Cloning and Characterization of an Uncoupling Protein Homolog

A Potential Molecular Mediator of Human Thermogenesis

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We have identified a novel cDNA encoding a protein highly homologous to the mammalian brown fat uncoupling protein (UCP). Unlike the known UCP, which is expressed specifically in brown adipose tissue, the UCP homolog (UCPH) mRNA is expressed in a variety of tissues, with predominant expression in human white adipose tissue and skeletal muscle. In the white adipose tissue of *ob/ob* and *db/db* mice, the UCPH transcript is induced approximately fivefold relative to lean littermate controls. Expression of murine UCPH in yeast results in growth inhibition under conditions that require aerobic respiration, but does not affect growth under anaerobic conditions. Furthermore, UCPH expression in yeast causes a decrease in the mitochondrial membrane potential, as judged by staining with the potential-sensitive dye $DiOC_6$. These observations suggest that UCPH, like UCP, uncouples oxidative phosphorylation. The possibility that the UCPH protein is an important mediator of human thermogenesis is discussed. *Diabetes* 46:900–906, 1997

The regulation of body fat in mammals is a complex process involving the regulation of not only appetite but also energy expenditure. An important component of energy expenditure is nonshivering thermogenesis (NST). In rodents, the majority of NST appears to occur in brown adipose tissue (BAT) via the uncoupling protein (UCP) (1,2). UCP is a proton channel located exclusively in the inner mitochondrial membrane of adipocytes of the BAT (3). By allowing protons to equilibrate across the inner mitochondrial membrane, UCP uncouples oxidative phosphorylation and thus converts stored energy into heat rather than work (4,5). UCP-mediated uncoupling not only is capable of increasing body temperature in cold-acclimatized rodents and hibernating animals, but also can dissipate surplus caloric energy (6–8). A number of studies have now implicated UCP and BAT as important regulators of body weight in rodents (8–11).

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BAT, brown adipose tissue; EST, expressed sequence tag; FACS, fluorescence-activated cell sorter; NST, nonshivering thermogenesis; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SC, synthetic complete; UCP, uncoupling protein; UCPH, UCP homolog.

In humans, regulated thermogenesis is also thought to be an important component of metabolism and body weight homeostasis (12–14). However, the importance of the UCP in adult humans is questionable because of the low levels of BAT and, consequently, the low levels of UCP expression (15–18). In adult humans and other animals that do not contain large amounts of BAT, a large portion of NST and regulated thermogenesis is thought to be mediated by muscle and, to a lesser extent, the white adipose tissue (14,19–23); however, the molecular mediators for this are currently unknown (24). In this report, we describe the identification and characterization of mouse and human cDNAs that encode proteins sharing functional properties and sequence similarity with the known UCP.

RESEARCH DESIGN AND METHODS

Cloning of murine and human UCP homolog. A mouse spleen cDNA library was constructed in the Uni-ZAP vector (Stratagene, La Jolla, CA). Random cDNA clones were isolated from the library and sequenced to generate a database of expressed sequence tags (ESTs) that were compared with the GenBank sequence database using the BLASTX algorithm (25). This analysis identified an EST that is predicted to encode a peptide that shares a high level of similarity to the brown fat UCP. A 1.6-kb cDNA containing the entire mouse UCP homolog (UCPH) open reading frame was isolated from the same library by screening with a ^{32}P-labeled DNA probe using standard high stringency conditions. A 5′-truncated human UCPH clone was isolated by screening a human spleen cDNA library (Stratagene) under low stringency conditions with the mouse UCPH cDNA probe. The 5′-end of the human UCPH cDNA was identified by 5′-RACE (rapid amplification of cDNA ends) using a commercially available RACE kit and human skeletal muscle 5′-RACE–ready cDNA (Clontech Laboratories, Palo Alto, CA). The full-length human UCPH cDNA was constructed by inserting the 5′-RACE product into the original 5′-truncated human UCPH clone using standard molecular biology techniques.

The accession numbers for the protein and nucleotide sequences reported in this paper are U94593 (mouse) and U94592 (human).

