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# Cloning and functional characterization of an uncoupling protein homolog in hummingbirds

CLAUDIA R. VIANNA,<sup>1</sup> THILO HAGEN,<sup>2</sup> CHEN-YU ZHANG,<sup>2</sup> ERIC BACHMAN,<sup>2</sup> OLIVIER BOSS,<sup>2</sup> BALAZS GEREKEN,<sup>3</sup> ANSELMO S. MORISCOT,<sup>4</sup> BRADFORD B. LOWELL,<sup>2</sup> JOSÉ EDUARDO P. W. BICUDO,<sup>1</sup> AND ANTONIO C. BIANCO<sup>3</sup>

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**Vianna, Claudia R., Thilo Hagen, Chen-Yu Zhang, Eric Bachman, Olivier Boss, Balazs Gereken, Anselmo S. Moriscot, Bradford B. Lowell, José Eduardo P. W. Bicudo, and Antonio C. Bianco.** Cloning and functional characterization of an uncoupling protein homolog in hummingbirds. *Physiol Genomics* 5: 137–145, 2001.—The cDNA of an uncoupling protein (UCP) homolog has been cloned from the swallow-tailed hummingbird, *Eupetomena macroura*. The hummingbird uncoupling protein (HmUCP) cDNA was amplified from pectoral muscle (flight muscle) using RT-PCR and primers for conserved domains of various known UCP homologs. The rapid amplification of cDNA ends (RACE) method was used to complete the cloning of the 5' and 3' ends of the open reading frame. The HmUCP coding region contains 915 nucleotides, and the deduced protein sequence consists of 304 amino acids, being ~72, 70, and 55% identical to human UCP3, UCP2, and UCP1, respectively. The uncoupling activity of this novel protein was characterized in yeast. In this expression system, the 12CA5-tagged HmUCP fusion protein was detected by Western blot in the enriched mitochondrial fraction. Similarly to rat UCP1, HmUCP decreased the mitochondrial membrane potential as measured in whole yeast by uptake of the fluorescent potential-sensitive dye 3',3'-dihexyloxycarbocyanine iodide. The HmUCP mRNA is primarily expressed in skeletal muscle, but high levels can also be detected in heart and liver, as assessed by Northern blot analysis. Lowering the room's temperature to 12–14°C triggered the cycle torpor/rewarming, typical of hummingbirds. Both in the pectoral muscle and heart, HmUCP mRNA levels were 1.5- to 3.4-fold higher during torpor. In conclusion, this is the first report of an UCP homolog in birds. The data indicate that HmUCP has the potential to function as an UCP and could play a thermogenic role during rewarming.

nonshivering thermogenesis; torpor; brown adipose tissue; birds

IN MOST ANIMALS THE ABILITY to sustain and adapt to food deprivation relates directly to the relative size of their

energy depot, mostly fat tissue, and inversely to their mass-specific metabolic rate. A few species, however, have evolved the capacity to reduce the resting metabolic rate during periods of food deprivation, a process known as torpor. This usually lasts a few hours and can occur on a daily basis. In addition, food shortage can be anticipated and adjusted to on an annual basis, by strategies such as in hibernation, a prolonged period of marked reduction in body temperature and metabolic rate (37).

Hummingbirds have one of the highest mass-specific metabolic rates recorded among vertebrates, requiring an abundant supply of energy substrates (43). The limited amount of adipose tissue (our own observation in *Eupetomena macroura*) impairs the upholding of the euthermic metabolic rate under conditions of food deprivation. To circumvent this, hummingbirds may enter torpor on a daily basis to conserve energy and survive through the night (4, 31). By doing so, their metabolic rate may fall to <10% of euthermic values, and body temperature may reach as low as 15°C (31). Subsequently, triggered by various signals, including ambient temperature and luminosity, both metabolic rate and body temperature rise rapidly, constituting the awakening process known as rewarming (31). The cellular mechanisms of rewarming in hummingbirds are poorly understood. Torpid and hibernating mammals rely mostly on nonshivering thermogenesis (NST) in the brown adipose tissue (BAT) for rewarming (42). This is a tissue that is specialized in heat production due to expression of an uncoupling protein 1 (UCP1), a physiological uncoupler of the mitochondrial oxidation from ATP production (26). Bird species studied so far, however, have no distinct BAT or a related thermogenic tissue (25, 39); therefore, shivering has been considered the primary thermogenic mechanism during rewarming or cold exposure (6, 14, 47). Electrical activity in skeletal muscles during cold exposure usually starts before the capacity for NST is exhausted, not excluding the possibility of concurrent NST (23, 40). On the contrary, there is now strong evidence that

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nonsivering mechanisms might also play an important role in birds during cold exposure, as in the case of cold-acclimated ducklings (1, 2). The source of this putative NST in birds has remained elusive, although it is clear that the skeletal muscle and the sarcoplasmic reticulum calcium pump (SERCA) are involved (15, 16). In hummingbirds, the pectoral and supracoracoideus muscles represent ~30% of body mass (3) and mitochondria fill ~35% of the fiber volume, giving these muscle groups a high rate of respiration (44).

The key protein responsible for the regulated loose coupling of BAT mitochondria, UCP1, occurs exclusively in BAT. However, similar UCPs were recently found in various tissues of mammals, such as UCP2, which is ubiquitously expressed (20), and UCP3, primarily expressed in skeletal muscle (8). When expressed in yeast mitochondria or reconstituted into liposomes, both UCP2 and UCP3 catalyze electrophoretic H<sup>+</sup> flux (22). The physiological role of these newly described UCPs is not yet defined, particularly because they have been found in tissues whose primary function is not heat related, e.g., spleen, kidney, and brain.

