Cloning, ontogenesis, and localization of an atypical uncoupling protein 4 in *Xenopus laevis*

Patrick A. Keller,1 Lorenz Lehr,1 Jean-Paul Giacobino,1 Yves Charnay,2 Françoise Assimacopoulos-Jeannet,1 and Natalia Giovannini1

1Department of Cell Physiology and Metabolism, University Medical Center 1, Geneva; and 2Division of Neuropsychiatry, Belle-Idée, Geneva University Hospital, Chene-Bourg, Switzerland

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Keller, Patrick A., Lorenz Lehr, Jean-Paul Giacobino, Yves Charnay, Françoise Assimacopoulos-Jeannet, and Natalia Giovannini. Cloning, ontogenesis, and localization of an atypical uncoupling protein 4 in *Xenopus laevis*. Physiol Genomics 22: 339–345, 2005. First published May 24, 2005; 10.1152/physiolgenomics.00012.2005.—Uncoupling protein 4 (UCP4) is the first UCP described. It belongs to the family of mitochondrial carrier proteins and is expressed mainly in brown adipose tissue. Recently, the family of the UCPs has rapidly been growing due to the successive cloning of UCP2, UCP3, UCP4, and UCP5, also called brain mitochondrial carrier protein 1. Phylogenetic studies suggest that UCP1/UCP2/UCP3 on one hand and UCP4/UCP5 on the other hand belong to separate subfamilies. In this study, we report the cloning from a frog *Xenopus laevis* (Xl) oocyte cDNA library of a novel UCP that was shown, by sequence homology, to belong to the family of ancestral UCP4. This cloning provides a milestone in the gap between *Drosophila melanogaster* or *Caenorhabditis elegans* on one hand and mammalian UCP4 on the other. XI UCP4 is already expressed in the oocyte, being the first UCP described in germ cell lineage. During development, it segregates in the neural cord, and, in the adult, in situ hybridization shows its expression in the neurons and also in the choroid plexus of the brain. By RT-PCR analysis, it was found that XI UCP4 is present in all the subdivisions of the brain and also that it differs from mammalian UCP4 by a very high relative level of expression in peripheral tissues such as the liver and kidney. The peripheral tissue distribution of XI UCP4 reinforces the hypothesis that UCP4 might be the ancestral UCP from which other UCPs diverged from.

UNCOPLING PROTEIN 1 (UCP1) is the first UCP described. It belongs to the family of mitochondrial carrier proteins and is expressed mainly in brown adipose tissue. UCP1 uncouples oxidative phosphorylation by dissipating the proton gradient generated by the activity of the respiratory chain and is considered as the main effector of adaptive thermogenesis in rodents (7).

Recently, the family of the UCPs has rapidly been growing due to the successive cloning of UCP2 (9), UCP3 (6), UCP4 (22), and UCP5 (also called brain mitochondrial carrier protein 1) (32, 40). Sequence alignment and analysis of energy transfer protein signatures suggested that UCP1/UCP2/UCP3 on one hand and UCP4/UCP5 on the other hand belong to separate subfamilies (2, 12). In rodents and humans, UCP2 was found to be expressed in almost all of the tissues studied (9), whereas UCP3 was expressed mostly in skeletal muscle (6). Several other UCPs also belong to this subfamily: carp and zebrafish UCP2, whose tissue distribution and possible role were not studied (35); an avian UCP that shares about 70% homology with mouse UCP2 and UCP3 and is expressed mostly in skeletal muscle (29, 37) but also to a lesser extent in the liver and heart (38); and plant UCP1 and UCP2 (3). Recently, marsupial UCP2 and UCP3 were cloned and found to be expressed ubiquitously for UCP2 and in skeletal muscle for UCP3, thus resembling the expression pattern found in rodents (14).