RNA preparation and Northern blotting. Mouse and human poly A$^+$ RNA blots were obtained from Clontech. Total RNA was isolated using guanidinium thiocyanate extraction and cesium chloride centrifugation (26) from the following tissues: Human skeletal muscle and white adipose tissue were obtained from a nondiabetic man with normal body weight (BMI 22.8) who underwent surgery for coronary artery disease. Mouse white and brown adipose tissue was obtained from three or four C57BL/6J animals. To determine the levels of UCPH expression in genetically obese animals, white adipose tissue was obtained from three or four animals each of C57BL/6J *ob/ob*, C57BL/6J +/?, C57BL/KsJ +*db*/+*db*, or C57BL/KsJ m+/m+ (Jackson Laboratories, Bar Harbor, ME). There was 15 μg of total RNA electrophoresed on a 1.3% agarose/formaldehyde gel and transferred onto nylon membranes. Blots were probed with ^{32}P-labeled DNA probes using the Rapid-Hyb buffer (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Expression of UCP and UCPH in *S. cerevisiae*. Yeast media were prepared and yeast molecular biology techniques were performed as described (27). Proteins were expressed in either the W303 genetic background (RGY12: *MATa/α ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 trp1-1/TRP1 ade2-100/ADE2 can1-100/can1-100* Gal$^+$; constructed

from strains obtained from G. Fink, Whitehead Institute, Boston, MA) or the S288C genetic background (CKY8: *MATα ura3-52 leu2-3,112* Gal$^-$; obtained from C.A. Kaiser, M.I.T., Boston, MA). Murine UCP or murine UCPH were expressed from the *GAL1* promoter in pYES2 (*URA3*, 2μ; Invitrogen, San Diego, CA) using a gap repair strategy (28). Polymerase chain reaction (PCR) primers were designed that amplified the open reading frame of UCP or UCPH fused to 30 nucleotides of the *GAL1* promoter (nucleotide −35 to −5) at the 5′-end and to 30 nucleotides of the *CYC1* terminator at the 3′-end. PCR products were cotransformed with linearized pYES2 (digested with *Hind*III and *Xba* I) into CKY8, and plasmids were recovered from several transformants and analyzed by restriction digestion and DNA sequencing. Typically, >80% of transformants contained plasmids with the expected inserts. Strains used for functional assays were generated by retransforming a plasmid whose entire insert sequence had been confirmed by DNA sequencing into CKY8 and RGY12.

Deletion mutants lacking amino acids 268–270 of murine UCP (UCPΔ3) or amino acids 270–272 of murine UCPH (UCPHΔ3) were constructed by site-directed mutagenesis (29). Mutants were fused to the *GAL1* promoter in pYES2, as described above, verified by DNA sequencing, and expressed in CKY8 or RGY12.

For growth assays, strains were pregrown overnight on synthetic complete (SC) medium lacking uracil and supplemented with 2% glucose. To induce expression from the *GAL1* promoter, strains were plated onto SC medium lacking uracil and supplemented either with 2% raffinose and 2% galactose (anaerobic growth) or with 3% glycerol and 2% galactose (aerobic growth). Growth was assayed at 30°C either under normal atmospheric conditions or in an anaerobic chamber (BBL Gas Pak Pouch, Becton Dickinson, Cockeysville, MD).

For measurements of the mitochondrial membrane potential, strains were pregrown in SC medium lacking uracil and supplemented with 2% raffinose. At a density of 4×10^6 cells/ml, galactose was added to 2% and strains were allowed to grow for an additional 5 h. The cell concentration was then adjusted to 2×10^6 cells/ml, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; Sigma, St. Louis, MO) was added to a final concentration of 10 ng/ml, and fluorescence was measured 10–30 min later in a fluorescence-activated cell sorter ([FACS] FACSCalibur; Becton Dickinson). Where indicated, 200 μmol/l of the chemical uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma) was added 5 min before addition of $DiOC_6$. A total of 10,000 objects were counted for each analysis. Dead cells were identified by staining with propidium iodide (2 μg/ml final concentration; Molecular Probes, Eugene, OR) and represented <2% of cells in all samples assayed. Debris and clumped cells were identified by forward and side scatter and were excluded from the analysis. Analysis was performed using the CellQuest software (Becton Dickinson).

