Proopiomelanocortin Gene Expression in the Ovary of the Frog, *Rana esculenta*[a]

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The presence of proopionmelanocortin (POMC)-like mRNA has been demonstrated in a variety of extrapituitary tissues including hypothalamus,[1] placenta,[2] ovary,[2] and testis.[3] In amphibians, the POMC gene is actively expressed in the pituitary, both in melanotrope cells of the pars intermedia and in corticotrope cells of the pars distalis.[4–6] POMC gene expression in peripheral organs has also been investigated in *Rana esculenta*,[7] indicating that POMC is actually synthetized in the ovary. Previous studies have shown that POMC-derived peptides are involved in local control of ovarian function and display seasonal changes.[8,9] The aim of the present work was to develop a competitive reverse transcriptase polymerase chain reaction (RT-PCR) method using a synthetic, deletion mutant of POMC cRNA as an internal standard in order to quantify the amount of POMC mRNA in the ovary of *Rana esculenta*.

METHODS

Total RNA was extracted, using Trizol RNA isolation reagent, from 5 g pooled frog ovaries collected during the postreproductive period. Final RNA concentration was determined by optical density reading at 260 nm, and integrity was verified by ethidium bromide staining of 28S and 18S ribosomal bands on a denaturing agarose gel.

POMC primers were designed based on the sequence of POMC cDNA from *Rana ridibunda.* The upstream primer (sense), 5′CCGAAGGAACAGCACCAGC 3′(19mer) was located in the γ-melanophore-stimulating hormone (γ-MSH) region and the downstream primer (antisense), 5′TCCTCCATACCTCTTGTC 3′(18mer) in the β-MSH/β-EP region. Primers were designed such that the predicted size of the PCR product was 436 bp for mutant POMC. Competitive cRNA template (7.5×10^5 molecules) was co-reverse transcribed with a serial dilution of total RNA (0.5 up to 3 μg) by 100 U M-MLV reverse transriptase. RT reactions were carried out at 37°C for 90 min followed by 10 min at 95°C. An aliquot (1 μl) of the resulting cDNA products was subsequently co-amplified with 2.5 U Taq DNA polymerase in 50 μl

[a]This work was supported by grants from MURST 60%, 40%, and CNR.

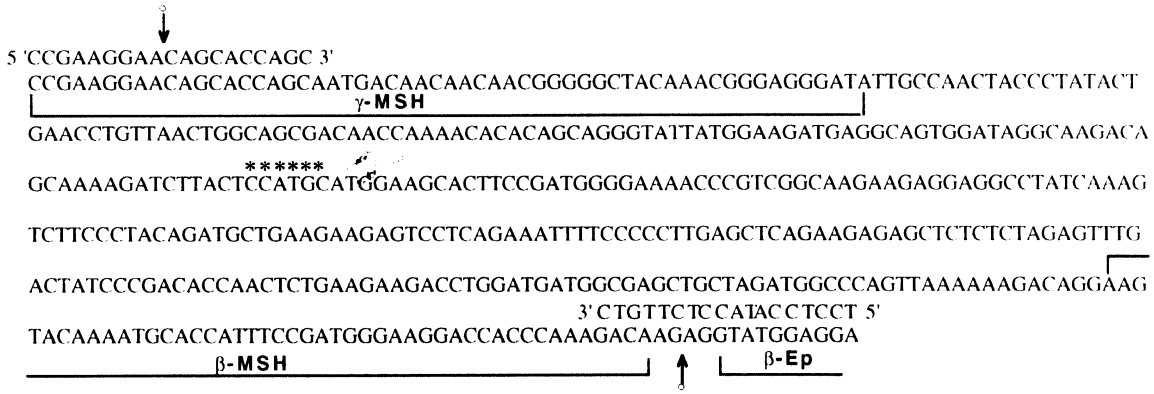

FIGURE 1. Deletion mutant of POMC cDNA. *Arrows* indicate the POMC primers and *asterisks* (*) the mutated Nco I restriction site.

master mix containing: 1 × PCR buffer, 1.5 mM $MgCl_2$, dNTP 2.5 mM and POMC primers (50 pmol each). PCR amplification was carried out for 35 cycles (94°C for 40 sec, 55°C for 40 sec, 72°C for 1 min) followed by a post-PCR incubation at 72°C for 7 min. Restriction enzyme analysis of PCR products was carried out in 50 µl reaction mixture containing: 1 × Nco I buffer and 1U Nco I for 1 hr at 37°C.

Southern blot analysis was performed using the downward alkaline capillary transfer[10] on Hybond-N+ membrane, with the Turboblotter system (Scheicher & Schuell). The filter was hybridized overnight at 68°C, with a 'random primed' digoxigenin-11-deoxyuridine triphosphate (DIG-11-dUTP)-labeled POMC cDNA. Hybridization and detection were performed using a DIG DNA labeling and detection kit (Boehringer, Mannheim).