UCP4 mRNA in mammals is exclusively expressed in the brain (22). UCP5 mRNA in humans and rodents is predominantly expressed in the brain (17, 32, 40), but, in humans, it was found to be also expressed in the testis, kidney, uterus, and heart (40). In rodents, it was found to be expressed in the testis, white adipose tissue, kidney, and heart (15, 40). UCP5 protein in rodents was the highest in the brain, where it was found to be almost exclusively neuronal (15). Recently, an UCP5 homolog has been cloned in *Drosophila melanogaster* and found to be predominantly expressed in the adult head (10). In addition, quantitative RT-PCR analysis has shown that UCP4 and UCP5 are the most abundant of all the UCP isoforms in the mouse brain cortex (20). Therefore, all the data available until now suggest that UCP4 is exclusively and UCP5 essentially expressed in the brain.

What about the biological function of UCPs? UCP2 and UCP3 do not seem to be involved in adaptive thermogenesis in opposition to UCP1 (4, 26, 31). On the other hand, *Solanum tuberosum* plant UCP (18, 21) and avian muscle UCP (29, 37, 38) have been found to be upregulated by cold exposure. Rodent spinal cord UCP2 and human brain UCP4 and UCP5 have also been shown to be upregulated by cold exposure (24, 40). Therefore, some UCPs might play a role in the protection against a cold environment. UCP2 and UCP3, considered as minor players in thermoregulation, have alternatively been proposed to be involved in the prevention of reactive oxygen species (ROS) accumulation (4, 26, 31). This role was also postulated in the brain for UCP2 (13) and UCP5 (15). Finally, UCP3 has been proposed to protect the mitochondria against lipotoxicity by functioning as an exporter of fatty acids outside the mitochondria (34).

Phylogenetically, it was postulated that UCP4 represents an ancestral UCP from which the other UCPs diverged and that UCP1, UCP2, and UCP3 have developed later during evolution. *D. melanogaster* and *Caenorhabditis elegans* are the closest UCP4 analogs described until now (12).

It is still unknown how the ancestral UCP4 evolved into a brain-specific protein. For this reason, the possible existence of

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UCP4 in a species that is intermediary between *D. melanogaster* or *C. elegans* and mammals was examined. The frog, being a poikilotherm, i.e., a vertebrate animal essentially devoid of thermoregulatory needs, seemed an interesting model for this study. We searched expressed sequence tag (EST) databases and found a *Xenopus laevis* (XI) oocyte EST (Accession No. AW147976) coding for a segment of putative UCP4. The present study describes the cloning of the XI oocyte UCP as well as its fate during development and tissue distribution.

**MATERIALS AND METHODS**

**Animals.** All experiments carried out conformed to the guidelines set by the Swiss Federal Veterinary Office. Animal protocols were approved by André Solaro (responsible for lines set by the Swiss Federal Veterinary Office. Animal protocols were approved by André Solaro (responsible for lines set by the Swiss Federal Veterinary Office). Adult brains were carefully removed from the skull and frozen on dry ice for hybridization in situ and real-time PCR studies. Tissue blocks of four different representative regions of the brain (see Fig. 1) were separated and used for real-time PCR studies. Samples of the liver, heart, kidney, and leg skeletal muscle were also collected.

**Cloning of the novel UCP gene.** An incomplete XI potential UCP EST was identified in GenBank (Accession No. AW147976). The cDNA encoding full-length XI UCP4 was cloned by a nested PCR from a XI oocyte cDNA library in pBluescript generously provided by Dr. Nigel Garrett (Wellcome/CRC Institute, Cambridge, UK.). Primers were chosen on the vector and on the incomplete UCP clone. For the first round, the primers were 5'-tcatgctcctgttttcc-3' (insert) and 5'-tacatgtaaaccattctc-3' (vector), and for the second round primers were 5'-tctgcccttttctggtg-3' (insert) and 5'-aagctcatacgcagaaccgccgccg-3' (vector). Three cDNAs stemming from independent PCRs were cloned in PCR 2.1 TOP (Invitrogen; Leek, The Netherlands) and sequenced (Univ. Medical Center Sequencing Unit, Univ. of Geneva, Switzerland). The overlaps were confirmed to match with both each other and with the AW147976 sequence. The consensus cDNA was then cloned in pcDNA 3.1 (Invitrogen) using *Bsi*XI as the restriction site.