RESULTS

Identification of a mouse cDNA encoding a protein highly homologous to the brown fat uncoupling protein. Screening of Millennium sequence databases for candidate obesity genes identified a partial cDNA clone that was predicted to encode a protein fragment with sequence similarity to a region of the mammalian brown fat UCP (see METHODS). A full-length cDNA was then isolated and sequenced. The deduced amino acid sequence of the encoded protein showed 56% identity to the known mouse UCP throughout its length (Fig. 1*A*). Many of the nonidentical amino acids are conservative substitutions and correspond to residues showing variation among the known uncoupling proteins from various mammalian species (30). We have designated this new protein the uncoupling protein homolog (UCPH). More recent searches of the GenBank sequence database revealed an unpublished mouse cDNA sequence (UCP2; GenBank accession number U69135) identical to UCPH; furthermore, a number of mouse and human expressed sequence tags likely to correspond to UCPH are now present in the database. The high degree of sequence similarity between UCP and UCPH suggests that the UCPH protein may perform a biochemical function similar to UCP.

Several other proteins showing more limited similarity to UCP have been identified previously, all of which are known or suspected to be mitochondrial inner membrane transporters. These proteins include the ATP/ADP transporter, the phosphate transporter, and the oxaloacetate transporter (30,31). Sequence alignment of these related transporters with UCP shows a much lower degree of homology than observed between UCP and UCPH (Table 1). The amino acid identities between UCP or UCPH and these transporters ranged from about 17 to 31%—significantly lower than the 56% identity observed between UCP and UCPH. This further indicates that the UCPH protein not only may be a mitochondrial inner membrane transport protein, but may be a second uncoupling protein with weight regulatory and thermogenic properties similar to the ones demonstrated for the known UCP.

Cloning of human UCPH. To identify a human UCPH and address the extent of sequence conservation, we isolated and sequenced a cDNA clone hybridizing to mouse UCPH from a human spleen library. The amino acid sequences between mouse and human UCPH are 95% identical (Fig. 1*B*). The degree of amino acid identity between mouse and human UCPH is even higher than that observed between mouse and human UCP (79%) (32,33). This high degree of amino acid identity, which is also reflected at the nucleotide level (see GenBank submission), strongly argues that the isolated cDNA encodes the human UCPH protein.

UCPH mRNA expression. Unlike UCP, which is expressed specifically in BAT, UCPH is expressed in a variety of tissues. Low levels of the human UCPH mRNA are detectable by poly-A$^+$ mRNA Northern blots in most tissues, including heart, placenta, lung, liver, kidney, and pancreas (Fig. 2*A*, left panel). Interestingly, much higher levels of UCPH mRNA are present in skeletal muscle, an important thermogenic organ in humans (14,20–22). Analysis of total RNA Northern blots (Fig. 2*A*, right panel) revealed that the level of UCPH transcript in white adipose tissue exceeded even that found in muscle.

The tissue distribution of UCPH was also examined in the mouse (Fig. 2*B*). Similar to human UCPH, mouse UCPH mRNA is expressed at low levels in most tissues examined. However, mouse UCPH mRNA is present at only low levels in skeletal muscle (Fig. 2*B*, left panel) and, in fact, was undetectable in skeletal muscle on total RNA blots (R.E.G., M.D., X.W., N.D., A.W.S., C.J.G., F.I., S.J.E., E.A.W., L.A.T., unpublished observations). As was the case in humans, the mouse UCPH transcript was readily detectable in white adipose tissue, even on total RNA Northern blots (Fig. 2*B*, right panel). UCPH RNA was also detected in BAT, but at levels severalfold lower compared with white adipose tissue (Fig. 2*B*, right panel).

To test whether the UCPH protein was regulated by obesity, we isolated total RNA from the white adipose tissue of *ob/ob* mice, *db/db* mice, and their lean littermate controls. There was a clear four- to sixfold increase in steady-state UCPH transcript levels in *ob/ob* and *db/db* mice relative to lean littermate controls (Fig. 2*C*). Thus, UCPH mRNA levels are upregulated in two genetic models of obesity.