In hummingbirds, the daily pattern of body temperature fluctuations and the high metabolic rate pose an interesting problem of heat generation and thermoregulation. Given the apparent widespread distribution of UCPs among eukaryotes and the lack of BAT in birds, hummingbirds become ideal models to test the hypothesis that body heat might be generated in the avian group through the mediation of an UCP. In the present study we cloned and functionally characterized a novel UCP homolog in hummingbirds (HmUCP) that, as with the other UCPs, is capable of lowering mitochondrial membrane potential when transiently expressed in yeast. Its mRNA is predominantly expressed in the pectoral muscle, myocardium, and liver. More importantly, HmUCP mRNA levels are predominantly higher during torpor, as occurs with UCP2 and UCP3 mRNAs in torpid and hibernating mammals (9), suggesting that HmUCP might play a physiological role in the rewarming process.

## MATERIAL AND METHODS

**Animals.** Swallow-tailed hummingbirds (*E. macroura*) were captured in Ribeirão Preto, SP, Brazil, and brought to our laboratory under license no. 120/99-DIFAS from the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis. This is a tropical nonmigratory species of hummingbird typical of the Brazilian southeast. Birds were kept in individual flight cages (dimensions: 2 m high × 2 m deep × 1 m wide), at ~24°C, under a 12:12-h light-dark photoperiod-controlled regimen, and were fed with a 20% sucrose solution ad libitum and *Drosophila*. In the experiments where torpor was induced, birds were studied within a week of their capture. Birds were killed by decapitation, and the pectoral muscle, heart, brain, liver, kidney, and lung were immediately excised, frozen in liquid nitrogen, and stored at -80°C.

**Amplification of the HmUCP cDNA.** Total RNA was isolated from hummingbird pectoral muscle using RNA Stat 60 (Tel-Test, Friendswood, TX) as described (11). Reverse tran-

scription was started with an oligo(dT) primer using SuperScript (Life Technologies, Palo Alto, CA). The RT-PCR was performed using the primers *A* and *B* designed by comparison of the nucleotide sequence of the UCPs' highly conserved domains (*primer A*, sense, 5' CCGCTGGACACCGCCAAAG 3'; *primer B*, antisense, 5' ATCGGGTCTTTACCACATCCAC 3'). PCR cycles were as follows: 95°C for 5 min; 95°C for 45 s, 63°C for 1 min 30 s, 72°C for 2 min, 35 cycles. The product was purified, cloned into pGEM-T Easy plasmid (Promega, Madison, WI), and sequenced. On the basis of the obtained sequence information, sense (*primer C*), and antisense (*primer D*) primers were designed and used to clone more 3' and 5' sequences using rapid amplification of cDNA ends (RACE) PCR performed by the Marathon cDNA amplification kit and Advantage 2 Polymerase from Clontech Laboratories (Palo Alto, CA), following the instructions of the manufacturer. Briefly, first- and second-strand cDNA synthesis were followed by the Marathon adaptor ligation. RACE reactions were performed using an adaptor primer for the Marathon adaptor and with the *C* or *D* HmUCP-specific primers for the 3' or 5' RACE, respectively (*primer C*, sense, 5' GCTGCAGCGCCAGATGAGCTTGG 3'; *primer D*, antisense, 5' GTGTGTCAGCCCCGCGAGTAACG 3'). For the 5' end, PCR cycles were as follows: 94°C for 30 s; 98°C for 30 s, 60°C for 1 min, 68°C for 2 min 30 s; 34 cycles, 68°C for 10 min. For the 3' end, PCR cycles were as follows: 94°C for 4 min; 94°C for 45 s, 65°C for 1 min, 72°C for 2 min, 34 cycles, 72°C for 10 min. The obtained fragments were purified, cloned into pGEM-T Easy, and sequenced.

**Assembling of the HmUCP coding region: epitope tagging.** HmUCP coding region was assembled by RT-PCR, as follows. The hummingbird cDNA used for the RACE was amplified by TaKaRaTaq Polymerase (PanVera, Madison, WI) using the primers *E* and *F*. *Primer E* introduced a *Hind*III and *Eco*RI site 5' to the start codon, while *primer F* introduced a *Not*I site 3' to the stop codon (non-HmUCP-specific tails are shown in lower case; *primer E*, sense, 5' aagcttgaattcATGGTGCTCTGAAATCC 3'; *primer F*, antisense, 5' ggcgcccgcTCAGGATAGGGCCAGGA 3'). PCR cycles were as follows: 94°C for 4 min; 94°C for 45 s, 63°C for 1 min, 72°C for 1 min 30 s, 34 cycles, 72°C for 10 min. The product was subcloned into pGEM-T Easy (5' at T7) and sequenced (HmUCPcd-1). The amino terminus of the HmUCP was fused to the human influenza virus hemagglutinin epitope (12CA5) in a pYES2 yeast expression vector (Invitrogen, Carlsbad, CA). The HmUCPcd-1 was cut by *Eco*RI and *Spe*I, and the fragment was inserted between the *Eco*RI and *Xba*I sites of the 12CA5-pYES2 fusion vector, which contained the sequence coding for the epitope (MYPYDVPDYA) between the *Hind*III and *Eco*RI sites [Zhang et al., in 1999 (49)]. The resulting construct (HmUCPcd-2) was mapped by *Hind*III-*Not*I and *Eco*RI-*Not*I digestion. The rat UCP1 construct was prepared as previously described (49).

**Sequencing and sequence analyses.** All sequencing was performed with automated sequencing (ABI PRISM dGTP BigDye Terminator Ready Reaction Kit and BigDye Terminator Ready Reaction kit; Perkin-Elmer, Foster City, CA). Sequences were analyzed by Sequence Analysis Software Package of the Genetics Computer Group (University Research Park, Madison, WI).

**Transient expression of rat UCP1 and HmUCP in yeast.** Diploid yeast (strain INVSC1; Invitrogen) was transformed with pYES2 expression vectors carrying the 12CA5-tagged HmUCP (HmUCPcd-2) or the rat UCP1, using the standard lithium acetate method. The empty pYES2 vector was transformed as a negative control. Yeast transformants were selected on plates lacking uracil. Single colonies were inocu-

lated into a preculture grown in synthetic complete (SC) medium lacking uracil and supplemented with 2% lactate and 0.1% glucose to an OD<sub>600</sub> of ~3.0. The preculture was then diluted to an OD<sub>600</sub> of 0.04 in 400 ml of SC medium without uracil, supplemented with 3% lactate and no glucose, and grown at 30°C with vigorous shaking. After ~24 h, 1% galactose was added to induce UCP expression, and the cells were harvested after ~12 h.

