RESEARCH COMMUNICATION

An uncoupling protein homologue putatively involved in facultative muscle thermogenesis in birds

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The cDNA of an uncoupling protein (UCP) homologue was obtained by screening a chicken skeletal-muscle library. The predicted 307-amino-acid sequence of avian UCP (avUCP) is 55, 70, 70 and 46 % identical with mammalian UCP1, UCP2 and UCP3 and plant UCP respectively. avUCP mRNA expression is restricted to skeletal muscle and its abundance was increased 1.3-fold in a chicken line showing diet-induced thermogenesis, and 3.6- and 2.6-fold in cold-acclimated and glucagon-treated ducklings developing muscle non-shivering thermogenesis respectively. The present data support the implication of avUCP in avian energy expenditure.

Key words: bioenergetics, mitochondria, mitochondrial carrier, skeletal muscle.

INTRODUCTION

As endotherms, birds actively regulate their body temperature in the cold by generating heat. The existence of facultative mechanisms of heat production in birds has long been denied, and shivering thermogenesis was thought to be the main avian thermogenic mechanism, although bodies of evidence for both diet-induced thermogenesis (DIT) and cold-induced regulatory non-shivering thermogenesis (NST) have accumulated in the literature (see [1] for a review).

Evidence for avian DIT was obtained by comparing two experimental Rhode Island Red chicken lines divergently selected for low and high efficiency of food utilization for growth [2]. For the same body weight and egg production, the inefficient line consumes 30–40 % more food than the efficient line, is leaner [3], and shows a larger (+133 %) heat production after a meal [4]. Enhanced capacity for regulatory NST, as assessed by simultaneous measurements of heat production and electromyographic activity, was reported in response to long-term cold exposure in young ducklings and king-penguin (*Aptenodytes patagonicus*) chicks [5,6]. Avian NST was also induced by chronic treatment of ducklings kept at thermoneutrality with glucagon, a thermogenic hormone in birds [6]. Avian NST mainly originates from skeletal muscle [7] and involves a loose coupling of mitochondrial oxidative phosphorylation under the control of fatty acids [8,9]. The molecular mechanisms of such uncoupling are not known.

In rodents, the importance of respiratory uncoupling has been well demonstrated, since maintenance of body temperature during exposure to cold is controlled by modulation of the proton electrochemical gradient across the inner mitochondrial membrane through uncoupling protein-1 (UCP1), which promotes the dissipation of oxidation energy in brown adipocytes [10–13]. Recently, homologues of the brown-fat UCP were characterized in mammals (see [14,15] for reviews), and also in plants [16]. Although these proteins show an uncoupling activity in recombinant systems, they are probably not important for the regulation of body temperature or adaptive thermogenesis under physiological conditions. UCPs have not been characterized so far in birds.

We report here the identification of a cDNA from chicken that encodes a protein highly homologous with mammalian UCP2 and UCP3. This *UCP* gene, referred to as *avUCP* (*avian UCP*), is uniquely expressed in avian skeletal muscle. Moreover, analysis of the avUCP mRNA level under several conditions suggested a role for this gene in facultative thermogenesis in chicken and duck.

MATERIALS AND METHODS

Animals

Two Rhode Island Red chicken (*Gallus gallus*) lines were divergently selected for high R+ or low R− strain (residual food consumption indicative of respectively low and high efficiency of food utilization for growth) [2]. Male chickens, 62 weeks old, were bred in individual cages and under standard conditions (20 °C ambient temperature, 14/10 h light/dark cycle). Animals were fed *ad libitum*. After cervical dislocation, several tissues (lung, brain, gizzard, kidney, muscle, adipose tissue, heart, liver, testis and intestine) were quickly removed, frozen in liquid nitrogen, ground and stored at −80 °C until use.

Male Muscovy ducklings (*Cairina moschata* L., pedigree R31, Institut National de la Recherche Agronomique, France) were obtained from a commercial stockbreeder (Ets Grimaud, Malafretag, France). They were kept in a constant photoperiod (8/16 h light/dark), were fed a commercial mash (Aliment Genthon Démarrage; Ets Genthon, Cheyssieu, France)

Abbreviations used: (av)UCP, (avian) uncoupling protein; DIT, diet-induced thermogenesis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NST, non-shivering thermogenesis; RT-PCR, reverse-transcription PCR.