The UCPH protein uncouples oxidative phosphorylation. The UCP protein is known to transport protons across the mitochondrial inner membrane, thereby reducing proton motive force and allowing caloric energy to be dissipated in the form of heat (4,5). The biochemical properties of UCP have previously been studied in yeast expressing mammalian UCP by measuring defects in aerobic growth (34) and by detecting decreases in the mitochondrial membrane potential using potential-sensitive dyes such as $DiOC_6$ (35). To test

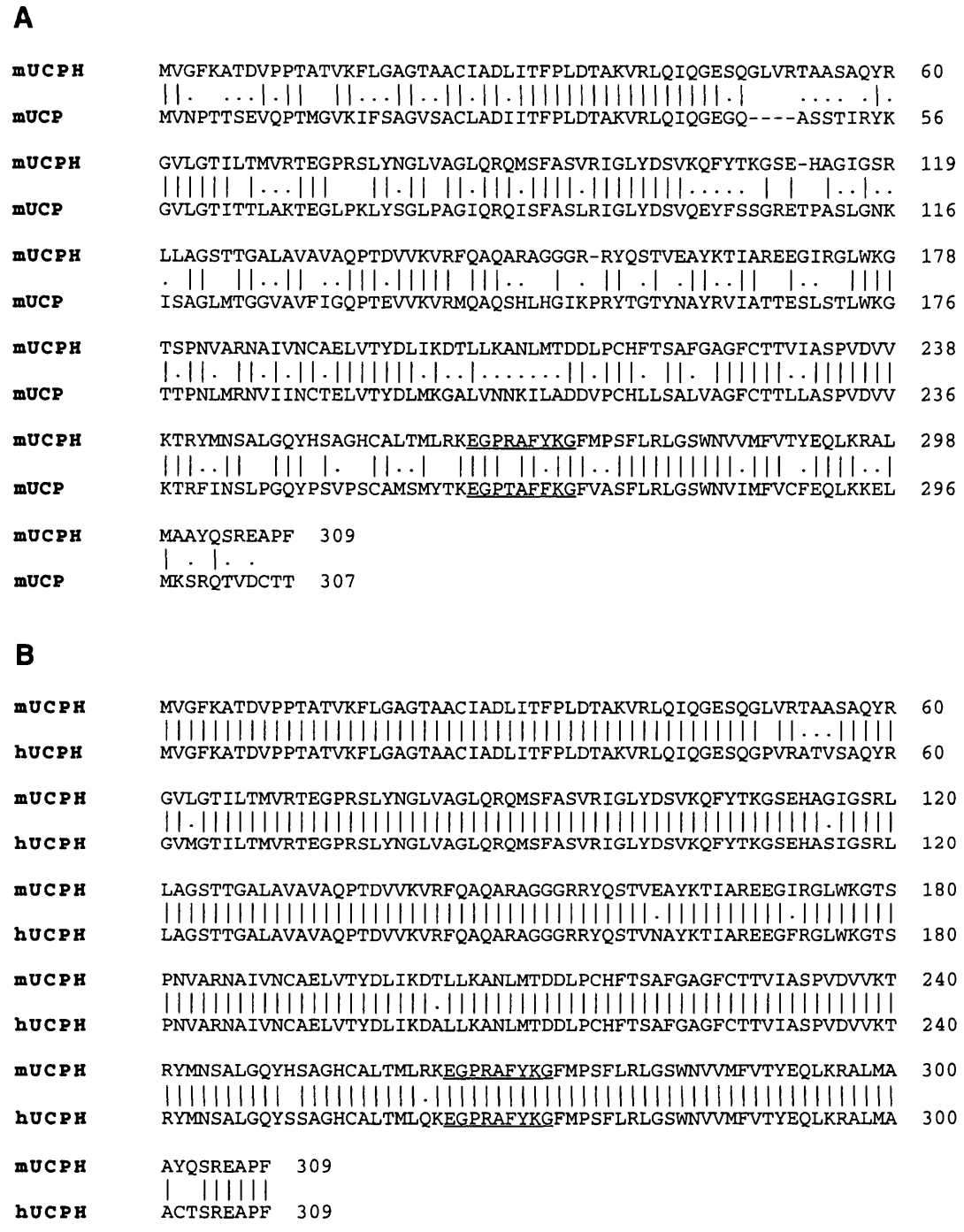

FIG. 1. Comparison of the amino acid sequences of murine UCP (mUCP) (32) and murine UCPH (mUCPH) (*A*), and mUCPH and human UCPH (hUCPH) (*B*). Amino acid identities are indicated by lines, conservative substitutions are indicated by dots. The putative nucleotide binding sites are underlined. Alignments were performed using the ALIGN program (40).