**Northern blot analyses.** Total RNA was purified by the method of Chomczynski and Sacchi (8), and 12–20 μg was electrophoresed on a 1.2% agarose gel containing formamide, as described by Lehra et al. (19), and transferred to Electran Nylon Blotting membranes (BDH Laboratory Supplies; Poole, UK) by vacuum blotting. The probe used, obtained by PCR (upstream primer: 5'-gggtgattctcagtcgc-3', downstream primer: 5'-ggcgcctgctgctgctg-3'), had a size of ~600 bp and was controlled by sequencing. It was labeled by random priming with [α-32P]dCTP (Amer sham; Bucks, UK) to a specific radioactivity of ~1 × 106 disintegrations·min⁻¹·μg⁻¹ DNA. Northern blots were performed as previously described (5). Housekeeping mRNAs levels are subject to changes during embryogenesis. They cannot therefore be used as internal references. The β-actin level, for instance, was found to be 3.2-fold higher in the tadpole than in the oocyte (results not shown). For this reason, we present the UCP4 results during development as arbitrary values obtained in the known amount of RNA loaded on the gel. The membrane was colored with methylene blue to check RNAs for degradation. The signals on the autoradiograms were quantified by scanning densitometry using ImageQuant Software version 3.3 (Molecular Dynamics; Sunnyvale, CA).

**Real-time PCR.** Oligo-dT primed first-strand cDNAs were synthesized using the Superscript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was performed using a ABI rapid thermal cycler system and a SYBR green PCR master mix for the quantification of UCP4 according to the manufacturer’s instructions. An assay performed without reverse transcriptase was always included to check for possible contamination of the RNA preparation by genomic DNA. GAPDH was used as a control to account for any variations in the efficiencies of the reverse transcriptase and PCR. The XI UCP4 oligonucleotide primers used were upstream 5'-ggacggccctggtgagc-3' and downstream 5'-gccgctcgtgcgct-3'. This primer pair covered nucleotides 724–790 of the XI UCP4 cDNA (GenBank Accession No. AY166600). All samples were analyzed in duplicate. The specificity of the reaction was shown by cloning of the PCR product in the vector pCR 2.1 TOPO and by sequencing. The GAPDH oligonucleotide primers used were upstream 5'-catcagctctccagcag-3' and downstream 5'-gccttcccacttaaagc-3'. This primer pair covered nucleotides 256–307 of the GAPDH cDNA (GenBank Accession No. X141753).

**In situ hybridization of whole mount embryos.** The PCR fragment described above for the Northern blot analysis was inserted in a TOPO II vector from Invitrogen and subcloned in the EcoRI site of the pBlueScript KSII phage (Stratagene (La Jolla, CA) for RNA digoxigenine (DIG) probe transcription. Plasmid DNA was linearized with BamHI or XhoI for the production of antisense or sense probes with T3 or T7 RNA polymerase, respectively (Promega; Madison, WI). The incorporation of the DIG nucleotides was monitored by spotting on N+ membranes followed by hybridization with an anti-DIG antibody coupled to alkaline phosphatase (AP) and staining with BM purple (Roche; Rothkreus, Switzerland) according to Peng (28). Entire XI embryos were fixed in methanol. The sense and antisense probes for XI UCP4 were hybridized at 42°C to the embryos and were detected with the anti-DIG antibody and BM as described below.