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The nucleotide sequence data referred to, but not actually shown, will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AF287144.

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mUCP1   MVNPTTSEVQPTMGVKIFSAGVSACLADIITFPLDTAKVRLQIQGEGQ----ASSTIRYKGVLGTITTLAKTEGLPKLYSGLPAGIQRQISFASLRIGLYDSV
mUCP2   MVGFKATDVPPTATVKFLGAGTAACIADLITFPLDTAKVRLQIQGESQGLVRTAASAQYRGVLGTILTMVRTEGPRSLYNGLVAGLQRQMSFASVRIGLYDSV
mUCP3   MVGLQPSEVPPTTVVKFLGAGTAACFADLLTFPLDTAKVRLQIQGENP----GAQSVQYRGVLGTILTMVRTEGPRSPYSGLVAGLHRQMSFASIRIGLYDSV
avUCP   MVGLKPPEVPPTAAVKFFSAGTAACIADLCTFPLDTAKVRLQIQGEVR-IPRSTNTVEYRGVLGTLSTMVRTEGPRSLYSGLVAGLQRQMSFASIRIGLYDSV
        **      * **  **   **  ** **  ****************           * *****  *   ***    * ** **  ** **** ********

mUCP1   QEYFSSGRETPASLGNKISAGLMTGGVAVFIGQPTEVVKVRMQAQSHLHGIK-PRYTGTYNAYRVIATTESLSTLWKGTTPNLMRNVIINCTELVTYDLMKGAL
mUCP2   KQFYT-KGSEHAGIGSRLLAGSTTGALAVAVAQPTDVVKVRFQAQARAGGG--RRYQSTVEAYKTIAREEGIRGLWKGTSPNVARNAIVNCAELVTYDLIKDTL
mUCP3   KQFYTPKGADHSSVAIRILAGCTTGAMAVTCAQPTDVVKVRFQAMIRLGTGGERKYRGTMDAYRTIAREEGVRGLWKGTWPNITRNAIVNCAEMVTYDIIKEKL
avUCP   KQLYTPKGAESTGLLARLLAGCTTGAVAVTCAQPTDVVKVRFQALGALPESN-RRYSGTVDAYRTIAREEGVRGLWRGTLPNIARNSIINCGELVTYDLIKDTL
                      **  **  **   *** ***** **          *  *  **  **  *    ** ** **  ** * ** * ****  *  *

mUCP1       VNNKILADDVPCHLLSALVAGFCTTLLASPVDVVKTRFINSLPGQYPSVPSCAMSMYTKEGPTAFFKGFVASFLRLGSWNVIMFVCFEQLKKELMKSRQTVDCTT
mUCP2       LKANLMTDDLPCHFTSAFGAGFCTTVIASPVDVVKTRYMNSALGQYHSAGHCALTMLRKEGPRAFYKGFMPSFLRLGSWNVVMFVTYEQLKRALMAAYQSREAPF
mUCP3       LESHLFTDNFPCHFVSAFGAGFCATVVASPVDVVKTRYMNAPLGRYRSPLHCMLKMVAQEGPTAFYKGFVPSFLRLGAWNVMMFVTYEQLKRALMKVQVLRESPF
avUCP       LRAQLMTDNVPCHFVAAFGAGFCATVVASPVDVVKTRYMNASPGQYRNVPSCLLALLLQDGIAGLYKGFVPSFLRLGSWNVVMFISYEQLQRVVMLARSAPP---
                  *  ***   *  **** *  **********  *   * *     *        *     ***  ****** *** **   ***    *
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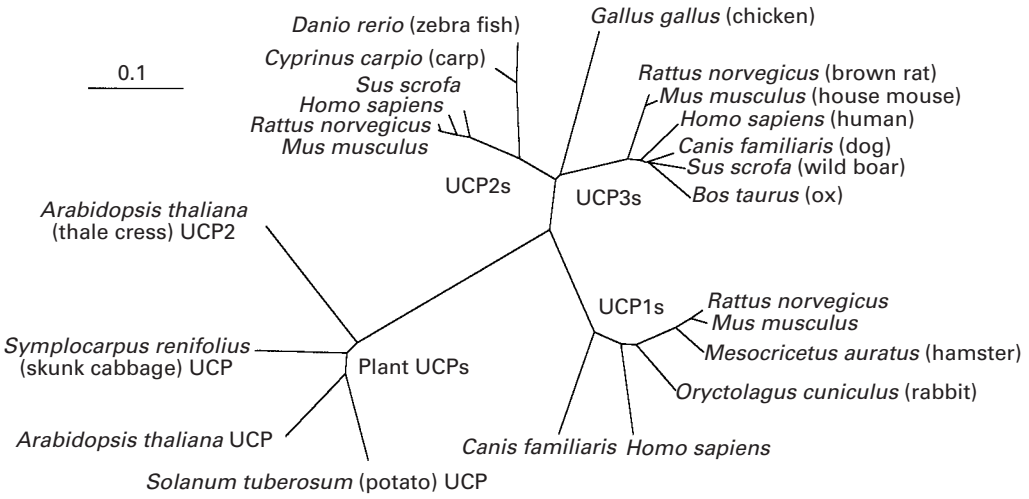