TABLE 1
Amino acid identities (%) between members of the mitochondrial carrier family

	UCP	UCPH	Phosphate	Oxoglutarate	ATP/ADP
UCP	—	56.3	16.8	29.7	19.2
UCPH	56.3	—	19.4	30.6	22.5
Phosphate	16.8	19.4	—	22.0	18.7
Oxoglutarate	29.7	30.6	22.0	—	23.5
ATP/ADP	19.2	22.5	18.7	23.5	—

Sequence comparisons were done using the ALIGN program (40). Sequences used were murine UCP (P12242), murine UCPH (U69135), rat phosphate carrier (P16036), human oxoglutarate/malate carrier (Q02978), and murine ATP/ADP carrier (423368).

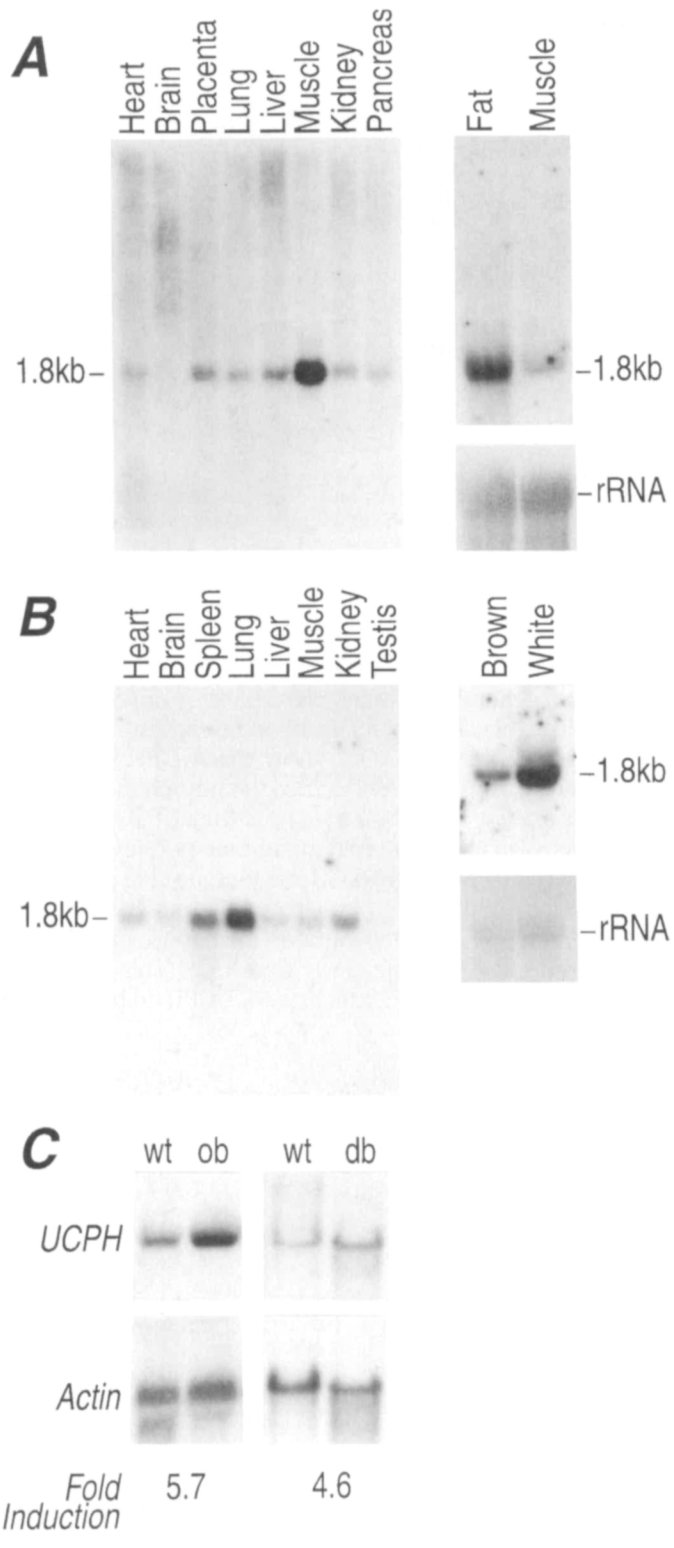

FIG. 2. Tissue distribution of UCPH. *A*: Northern blot analysis of UCPH expression in human tissues. Left panel: 2 µg poly-A⁺ mRNA. Right panel: 15 µg total RNA. 28S rRNA is shown as a loading control. *B*: Northern blot analysis of UCPH expression in mouse tissues. Left panel: 2 µg poly-A⁺ mRNA. Right panel: 15 µg total RNA. 28S rRNA is shown as a loading control. *C*: UCPH expression is upregulated in the white adipose tissue of *ob/ob* and *db/db* mice. There was 15 µg of total RNA loaded in each lane. The relative amounts of UCPH and control actin transcripts were quantitated by densitometry, and fold-induction was calculated as: [amount UCPH (*ob,db*)/amount UCPH (wt)] × [amount actin (wt)/amount actin (*ob,db*)].

whether UCPH shares uncoupling properties with UCP, we expressed the mouse UCP and UCPH proteins in yeast and compared their ability to inhibit aerobic growth. Proteins were expressed from the strong inducible *GAL1* promoter in two different yeast genetic backgrounds (W303 and S288C). UCPH was expressed at levels similar to UCP in both strain backgrounds, as judged by Western blotting of C-terminally myc-tagged variants of UCP and UCPH (data not shown). When grown on media where ATP is derived primarily by aerobic respiration (glycerol/galactose) (36,37), strains expressing UCPH had a dramatically reduced growth rate relative to strains containing expression vector only (Fig. 3*A*). Importantly, the extent of growth inhibition by UCPH was similar to that observed with the known UCP (Fig. 3*A*). To control for the possibility that the