**In situ hybridization of brain slices.** The pCR 2.1 TOPO XI UCP4 plasmid was used as a cDNA template for PCR in a ReadyMix PCR (Sigma). The oligonucleotide primers used were upstream 5'-acccagtcgctctggtcctg-3' and downstream 5'-aacagtgctctcagcgg-3'. This primer pair covered nucleotides 123–460 of the XI UCP4 cDNA. The PCR product was then directly cloned in the plasmid vector pCR-TOPO (Invitrogen). Plasmid DNA was linearized with *Not*I or *Pme*I for the production of antisense and sense probes with T3 or T7 polymerase, respectively (Invitrogen). Labeled riboprobes were obtained by the incorporation of DIG-UTP (Roche Diagnostics) during an in vitro transcription reaction according to the manufacturer’s instructions (Maxscript T7/T3; Ambion). The riboprobes were purified by Sephadex G50 (Quickspin columns, Roche) and checked by agarose-agarose gel electrophoresis with ethidium bromide staining.

XI frogs were deeply anesthetized by immersion in 0.2% 3-aminobenzoic acid ethyl ether methanesulfonate salt before decapitation. The brains were carefully removed, and sagittal and frontal cryostat sections (15 μm) of representative brain regions (from the cortex to the medulla) were collected onto poly-L-lysine-coated glass slides. They were fixed by immersion in a fixative containing 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 15 min at 4°C and washed in 2× standard saline citrate (SSC) acetylated in 0.5% acetic anhydride.
in 0.9% NaCl containing 100 mM triethanolamine (pH 8). Sections were then dehydrated in an ascending series of ethanol washes, delipidated in chloroform, and air dried before hybridization.

Probes were heat denatured (5 min at 65°C) and then placed on ice. They were added to a final concentration of 0.5 μg/ml to the hybridizing buffer containing 1× Denhardt’s solution, 50% formamide, 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 1 mM EDTA, 10% dextran sulfate, 50% formamide, 100 μg/ml salmon testes DNA, and 250 μg/ml yeast tRNA. After hybridization overnight at 60°C, the sections were washed at room temperature (RT) 4× 5 min in 4× SSC, incubated with RNase A (50 μg/ml, Sigma) for 30 min at 37°C, and then washed successively 2× 5 min in 2× SSC, 10 min in 1× SSC, 10 min in 0.5× SSC at RT, 30 min in 0.1× SSC at 50°C, and finally 15 min in 0.1× SSC at RT.

Slides were washed in Tris saline buffer (150 mM Tris-HCl and 100 mM NaCl, pH 7.5) and incubated for 1 h in blocking solution (Roche). They were then incubated for 1 h at 4°C in AP-conjugated anti-DIG antibodies (working dilution 1:100, DAKO Diagnostics). The immunoreactivity was revealed by a dark blue purple staining in the presence of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Dakocytomation).

Phylogenetic analysis. The amino acid sequence of XI UCP4 was aligned with other known UCPs, and the phylogenetic tree (Megaalign of the Lasergene 5.0 sequence analysis program) was constructed using the Jotun Hein method as described by Hanak and Jezek (12).

Statistical analysis. Results are given as means ± SE. For statistical comparison, one-way ANOVA followed by an unpaired Student’s t-test were done on Origin 7.0 (OriginLab).

RESULTS

An EST coding for a segment of a putative UCP4 was found in Genbank, and the full-length cDNA was cloned and sequenced as described in MATERIALS AND METHODS. As shown in Fig. 2A, XI UCP displays structural features of mitochondrial carriers, i.e., six putative transmembrane domains and three mitochondrial energy transfer protein signatures that can be identified at the border and downstream of the first, third, and fifth potential transmembrane domains. It also shares a purine nucleotide-binding domain with other UCPs. XI UCP was
found to display the highest level of amino acid identity with mouse and human UCP4 (70.8 and 70.6%, respectively). The identities of the XI UCP with mouse (m)UCP5, mUCP3, and mUCP2 were 32.6, 31.7, and 29.1%, respectively. The presence of the amino acids (position in brackets) valine (96), serine (98), glutamine (194), alanine (196), proline (298), serine (300), and phenylalanine (303) suggests that the XI UCP belongs to the UCP4 family (12). The phylogenetic analysis of Fig. 2B, which compares XI UCP to other UCPs, shows that XI UCP4 is positioned at the basis of its mammalian orthologs. Despite a high phylogenetic distance between amphibian and mammals, UCP4 orthologs are relatively similar (about 70% amino acid identity). It is noteworthy that at the same phylogenetic distance, UCP2 orthologs are more similar (80% amino acid identity) (14). These differences reveal different evolutionary speeds of the UCP4 and UCP2 families.

