis and we found that Gαo mRNA levels did not vary during maturation but decrease gradually during the early embryo stages analyzed, reaching a level of 0.68 pg at the blastula stage (Table I).

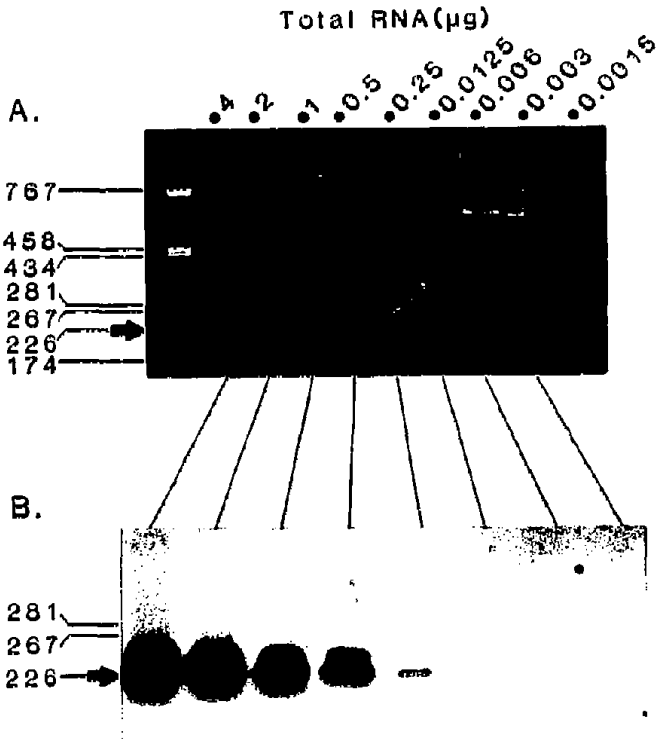

Fig. 4. Gαo mRNA detection from different amounts of total RNA. Different amounts of total RNA from stage-VI oocytes were subjected to PCR amplification with the pair of primers for Gαo. PCR products were analyzed by agarose gels and ethidium bromide staining (part A) or autoradiography of labeled products (part B). The black arrow indicates the size of the Gαo PCR product (226 base pairs).

4. DISCUSSION

The function of Gαo in oocyte maturation and early embryogenesis in *Xenopus* is unclear. Acetylcholine administered to *Xenopus* oocytes stimulates the maturation process triggered by progesterone, but the muscarinic ligand by itself does not have any effect. This phenomenon is clearly pointing in the direction of some kind of crosstalk between cAMP and the phosphatidylinositol pathways. We recently identified cDNAs encoding a Gαo subunit and a receptor for acetylcholine (Olate et al., manuscript in preparation) [27]. These