The *Rana ridibunda* POMC cDNA-inserted pGEM-3Zf vector was used. Computer analysis indicates that POMC cDNA contains a unique single site of Nco I (478 bp), so the plasmid for the preparation of synthetic internal control RNA (cRNA) was constructed by deletion of the Nco I restriction site. The deletion mutant of POMC cDNA was subsequently sequenced (FIG. 1). The plasmid containing deletion mutated POMC cDNA was linearized and transcribed by T7 RNA polymerase to generate internal cRNA. The cRNA was purified as described by Gurevich *et al.*[11] The absolute number of cRNA molecules was calculated using spectrophotometric absorbance at 260 nm, the molecular weight of the cRNA molecule (398310 g/mol) and Avogadro's number. The cRNA solution was diluted to 7.5×10^5 molecules/µl, and stored at −80°C

Sequencing. The plasmid containing deletion mutant POMC cDNA was purified using Qiaprep plasmid kits (Qiagen), and DNA sequence analysis was performed in the ABIPRISM 310 Genetic Analyzer (Perkin Elmer), by dye-labeled terminators using a DNA sequencing kit (Perkin Elmer). The nucleotide sequence was read in both directions.

RESULTS AND DISCUSSION

Quantification of gene expression requires that an accurate measurement of a specific transcript be made, and RT/PCR can be used for this purpose, mostly when the target gene expression is very low.[12,13] Our competitive PCR used a synthetic deletion mutant cRNA as an internal standard that is co-reverse transcribed together with the target POMC mRNA. Since the same primer set was used in the PCR amplification on both templates, a possible difference in the primer efficiency is minimized. Restriction enzyme analysis allows discrimination between DNA derived from endogenous target mRNA and that from internal standard cRNA. In a dilution series containing added mRNA, the resultant PCR products show progressively more endogenous DNA fragments and fewer exogenous ones. Only one aliquot contains equal or nearly equal starting amounts of the corresponding RNA species (FIG. 2). The known amount of *in vitro* transcript added to this sample can be taken as equivalent to the amount of specific target mRNA present. In the RT/PCR of cRNA and total RNA from *Rana esculenta* ovary, three different fragments corresponding to cRNA (436 bp) and POMC mRNA (287 bp and 149 bp) were found. The amount of PCR product derived from endogenous RNA corresponds to 8.3×10^5

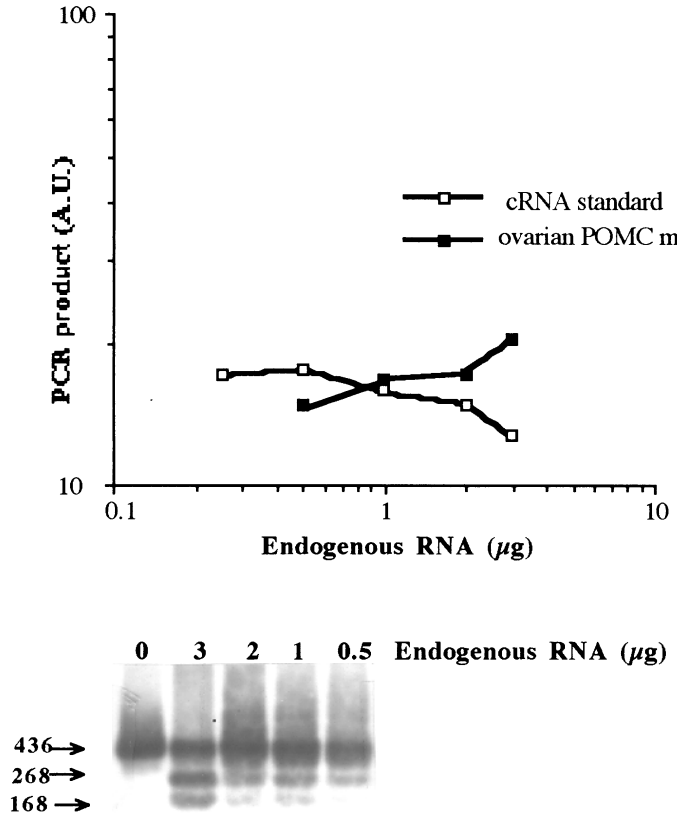

FIGURE 2. Quantification using chemiDNA probe. PCR reactions were performed in duplicate. cDNA was synthesized from a mixture containing 7.5×10^5 standard molecules indicated, and a serial dilution of total RNA from *Rana esculenta* ovary. Following 35 cycles of PCR and restriction enzyme analysis, the sample was run on a 2% agarose gel. The gel was processed for Southern blot analysis. The chemiluminescent signal was quantified by densitometric analysis. Each point represents the average of duplicate reactions.

molecules/μg of total RNA (FIG. 2). This technique allows low contents of POMC mRNA to be determined; therefore, the role of ovarian POMC in the reproductive functions of the European green frog can be clarified.

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