**Western blot.** Yeast was broken by enzymatic digestion, and the enriched mitochondria fraction was isolated as described previously (49). Briefly, spheroplasts were prepared by digestion with zymolyase, and mitochondria were isolated by differential centrifugation after homogenization of the spheroplasts in a buffer containing 0.6 M mannitol, 10 mM Tris, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1% BSA. BSA was omitted during the last washing step. A quantity of 40 µg of protein was run in a 12% SDS-PAGE. Western blotting of 12CA5-fused UCPs was performed using a mouse monoclonal antibody against the 12CA5 epitope (Boehringer Mannheim, Indianapolis, IN), and the signal was detected with enhanced chemiluminescence (ECL) Western blotting detection reagents, according to the instructions of the manufacturer (Amersham Pharmacia Biotech, Uppsala, Sweden).

**Measurement of mitochondrial membrane potential in yeast cells.** The mitochondrial membrane potential was measured in intact yeast cells with the potential-sensitive dye 3',3'-dihexyloxycarbocyanine iodide (DiOC<sub>6</sub>) (Molecular Probes, Eugene, OR) using a FACScan instrument (Becton-Dickinson, Cockeysville, MD), as previously described (49).

**Northern analysis.** Total RNA from various hummingbird tissues was isolated as previously described (11). For the Northern analysis, 15 µg of total RNA was resolved in a 1.2% agarose gel containing 5.4% formaldehyde, as described (32). The RNA was transferred to nylon blotting membranes, fixed by ultraviolet light (Stratalinker; Stratagene, La Jolla, CA), and stained with methylene blue to check loading and transfer. As a probe, a full-length HmUCP coding region PCR fragment was used (described above). The fragment was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (NEN, Boston, MA) using the Prime-a-Gene labeling System (Promega, Madison, WI). Hybridization was carried out for 2 h at 65°C in QuikHyb (Stratagene), then washed in a solution of 2× SSC/0.1% SDS at 50°C twice for 5 min, and finally in 0.1× SSC/0.1% SDS at 50°C for 5 min. Size estimates of RNA were determined using an RNA ladder as reference. The films were then scanned, and the intensity of the bands quantified using the Iq software (v. 3.22; Molecular Dynamics, Sunnyvale, CA).

**Oxygen consumption and temperature measurements.** Oxygen consumption was measured by open-flow respirometry (48). Body temperature (T<sub>b</sub>) was measured using a thermocouple (TT-T-40, copper/constantan, Omega Engineering) that was placed on the lateral thorax of the animal under the wing and fixed with surgical tape. A second thermocouple (TT-T-30) was used to read ambient temperature (T<sub>a</sub>). All the experiments were conducted inside a temperature-controlled room, and all animals were unrestrained and kept at ~24°C up until the beginning of the experiments. Torpor was induced by lowering the ambient temperature to ~15–17°C, and rewarming was induced by raising room temperature to 23°C. Groups of two animals were killed at each physiological state, i.e., euthermia, torpor, and rewarming.

**Statistical and phylogenetic analysis.** Results are expressed as means ± SD or the range as indicated. Multiple comparisons were performed by one-way ANOVA followed by the Student-Newman-Keuls test. The amino acid sequence for the hummingbird UCP was compiled into a phylogenetic

tree by comparing its sequence to many of the known UCPs and mitochondrial carriers using Protpars program from the PHYLIP package. The assumptions of this program are based on a previous publication (19).

## RESULTS

**Cloning of the HmUCP.** The RT-PCR of total RNA from hummingbird pectoral muscle, using the *A* and *B* primers, generated a fragment of ~630 bp. This *AB* fragment was then aligned to coding regions of other UCPs, showing ~73% homology to rat UCP3 (GenBank accession no. AF030163), ~70% to rat UCP2 (AF039033), and ~63% to rat UCP1 (NM012682). The high degree of similarity indicated that the cloned fragment contained a portion of the putative HmUCP coding region and that the 5' and 3' ends of the coding region were missing. The 5' RACE generated a ~580-bp fragment that had an ~280-bp-long overlap with the *AB* fragment. The length of the 3' RACE product and its overlap with *AB* were ~750 bp and ~480 bp, respectively (Fig. 1). The assembled HmUCP coding sequence was deposited into the GenBank under accession number AF255729. The HmUCP open reading frame has a strong translational initiation sequence (AGGATGG).

**HmUCP amino acid sequence.** The deduced amino acid sequence of HmUCP consists of 304 amino acids, with a molecular mass of 32.8 kDa as estimated by the GCG Peptidesort. The HmUCP has three domains of ~100 amino acids. Each one possesses a mitochondrial carrier signature motif (Fig. 2) that is well conserved among all known mitochondrial carriers. In the HmUCP, only one histidine residue, equivalent to His<sup>145</sup> or His<sup>147</sup> in UCP1, is present. A number of other residues, equivalent to Arg<sup>83</sup>, Arg<sup>182</sup>, Arg<sup>276</sup>, Glu<sup>190</sup>, and His<sup>214</sup> in UCP1, are also present in HmUCP. The predicted amino acid sequence of HmUCP has higher identities to human UCP3 and rat UCP2 isoforms, of ~72 and ~70%, respectively. The identity to rat and human UCP1 is ~55% (Fig. 2).