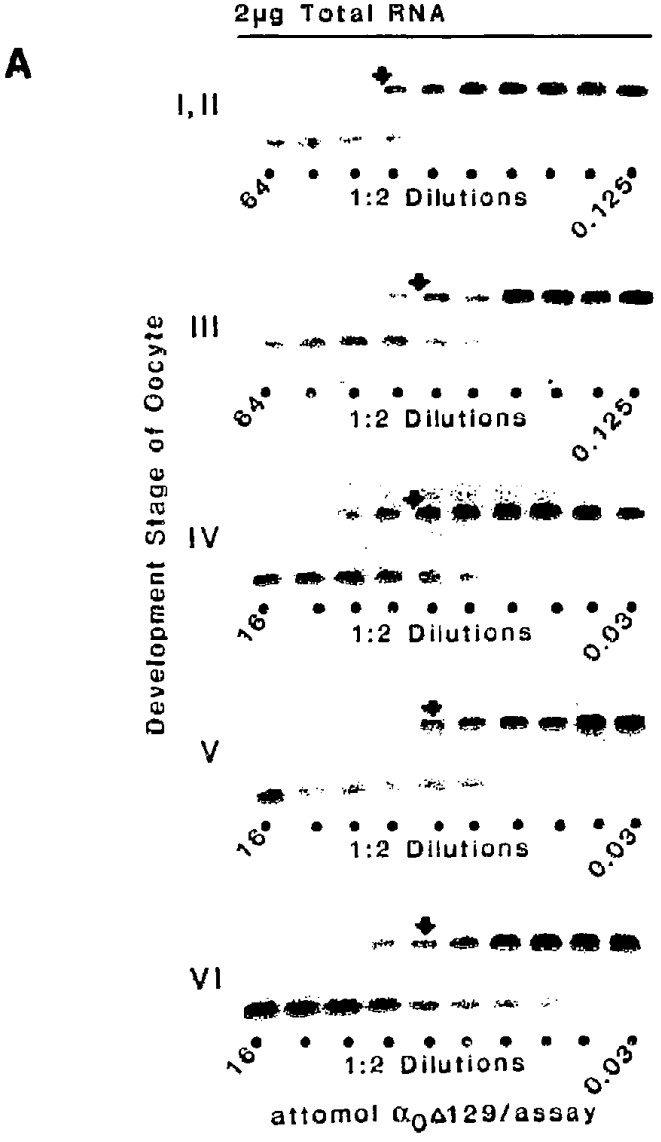

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Fig. 5. Quantitation of mRNA of *Xenopus* Gαo during oogenesis. Part A shows when serial dilutions of the internal standard *Xenopus* Gαo mRNA and 2 μg of total RNA from different oocyte stages were subjected to the reverse PCR amplification reaction. 10 μl of each reaction was analyzed by agarose gels and the labeled PCR products visualized by autoradiography. Black arrows indicate the equivalence point between both PCR products (226 and 97 base pairs). Part B shows the respective plots corresponding to each quantitation and the broken lines indicate the amount of mRNA present in each stage extrapolated from ratios 1:1.

Table I

Gαo mRNA quantitation during oogenesis, maturation and early embryogenesis

Stage	pg Gαo mRNA/stage
Oocyte	
I–II	3.75
III	3.61
IV	3.80
V–VI	3.20
Maturation	3.28
Embryo	
4 cells	3.11
8 cells	3.10
16 cells	2.23
32 cells	2.70
Morula	0.93
Blastula	0.68

findings have confirmed previous electrophysiologic studies showing that muscarinic receptors are present in developing oocytes and that phospholipase C is the enzyme probably regulated by Gαo in response to acetylcholine [42,43]. All these suggest a crucial role for Gαo during these early stages of development.

In this work we started to study the expression of different Gα subunits during early development of *Xenopus* oocytes and especially we focused our attention on Gαo. The finding that all Gα transcripts are present in stage V–VI oocytes indicate that they are transcribed very early during oogenesis, maintained through the rest of oocyte stages and in hormone-matured oocytes. Using the highly sensitive reverse PCR technique we have shown that Gαo mRNA was maternally expressed and that a *Xenopus* oocyte contains about 5×10^5 copies. It has been shown, also in *Xenopus* oocytes, that important genes like the protein factors insulin, TGFB, PDGF, bFGF, and myoD, and proto-oncogenes like c-ras, c-myc, c-mos, c-ets-2, c-rel and raf [13,44–53] are expressed in a similar way and it is known that the expression of these genes is necessary for normal embryo development.

Localized maternal RNAs play important roles during early embryogenesis and their protein products might turn on signals on just some regions of the oocyte or the embryo. Recently Otte et al. [54] have shown that overexpression of *Xenopus* Gαo inhibits the normal disappearance of the blastocoel during gastrulation, suggesting a role for this protein in regulating this process through the phosphatidyl inositol cascade.

The work we have reported here represents the first simultaneous analysis of the expression of different Gα subunits in a single germ-line type of cell, making it possible to further study the role of each of these Gα subunits in early embryogenesis.

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