What is the fate of UCP4 during development? To answer this question, the UCP4 mRNA level was measured at various stages of XI development from the oocyte to tadpole. As shown in Fig. 3A, UCP4 mRNA is already present in the oocyte. The level of UCP4 mRNA is not modified by fertilization, decreased by 50% in the gastrula stage, and increased 3.3-fold in the tadpole stage. Thus the basal level of expression of UCP4 in the oocyte increases during development. Does UCP4 mRNA expression colocalize with a peculiar structure during development? In situ hybridization of the embryo showed that, at the neurula stage, UCP4 mRNA expression segregates in the neural cord (Fig. 3B).

In the adult XI frog, UCP4 mRNA expression was detected by in situ hybridization in most of the subdivisions of the brain. As illustrated in Fig. 4, the hybridizing signal given by the antisense probe was clearly detected in nerve cells (A, C, and E) and in the choroid plexus (C). No significant signal was observed in tissue sections incubated with the sense probe (Fig. 4, B, D, and F). Considering the size and the morphology of the UCP4-positive nerve cells, it can be assumed that most of them represent neuronal cells.

In a first approach to get a quantification of UCP4 mRNA expression in the brain, four blocks of brain including the cerebral cortex, optical tegmentum, cerebellum, and spinal cord (Fig. 1) were dissected, and UCP4 mRNA was measured in each of them by real-time PCR. UCP4 mRNA was expressed in the four regions and is therefore expressed in the whole brain. UCP4 mRNA was also found in peripheral tissues. Figure 5 shows the distribution of UCP4 mRNA in the brain subdivisions and peripheral tissues tested. The level of expression of UCP4 in the heart has been defined as representing one arbitrary unit. The level of UCP4 mRNA in the cortex

Fig. 3. A: UCP4 mRNA expression during X. laevis development. Levels of XIUCP4 in laid and fertilized oocytes as well as gastrula (stages 11 and 12), neurula (stages 16–18), and tadpole (stages 22 and 23). Total RNA was extracted and Northern blots were performed as described in MATERIALS AND METHODS. A photodensitometric comparison of signals obtained from total RNA hybridized with a 32P-labeled XI UCP4 probe is shown. The results were obtained from two different Northern blots. They were expressed in each blot as percentages of the mean laid oocyte value. The results are expressed as percentages of the laid oocytes values taken as 100%. Values are means ± SE of 4–7 experiments. ***P < 0.005 and **P < 0.002 vs. laid oocytes; °°P < 0.02 vs. gastrula. B: in situ hybridization of neurula (stages 16–18). Antisense (left) and sense (right) probes were hybridized and detected as described in MATERIALS AND METHODS.
is 6.4-fold, in the optical tegmentum 6.3-fold, in the spinal cord 5.2-fold, in the cerebellum 7.2-fold, in the liver 38.1-fold, and in the kidney 45.5-fold that of in the heart. No UCP4 mRNA was detected in skeletal muscle and adipose tissue (data not shown).

To answer the question of a possible thermoregulatory role of Xl UCP4, we studied the effect of an exposure to 4°C during 30 h in Xl oocytes or during 36 h in the adult Xl frog. These treatments did not change UCP4 mRNA expression in oocytes or in the adult Xl brain (data not shown).