The phylogenetic analysis infers an unrooted phylogeny and found one most parsimonious tree, which in-

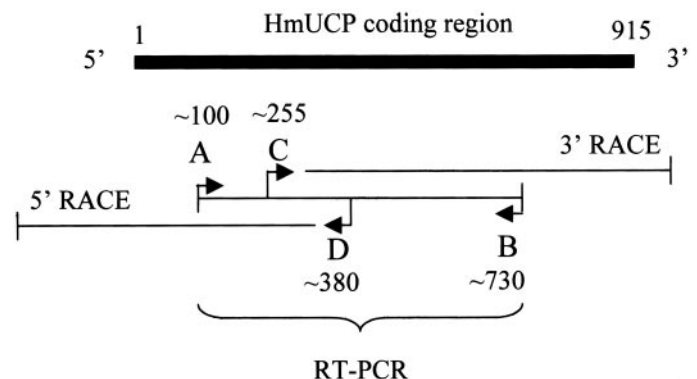


Fig. 1. Schematic diagram of the cloning strategy used to obtain the hummingbird uncoupling protein (HmUCP) coding region. The RT-PCR using the *A* and *B* primers provided an ~630-bp fragment, which was expanded into 3' and 5' directions using the oligo primers *C* and *D* in a 3' and 5' RACE reaction, respectively. RACE, rapid amplification of cDNA ends.

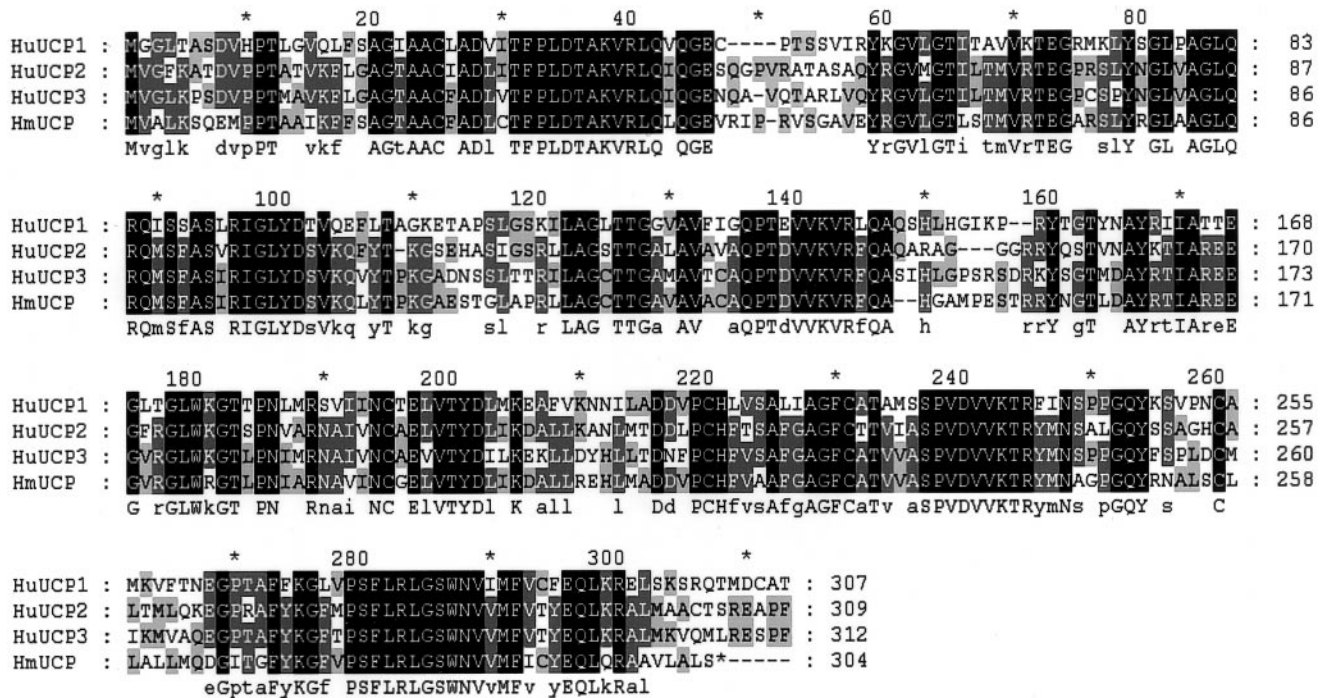


Fig. 2. Comparison of the deduced amino acid sequence of HmUCP and human UCP proteins. Sequences of HmUCP, HuUCP1 (GenBank accession no. U28480), HuUCP2 (U82819), and HuUCP3 (U84763) were compared by GCG Pileup, and the consensus sequence was prepared by GeneDoc. Darker shades of gray indicate a higher degree of sequence homology.

indicated that HmUCP, mammalian UCP2 and UCP3, and fish UCPs form a group that is closely related in terms of inferred genetic distance (Fig. 3).

**Effect of HmUCP in yeast mitochondrial membrane potential.** To test whether HmUCP could be efficiently expressed in *Saccharomyces cerevisiae* and used in functional studies, a pYES2 plasmid containing the 12CA5 epitope-tagged HmUCP was used to transform yeast cells. Galactose (1%)-induced HmUCP expression was maximal at 12 h as assessed by Western blot analysis of cell lysates using anti-12CA5 antibodies (data not shown). The Western blot revealed a single ~34-kDa protein, compatible with the predicted molecular weight in the enriched mitochondria fraction (Fig. 4A). In these cells, transiently expressed HmUCP decreased the DiOC<sub>6</sub> uptake as measured by flow cytometry to a degree similar to that of UCP1, demonstrating that HmUCP is capable of lowering the mitochondrial membrane potential (Fig. 4B).

**HmUCP mRNA transcripts and tissue distribution.** The Northern blot of total hummingbird RNA revealed a prominent transcript of ~1.6 kb and a less intense one of ~3.5 kb. The 1.6-kb band indicates that the combined 5'- and 3'-untranslated regions have ~0.7 kb. The analysis of HmUCP mRNA expression in various tissues indicated a high expression in the pectoral muscle. Heart and liver have both slightly lower expressions, followed by the lung and kidneys. Brain was the only tested tissue in which HmUCP mRNA was not detected (Fig. 5).