Figure 1 Predicted amino acid sequence and alignment of avUCP, an avian homologue of mammalian UCPs, and phylogenic tree

Mouse UCP (mUCP) sequences presented in the upper panel were obtained from GenBank®. The grey box is the PS00215 Prosite consensus motif for mitochondrial carriers. The asterisks (*) indicate identical amino acid residues. The phenogram rootless evolutionary tree presented in the lower panel describes the biochemical distances between the different animal UCP1, UCP2 and UCP3 and plant proteins. Sequences present in GenBank® were used. The bar indicates 0.1 amino acid residue change per site.

ad libitum and had free access to water. From the age of 1 week, ducklings were caged in groups of six for a period of 4 weeks at 25 °C (thermoneutral controls) or exposed to 4 °C (cold-acclimated animals) to induce the development of skeletal-muscle NST [5,7]. Another batch of ducklings received daily injections of glucagon (100 $\mu g \cdot kg^{-1}$, intraperitoneally twice daily) for 4 weeks starting from the age of 1 week. This pharmacological treatment was shown to promote glucagon-induced NST from muscle origin with loose-coupled mitochondria [17]. Animals were killed in a post-absorptive phase by decapitation and gastrocnemius muscle was sampled, frozen in liquid nitrogen and kept at −70 °C until analysis.

Cloning of avUCP cDNA

A 1 μg portion of total RNA from R+ chicken leg muscle was poly-T-primed, and reverse transcription (RT) was carried out using reverse transcriptase (Superscript II; Life Technologies, Rockville, MD, U.S.A.). The resulting cDNAs were submitted to PCR amplification, using forward primer 5′-GCGGTCGAC-ATCACCTTCCCGCTGGACAC-3′, reverse primer 5′-CATG-TCGACGTTCCAGGATCCGAGTCGC-3′, and PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, U.S.A.). The primers were calculated from alignment of nucleotide sequences of various UCP subtypes (GenBank®). Annealing was carried out at 45 °C during the first four cycles of PCR, whereas the following 35 cycles were run each at 94 °C for 30 s and 72 °C for 75 s. A rare restriction site, *Sal*I, was added to *Sal*I-linearized dephosphorylated pSL1180 vector (Amersham Pharmacia Biotech, Uppsala, Sweden) for easier ligation of the obtained PCR products. This PCR procedure led to the amplification of a partial 667 bp-long UCP cDNA that was used to further clone a full-length cDNA. To do that, we constructed a cDNA library from chicken skeletal muscle. Polyadenylated [poly(A)$^+$] RNA was purified from R+ chicken muscle using the PolyA tract kit (Promega, Madison, WI, U.S.A.). A 100 μg portion of poly(A)$^+$ RNA was used to construct a cDNA library according to the SMART cDNA synthesis and library construction kit (Clontech, Palo Alto, CA, U.S.A.). The cDNAs were ligated into lambda TriplEx2, and the lambda packaging kit (Amersham–Pharmacia Biotech) was used. The titre of the library was calculated to be around 2×10^6 independent recombinant clones. After amplification, 10^6 clones were screened using a ^{32}P-labelled PCR probe corresponding to the partial cDNA obtained (see above).