growth inhibition is due to nonspecific toxicity, as opposed to a disruption of aerobic respiration, we examined the same yeast strains under conditions of anaerobic growth (Fig. 3*B*). Under these conditions, strains containing vector only and strains expressing UCPH or UCP grew equally well (Fig. 3*B*). Thus, UCPH, like UCP, specifically affects growth under aerobic conditions, consistent with a dissipation of the proton motive force by UCPH.

To demonstrate more directly that the UCPH protein can dissipate the proton motive force, we compared the ability of yeast strains expressing UCPH or UCP and a strain containing vector only to be stained by a fluorescent dye ($DiOC_6$) that is sensitive to the electrochemical gradient across the mitochondrial inner membrane (36,38). A previous study showed that while wild-type UCP expressed in yeast does not affect $DiOC_6$ staining, a three amino acid deletion in the putative nucleotide binding site of UCP (UCPΔ3, lacking amino acids 268–270) leads to activation of UCP and a significant decrease in the mitochondrial membrane potential of the host strain (35). We constructed the equivalent deletion in UCPH (UCPHΔ3, lacking amino acids 270–272). UCPΔ3 and UCPHΔ3 inhibited aerobic growth to an extent similar to wild-type UCP or UCPH (Fig. 3*A*). Consistent with previously published data, we found that UCPΔ3 and, to a lesser extent, UCP decreased $DiOC_6$ staining (Figs. 4*A* and *B*). The difference between UCP and UCPΔ3 was more pronounced in the S288C background (Fig. 4*A*), but was also detectable in the W303 background (Fig. 4*B*). Expression of both wild-type UCPH and the UCPHΔ3 mutant decreased the mitochondrial membrane potential to a similar or even larger extent compared with UCPΔ3 in both backgrounds examined (Figs. 4*A* and *B*). The effects of UCPH or UCPHΔ3 expression were most obvious in the W303 genetic background; in this background, a portion of the cells showed a complete dissipation of the mitochondrial membrane potential (Fig. 4*B*). These data indicate that UCPH can dissipate the proton motive force and thus uncouple oxidative phosphorylation.