**Changes in HmUCP mRNA levels during torpor and rewarming.** To test whether HmUCP mRNA levels change during various physiological conditions, six hummingbirds were studied at euthermia, torpor, and rewarming. At each phase, two animals were killed, and various tissues were processed for Northern blot. These phases are shown in a typical profile of oxygen consumption of an *E. macroura* specimen that experienced all activity states (Fig. 6A). During the euthermia phase, Ta was kept between 15–17°C, while Tb remained 33–40°C. Oxygen consumption is high and shows a clear oscillatory pattern. During this phase, the respiratory quotient (RQ) started at 1–1.2 and rapidly declined to 0.6–0.7. In the torpor phase, both oxygen consumption and Tb drop precipitously. Tb varied between 15–23°C, while Ta was kept between 13–14°C. Oxygen consumption showed less oscillation and remained at only 1/10 of its normal values, while the RQ values were maintained at about 0.6–0.7. The rise of Ta to 23°C triggered the rewarming process (rewarming phase), during which both oxygen consumption and Tb increased, reaching euthermic levels. Throughout this phase, RQ values oscillated between 0.7 and 0.8.

The Northern blot analysis indicates that in the pectoral muscle and heart, HmUCP mRNA changed significantly during the torpor-rewarming process (Fig. 6, B and C). Accordingly, the heart of torpid animals showed an ~3.4-fold increase in HmUCP mRNA levels compared with euthermic animals. During the re-

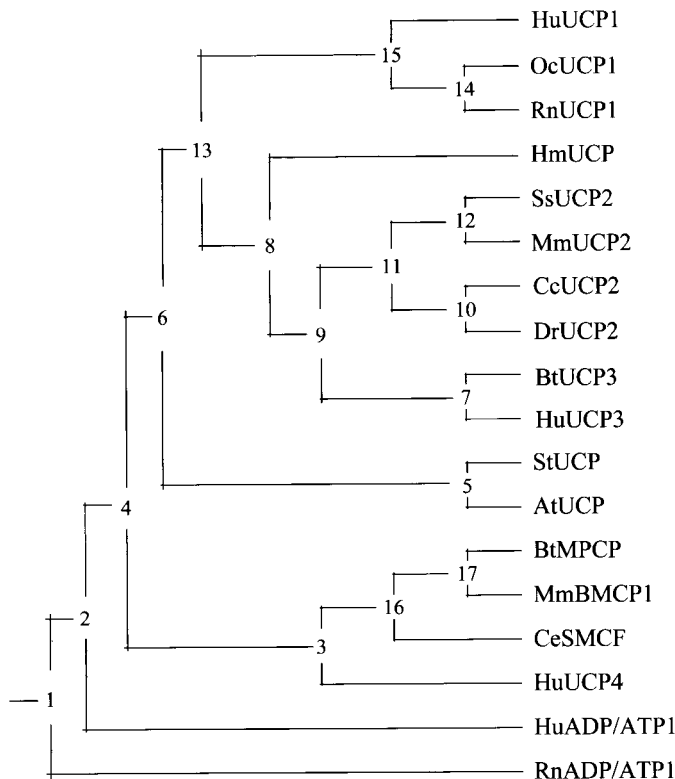


Fig. 3. Evolutionary tree of UCPs and other mitochondrial carriers. Unrooted evolutionary tree representing inferred genetic distances (the numeric values indicate base substitutions that change the amino acid sequences) between UCP sequences obtained from GenBank (Protpars program from PHYLIP package, Ref. 19). Abbreviations, species, and accession numbers are as follows: HuUCP1 is *Homo sapiens* (human) UCP1, P25874; OcUCP1 is *Oryctolagus cuniculus* (rabbit) UCP1, P14271; RnUCP1 is *Rattus norvegicus* (rat) UCP1, P04633; HmUCP is *E. macroura* (hummingbird) UCP, AF255729; SsUCP2 is *Sus scrofa* (pig) UCP2, AAD05201; MmUCP2 is *Mus musculus* (mouse) UCP2, P70406; CcUCP2 is *Cyprinus carpio* (carp) UCP2, CAB46248; DrUCP2 is *Danio rerio* (zebrafish) UCP2, CAB46268; BtUCP3 is *Bos taurus* (cow) UCP3, AAC61762; HuUCP3 is *H. sapiens* (human) UCP3, P55916; StUCP is *Solanum tuberosum* (potato) UCP, CAA72107; AtUCP is *Arabidopsis thaliana* (thale cress) UCP, CAA77109; BtMPCP is *B. taurus* (cow) mitochondrial phosphate carrier protein, P12234; MmBMCP1 is *M. musculus* (mouse) brain mitochondrial carrier protein, AAD03674; CeSMCF is *Caenorhabditis elegans* (worm) similar mitochondrial carrier family, AAB54239; HuUCP4 is *H. sapiens* (human) UCP4, AAD16995; HuADP/ATP1 is *H. sapiens* (human) ADP/ATP translocator, P12235; RnADP/ATP1 is *R. norvegicus* (rat) ADP/ATP carrier, Q05962.

warming phase, the induction of the HmUCP mRNA levels was only 2.2-fold compared with euthermic animals but did not reach statistical significance. In the pectoral muscle, a tissue that has fivefold higher levels of HmUCP mRNA than heart, similar, but less intense, changes were detected. It is interesting to note, however, that both heart and pectoral muscle exhibited their highest levels of HmUCP during torpor.

**DISCUSSION**

The presence of a tripartite structure in the HmUCP predicted amino acid sequence, with each possessing a mitochondrial carrier signature motif (PLDTAKVR, PTEVVKVR, and PVDVVKTR), demonstrates that

this protein belongs to a larger family of inner mitochondrial membrane proteins that share a number of common features (35, 46). HmUCP has 34% similarity to the 2-oxoglutarate/malate carrier, which is one of the most homologous mitochondrial carriers for UCPs and thought to lack uncoupling activity. In comparison, the human UCP2 and UCP3 are ~32% similar to 2-oxoglutarate/malate carrier. The HmUCP has the highest sequence homology (~70%) to human UCP3L and rat UCP2 compared with the other members of the mitochondrial carrier protein family. This high homology of HmUCP to other known UCPs demonstrates that this novel protein is part of the UCP family.