Northern-blot and RT-PCR analysis

Total RNA (100 μg samples from different tissues) was extracted using RNA Insta pure (Eurogentec, Angers, France) according to manufacturer's recommendations. Northern-blot analysis was done using standard protocols. To monitor RNA loading, the membrane was also hybridized with ^{32}P-labelled probe encoding 18 S rRNA. For RT-PCR analysis, 1 μg of total denatured RNA

from different tissues was reverse-transcribed with 20 units of avian-myeoloblastosis-virus reverse transcriptase in the presence of random hexamer primers (1 μg/μl; Promega). RT was carried out in the presence of dNTP Mix 0.5 mM (Sigma, Saint-Quentin Fallavier, France) and RNA guard RNase inhibitor from human placenta (0.5 μl; Amersham Pharmacia Biotech, Les Ulis, France). The reaction was assessed at 25 °C for 15 min and 42 °C for 50 min. PCR was carried out in the presence of three sets of primers, flanking: a 667 bp fragment of *avUCP* (sense, 5′-GTGAGGATCCCACGCAGCACC-3′; antisense, 5′-CAGCA-ATGCCATCCTGCAGCA-3′), a 515 bp fragment of 18 S RNA (sense, 5′-CTGCCCTATCAACTTTCG-3′; antisense, 5′-CATT-ATTCCTAGCTGCGG-3′) and a 766 bp fragment of glyceral-dehyde 3-phosphate dehydrogenase (GAPDH) (sense, 5′-TTTG-GCCGTATTGGCCGCCGCCT-3′; antisense, 5′-CAGCAGC-CTTCACTACCCTC-3′). Annealing and extension were carried out at 60 and 72 °C respectively during 25 cycles, followed by 7 min at 72 °C. Amplified products were transferred to nylon membranes and hybridized with adequately labelled *avUCP*, 18 S or GAPDH probes. The intensity of RT-PCR bands was determined by using a STORM apparatus (a PhosphorImager made by Molecular Dynamics).

RESULTS AND DISCUSSION

The cDNA of an UCP homologue was obtained by screening a chicken skeletal-muscle library with a RT-PCR product obtained from chicken mRNA (see the Materials and methods section). The nucleotide sequence of the cDNA was determined and was used to predict the amino acid sequence. The deduced 307-amino-acid protein was referred to as 'avUCP'. A triplicated motif present in all mitochondrial transporters (see [18] for a review) was identified in avUCP, indicating that it belongs to the mitochondrial anion-carrier family (Figure 1). An alignment of amino acid sequences of mouse UCP1, UCP2 and UCP3 and avUCP is shown in Figure 1. The predicted amino acid sequence of avUCP deduced from the nucleotide sequence is 55, 70, 70 and 46 % identical with mammalian UCP1, UCP2 and UCP3 and plant UCP respectively. To illustrate the evolutionary relationship between all UCPs, a phenogram was constructed for the set of animal and plant UCPs. It showed that this avian UCP homologue was equidistant from mammalian UCP3 and UCP2 and belongs to the subfamily of the UCPs (Figure 1). In mammals, *UCP2* and *UCP3* are adjacent genes and one out of these two genes probably results from the duplication of the other one [19,20]. However, it is unknown whether birds have adjacent *UCP* genes on a chromosome and when the duplication event occurred during species evolution.

Analysis of avUCP mRNA in chicken tissues revealed a 1.8 kb transcript uniquely present in skeletal muscle (Figure 2a). The avUCP probe also hybridized with a muscle-specific duckling homologue of similar size (results not shown). The pattern of expression of *avUCP* therefore resembles that of the mammalian *UCP*3, which is predominantly expressed in skeletal muscles [21–23] and differs from that of the ubiquitous UCP2 [24].

A Northern-blot analysis of total RNA from two experimental Rhode Island Red chicken lines divergently selected for their low and high efficiency of food utilization for growth was carried out. The relative abundance of avUCP mRNA was calculated to be 1.3-fold higher in skeletal muscle of the inefficient line (Figure 2b). It suggested a possible role for this UCP and mitochondrial oxidation in the increased energy dissipation of the inefficient chickens resistant to obesity. However, the lack of experimental data supporting a lower respiratory coupling of skeletal-muscle mitochondria from inefficient relative to efficient chickens does not allow us to put forward this hypothesis. Further biochemical studies are required to examine this point and assess whether the higher abundance of avUCP mRNA may be related to alterations of muscle mitochondrial energetics in the inefficient line.