Fig. 4. UCP4 mRNA expression in the brain of X. laevis frogs. The antisense digoxigenin (DIG)-labeled probe revealed UCP4 mRNA expressing neurons (dark labeling) in most of the brain subdivisions. A: representative result throughout the thalamic region. C: high hybridization signal in the brain choroid plexus. Note also the hybridization signal in the neurons of the cerebral cortex (arrow). E: superficial layer of the parietal cortex. Note the high density of cortical neurons expressing UCP4. The sense DIG-labeled probe did not reveal any significant labeling in the respective adjacent sections (B, D, and F). Scale bars = 260 μm in A–D and 100 μm in E and F.

Fig. 5. UCP4 mRNA expression in four blocks of brain and in some peripheral tissues of X. laevis frogs. Quantitative real-time PCR determinations were performed as described in MATERIALS AND METHODS. The results are expressed as arbitrary units, with the heart value being considered as 1. Values are means ± SE of 6 samples for each tissue. ***P < 0.001 for peripheral tissues (liver and kidney or heart) vs. brain regions.
**DISCUSSION**

The novel XI UCP that we describe in this study is the first member of the UCP4 family identified in the evolution between *D. melanogaster* or *C. elegans* and mammals (12). The absence of an UCP4 mRNA upregulation in oocytes and the XI brain upon exposure to cold is in contrast with the results obtained in the rodent whole brain, where UCP4 was found to be increased in response to cold exposure (40).

UCP2, UCP3, and UCP5 have been proposed to be involved in the prevention of ROS accumulation (4, 13, 15, 27, 32). It is therefore interesting to discuss the possible role of XI UCP4 in the context of this prevalent hypothesis.

XI UCP4 is the first UCP found to be expressed in germ cell lineage. This finding suggests a new role for UCPS in oocyte maturation and preimplantation embryo development. What could be the role of UCP4 in XI during development? ROS, like H₂O₂, O₂⁻, and OH⁻, are chemical mediators that act on signaling pathways to modulate, among others, growth and differentiation (33). Metabolic gradients are established at very early stages of development that, by inducing the formation of redox and ROS gradients, influence the expression and activity of proteins involved in development (1). Although ROS are necessary during embryogenesis, they can also induce defective embryo development. The internal protection against deleterious ROS accumulation comprises antioxidant enzymes like superoxide dismutase, glutathione peroxidase, and γ-glutamylcysteine synthetase, whose transcripts are already present in oocytes and embryos (11). UCP4 could constitute an additional protection mechanism against potential deleterious molecules like ROS during oocyte maturation and embryogenesis. Thus the variation of XI UCP4 expression during development could be explained by the fact that the embryo may display different sensitivities to ROS at different developmental stages (25). Recently, UCP2 was found to be expressed in mouse ovarian cells and postulated to contribute to minimize the inflammatory response accompanying changes in follicular structure (30). The possible role of UCPS at early stages of development is a field that deserves further study.

UCP4 is localized not only in the neurons, as reported for UCP5 (15), but also in the choroid plexus, and this suggests a protective role against endogenous accumulation in brain vital cells or against penetration into the brain of potential deleterious molecules like ROS. This hypothesis is supported by the recent findings that in the mouse another UCP, UCP2, might act as a cell death-suppressing protein (13) and prevent ischemic neuronal death by decreasing ROS accumulation (23).

The tissue distribution of the XI UCP4 is atypical compared with that of mammalian UCP4 (22, 40). Whereas in mammals UCP4 mRNA is expressed only in the brain, it was also found in XI in other tissues. In adult XI, indeed, very high levels of expression of UCP4 mRNA compared with the brain are observed in the liver and kidney. Our study therefore shows that during phylogenesis, UCP4 disappears from the periphery, where it might be redundant with ubiquitous UCP2, also present in XI (16). It would be interesting to determine whether UCP4 is also expressed in the periphery in invertebrates, like *D. melanogaster* and *C. elegans*.

In conclusion, we showed the existence in XI of UCP4 already present in the oocyte, which is also expressed in the adult animal in the choroid plexus and neurons of the brain as well as in the periphery. The exact function of UCP4 remains to be determined; it might play a role in the protection against ROS accumulation during development and in the adult animal.

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