Two histidine residues, His<sup>145</sup> and His<sup>147</sup>, are considered important (5) but not an absolute requirement (49) for UCP1-mediated H<sup>+</sup> transport in proteoliposomes. Accordingly, an equivalent histidine pair is absent in UCP2, and in HmUCP as well as human UCP3 only one equivalent histidine is present. Nevertheless, it has been repeatedly shown that UCP2 and UCP3 decrease the mitochondrial membrane potential (7, 20, 49), indicating that absence of this histidine pair by itself does not rule out uncoupling activity in these proteins.

Following the cloning and characterization of UCP2 and UCP3, investigators expressed these homologs in yeast and detected a drop in mitochondrial membrane potential (21, 36). Even though lowering membrane potential does not necessarily mean that proton conductance is increased, in this yeast system the oxoglutarate carrier, which lacks uncoupling activity, has no effect, or only a very small effect, on DiOC<sub>6</sub> uptake in yeast mitochondria (49), arguing against an unspecific effect on the membrane potential of transiently expressed proteins. In addition, the finding that, as with UCP1, purified UCP2 and UCP3 catalyzed an electrophoretic flux of protons in liposomes in the presence of fatty acids (22) strongly suggests that UCP2 and UCP3 might function as true UCPs. In the present studies the same (49) yeast transient expression system was used, and it was found that the mitochondrial membrane potential was reduced in yeast expressing HmUCP, as well as in yeast expressing UCP1. This is an important finding that supports proton conductance by this new UCP1 homolog.

Typically, the activity of UCP1 is regulated by various purine nucleotides. A number of residues in UCP1, including Arg<sup>83</sup>, Arg<sup>182</sup>, Arg<sup>276</sup>, Glu<sup>190</sup>, and His<sup>214</sup>, have been shown to be important for this nucleotide regulation (17, 18, 34). Interestingly, all the equivalents of these residues are present in HmUCP (Fig. 2), indicating that its uncoupling activity is potentially regulated by nucleotides. However, according to results obtained with UCP2 and UCP3, the presence of these residues does not guarantee high sensitivity to purine nucleotide inhibition (22), and further in vitro studies are necessary to clarify this point in HmUCP.

The tissue distribution of HmUCP mRNA shares similarities with that of UCP2 and UCP3. For example, the high expression of HmUCP mRNA in the skeletal muscle resembles the distribution pattern observed for

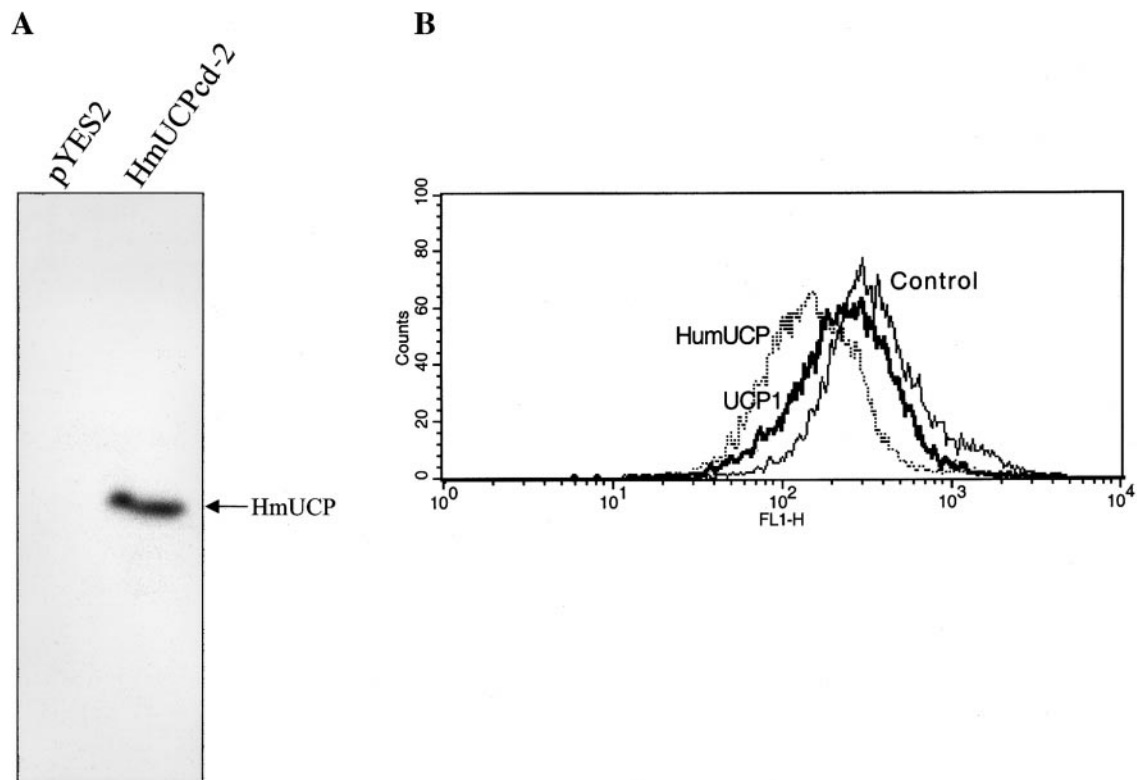


Fig. 4. HmUCP expression in yeast and HmUCP-induced mitochondrial uncoupling. **A:** Western blot detection of 12CA5-tagged HmUCP in isolated yeast mitochondria after 12 h of induction with 1% galactose using anti-12CA5 antibodies. Cells transformed with empty pYES2 vector were used as negative controls. **B:** flow cytometry analysis of membrane potential following the expression of HmUCP in yeast. Rat UCP1 and empty pYES2 vector were used as positive and negative controls, respectively. Yeast cells were incubated in the fluorescent potential-sensitive dye, DiOC<sub>6</sub>. The x-axis is a logarithmic scale of fluorescence intensity, and the y-axis represents the number of cells (counts). A shift of the curve toward the *left* indicates a decreased mitochondrial potential. DiOC<sub>6</sub>, 3',3'-dihexyloxycarbocyanine iodide. FL1-H, fluorescence parameter used in the flow cytometer.

the UCP3 in mammals. However, the high expression of HmUCP in the heart differs from mammals, and given the relatively large cardiac size in hummingbirds (2.0–2.4% as opposed to only ~0.6% in mammals), its presence might have physiological relevance. Heart rates recorded for hummingbirds are in the order of 1,250 beats/min (30), which is among the highest heart rates recorded for endotherms. It is conceivable that the high workload and ATP turnover observed in the hummingbird's heart might be associated to elevated HmUCP mRNA levels.