DISCUSSION

We have identified and cloned murine and human cDNAs encoding proteins 56–60% identical to the known UCP, an important mediator of NST. We have designated this novel protein the uncoupling protein homolog (UCPH). Two lines of evidence suggest that UCPH is not simply a new member of the mitochondrial inner membrane transporter family, but is a novel uncoupling protein that may have weight regulatory and thermogenic properties similar to the known UCP. First, the degree of sequence similarity between UCP and UCPH

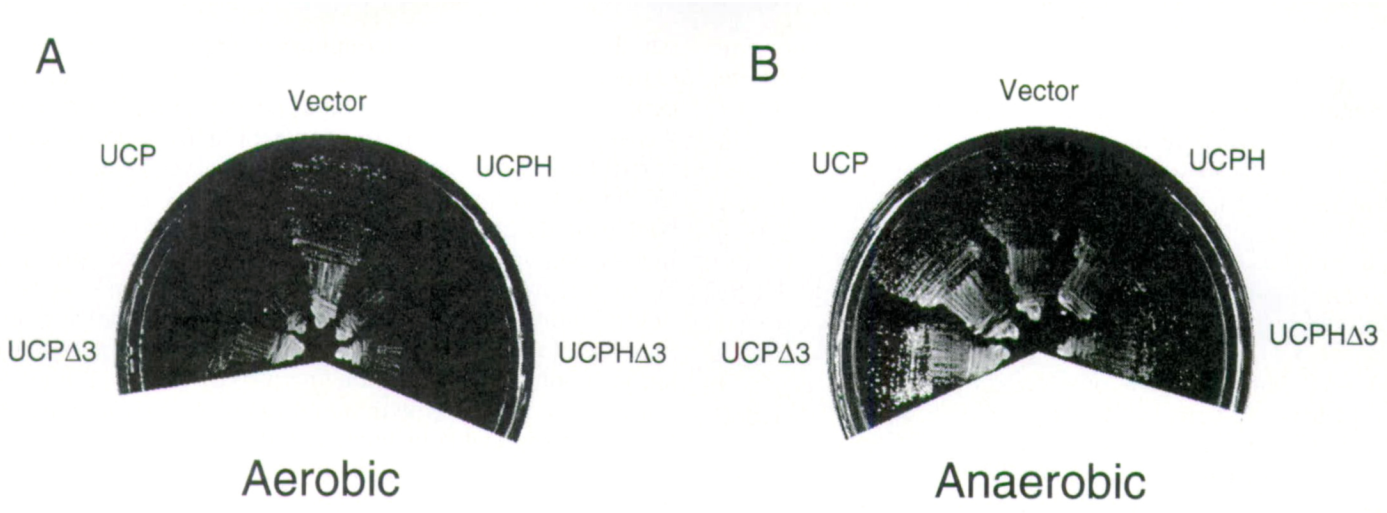

FIG. 3. UCPH expressed in *S. cerevisiae* inhibits growth under aerobic, but not under anaerobic, conditions. The yeast strain RGY12 containing the indicated expression constructs or vector only was grown at 30°C on selective medium containing 3% glycerol/2% galactose (*A*) or 2% raffinose/2% galactose (*B*). Growth was for 48 h under normal atmospheric conditions (*A*) or for 10 days in an anaerobic chamber (*B*). Similar results were obtained when proteins were expressed in CKY8 (not shown).

(56–60%, depending on species) is considerably higher than that observed among other members of the mitochondrial transporter family (17–31%). This high degree of amino acid identity is particularly striking when compared with the ~80% identity found among UCPs from different species (5) and suggests that the biochemical functions of UCP and UCPH are similar. Second, a series of yeast experiments that had previously been shown to successfully detect the uncoupling activity of the known UCP (34,35) reveal a similar activity for UCPH. As observed for UCP, expression of UCPH in yeast results in growth inhibition on a carbon source that promotes growth mainly through aerobic respiration. This growth inhibition is probably due to a specific disruption of respiration rather than generalized toxicity because it is not observed in yeast cells grown anaerobically. In addition, studies with the potential-sensitive dye $DiOC_6$ show that UCPH expression disrupts the proton gradient across the mitochondrial membrane to a similar or even larger extent than UCP expression. The decrease in mitochondrial membrane potential together with the growth phenotypes strongly argue that UCPH has uncoupling activity.