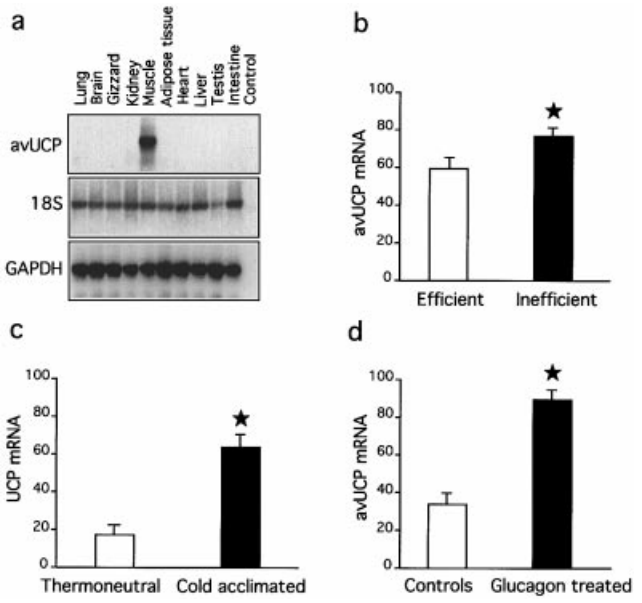

Figure 2 Pattern of expression of avUCP mRNA in chicken tissues (a), and relative abundance in leg skeletal muscle of energetically efficient or inefficient chickens (b), thermoneutral (25 °C) control or cold-acclimated (4 °C) 5-week-old Muscovy ducklings (c), and vehicle- or glucagon-treated (100 μg/kg intraperitoneally twice daily) 5-week-old ducklings reared at thermoneutrality (d)

In (**a**), expression was investigated by RT-PCR followed by a Southern blot with specific avUCP, 18 S or GAPDH probes ($n = 5$); similar data were obtained using Northern-analysis (results not shown). In (**b**), (**c**) and (**d**), relative expression was assessed by Northern blots and expressed in arbitrary units normalized to the 18 S rRNA level. Values are means ± S.E.M. The asterisks (*) indicates a significant ($P < 0.05$, $n = 5$, Student's t test) effect of line or treatment.

We analysed the expression of *avUCP* in cold-acclimated or chronically glucagon-treated ducklings, two avian models showing increased muscle NST based in part on an uncoupling of mitochondrial respiration [9,17]. These treatments markedly increased the relative expression of the duckling homologue of avUCP in skeletal muscles by 3.6- and 2.6-fold after cold-acclimation and glucagon treatment, respectively (Figures 2c and 2d). avUCP therefore resembles mammalian UCP1, which is strongly induced during exposure to cold [13–15] and is essential for resistance to cold in mice [12]. However, no induction of avUCP mRNA was observed in adult chickens exposed to the cold for only 4 h (results not shown). The presence and regulation of the duckling homologue of avUCP therefore provides a putative molecular mechanisms for the loose coupling of mitochondrial respiration observed in the skeletal muscles from cold-acclimated or glucagon-treated ducklings. Similarly, overexpressing human UCP3 in mice skeletal muscle induces hyperphagia (over-eating) and leanness and is associated with mitochondrial uncoupling [25].

Previously, it was shown that the expression of UCP1 [26,27] or UCP2 [24,28] in baker's yeast (*Saccharomyces cerevisiae*) induced an uncoupling of respiration from ATP synthesis. To further delineate the biochemical activity of avUCP, we tried to overexpress it in *S. cerevisiae*. However, for unknown reasons,

we failed to detect a significant level of the chicken UCP in yeast and therefore no functional data were obtained.

At this stage, we cannot demonstrate that avUCP has an uncoupling activity. However, the unique expression of this gene in skeletal muscles of birds, as well as its up-regulation after cold acclimation, or following treatment of animals by glucagon, two treatments inducing muscle NST, or in association with avian DIT, support a role for avUCP in energy expenditure in birds. Interestingly, avUCP shares certain properties with UCP1 and *Solanum tuberosum* (potato) UCP (i.e. induction by cold exposure), and with UCP3 (i.e. expression restricted to skeletal muscles). The greater identity of *avUCP* with mammalian UCP2 and UCP3 than with UCP1 may suggest that the function of *Gallus gallus* UCP is more related to those of UCP2 and UCP3 rather than specifically to UCP1. Nevertheless, the present findings will serve to stimulate an understanding of the mechanisms of facultative thermogenesis in birds. The functional activity and control of expression of avUCP now remain to be determined.

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