An additional feature that contrasts HmUCP to mammalian UCPs is high expression in liver, at levels similar to heart. In mammals, this is not the case. UCP2 expression is restricted to Kupffer cells (29) and can only be seen in hepatocytes of mice with fatty liver (13). It is interesting to note, however, that hummingbird hepatocytes contain ~20% fat as opposed to normal mouse hepatocytes, which contain about 0.2%. (4). It is tempting to speculate that the fat-induced UCP2 expression in mammals is similar to a mechanism also present in birds, linking food availability and uncoupling of the proton entry from ATP synthesis.

The physiological studies performed in the present investigation were limited by the difficulties in obtaining a large number of animals. Keeping that in mind,

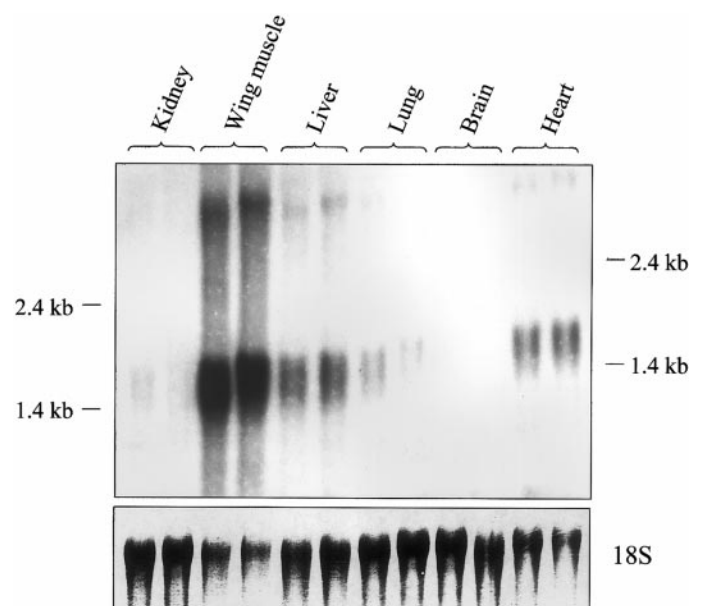


Fig. 5. Tissue distribution of HmUCP mRNA. Northern blot analysis of total RNA (15 µg/lane) extracted from kidney, pectoral muscle, liver, lung, brain, and heart of two different hummingbirds. The blot was probed with [ $\alpha$ -<sup>32</sup>P]dCTP-labeled HmUCP coding region. The predominant transcript of HmUCP mRNA was ~1.6 kb. Ribosomal RNA was stained with methylene blue.

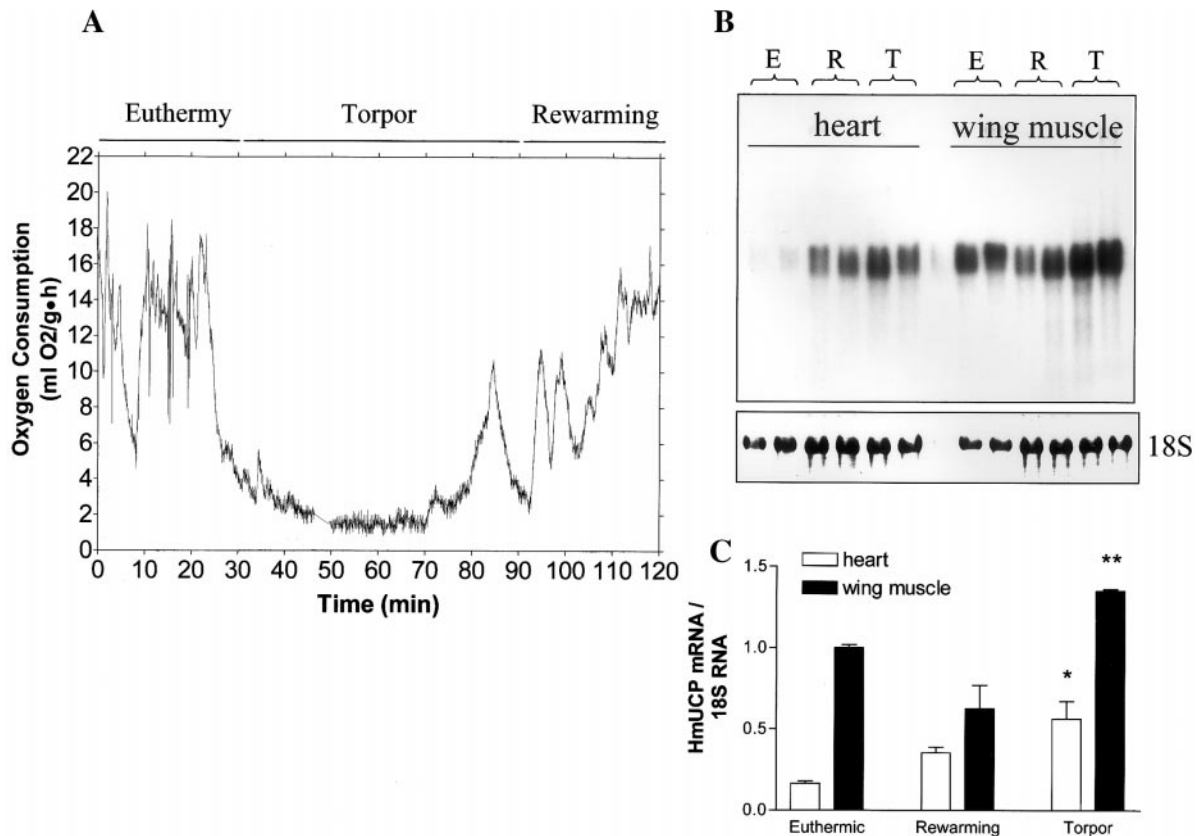


Fig. 6. Changes in HmUCP mRNA levels during torpor and rewarming. *A*: profile of oxygen consumption of an *E. macroura* specimen during euthermy (E), torpor (T), and rewarming (R). Body temperatures in the various animals were 33–40°C and 15–23°C during euthermy and torpor, respectively. *B*: Northern blot of total RNA (probed with [ $\alpha$ -<sup>32</sup>P]dCTP-labeled HmUCP coding region) obtained from heart and pectoral muscle of birds killed at the indicated times of the torpor/rewarming cycle. The ribosomal RNA stained with methylene blue. *C*: ratio between HmUCP mRNA and ribosomal RNA obtained after densitometry. Values are means  $\pm$  the range. Two animals were used per activity state. \**P* < 0.05 vs. euthermy. \*\**P* < 0.05 vs. rewarming (ANOVA).

HmUCP mRNA levels in the heart and pectoral muscle were found to be elevated during torpor compared with rewarming or euthermy. Because the rise in mRNA levels necessarily precedes the rise in protein levels, it makes sense that HmUCP mRNA levels are higher when animals are entering the rewarming phase. These characteristics show remarkable similarity to the ground squirrel, a hibernating mammal in which UCP2 and UCP3 mRNA levels are also maximal during hibernation (9). Unique to the hummingbird, however, was the ~3.4-fold upregulation of HmUCP in the heart. As discussed above, the hummingbird heart has singular characteristics, and, from torpor to euthermy, rates can vary by up to two orders of magnitude (30). Given the larger relative size and high frequency achieved, it is not possible to discard the possibility that the hummingbird's heart might have a strategic thermogenic role during rewarming.

Based on the uncoupling activity of UCP2 and UCP3, it has been proposed that, as with UCP1, these, too, could be thermogenic. The recent finding of increased thermogenesis in transgenic mice overexpressing UCP3 (12) strongly favors a role for UCP3 in adaptive thermogenesis. However, the physiological role of UCP2 and UCP3 is still highly controversial, with

several studies supporting or antagonizing their thermogenic role (38). Despite its proton conductance capacity, UCP3 expression in skeletal muscle does not change in response to 48 h of cold exposure, a condition known to increase UCP1 mRNA levels by three- to fourfold. Furthermore, UCP2 and UCP3 mRNA levels are upregulated during starvation due to increased circulating free fatty acids (FFA), a situation known to decrease energy expenditure (41). In the present investigation we also found evidence of increased oxidation of fatty acids as indicated by the low RQ values detected in the torpid hummingbirds. Similarly to mammals, increased circulating FFA in hummingbirds could play a role in stimulating HmUCP gene expression. However, it is important to note that RQ values of ~0.7 were also observed in the animals that did not enter torpor and were killed at the euthermy phase, a time when HmUCP mRNA levels were lowest. Contrary to starving mammals, however, the rise in HmUCP was followed by an elevation in oxygen consumption, typical of the rewarming phase.

Even if the primary role of UCP2 and UCP3 in mammals is not adaptive thermogenesis, this might not necessarily be the case for HmUCP in birds. Mammals have BAT whose temperature can rise 3–5°C

within minutes of adrenergic stimulation (10). However, unlike mammals, birds do not have BAT or a similarly thermogenic tissue and so far seem to possess only one UCP1 homolog. The presence of HmUCP mRNA in the pectoral muscle and heart and its increase during torpor suggest a thermogenic role during rewarming. The presence of HmUCP in the skeletal muscle may also be strategic for shivering thermogenesis by decreasing the thermodynamic efficiency of mitochondrial ATP synthesis. The shivering-induced ATP breakdown stimulates mitochondrial oxidation and ADP phosphorylation that, in the presence of HmUCP, will dissipate greater amounts of heat.

The cloning of a bird UCP adds an important new protein to a rapidly growing number of UCPs found throughout eukaryotes. A mitochondrial UCP-like protein was recently identified by Western blot in the nonphotosynthetic soil amoeboid protozoan, *Acanthamoeba castellanii* (24). In plants, StUCP and AtUCP have been cloned in *Solanum tuberosum* (potato) and *Arabidopsis thaliana*, respectively (28, 33). StUCP probably encodes the biochemically purified plant UCP, PUMP (45), and its activity as a mitochondrial uncoupler suggests a role in thermogenesis and resistance to chilling (28, 33, 38). The evolution of homeothermy in mammals and birds from ectothermic synapsid and diapsid ancestors, respectively, is presumed to have occurred independently, ~310 million years ago (27). The estimated divergence of fish from mammals and birds occurred ~450 million years ago. The finding that the UCPs from fish and birds and the mammalian UCP2 and UCP3 fall into a single arm, when analyzed in terms of inferred genetic distances, opens the possibility of an early evolutionary origin for UCP2 and UCP3. This scenario would suggest that UCP1 was adapted for its specific function in mammals (BAT) as a later evolutionary event. Given the apparent widespread distribution of UCPs, one might speculate on the existence of a common early ancestral gene for UCPs. However, they may have been the object of selection at different taxa, leading to convergent evolution with respect to thermogenesis.

#### NOTE ADDED IN PROOF

Since the acceptance of this manuscript, S. Raimbault, S. Dridi, F. Denjean, J. Lachuer, E. Couplan, F. Bouillaud, A. Bordas, C. Duchamp, M. Taouis, and D. Ricquier have published a chicken UCP cDNA sequence (AF287145; *Biochem J* 353: 441–444, 2001), which was deposited in the GenBank database 3 mo after the HmUCP (AF255729).

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The sequence reported in here has been deposited in the GenBank database (accession no. AF255729).

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