Interestingly, UCPH was found to have more pronounced effects in the dye-staining assay than UCP. This difference could reflect a higher specific activity of UCPH compared with

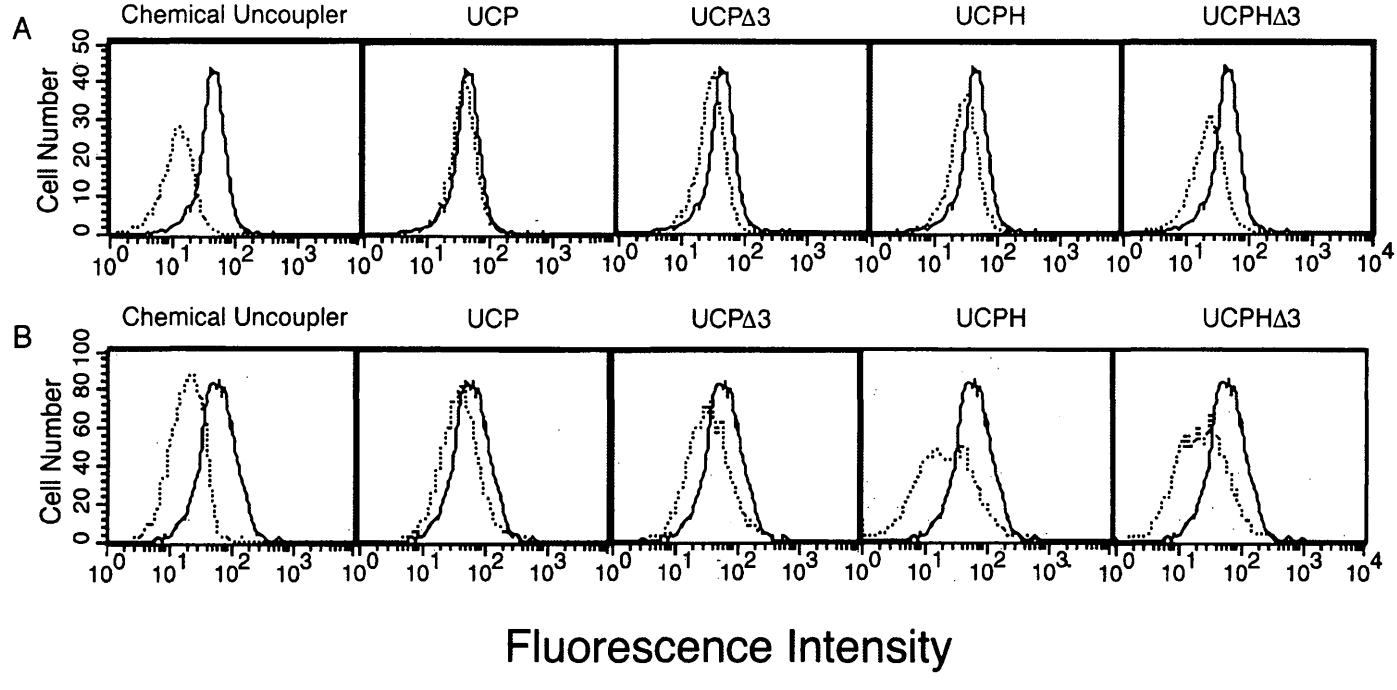

FIG. 4. UCPH expressed in *S. cerevisiae* decreases the mitochondrial membrane potential. Yeast strains CKY8 (*A*) or RGY12 (*B*) containing the indicated expression constructs or vector only were stained with the potential-sensitive dye DiOC6 and analyzed by FACS, as described in METHODS. Solid lines indicate strains containing vector only; dotted lines indicate strains containing the indicated expression plasmids or a vector only strain treated with 200 μmol/l of the chemical uncoupler CCCP.

UCP, or it could indicate that the activity of the two proteins is regulated differently. A single amino acid mutation in the inhibitory nucleotide binding site of UCP (Phe_{268} to Tyr) creates a UCP molecule that has higher uncoupling activity in the dye-staining assay (35); both mouse and human UCPH naturally contain a tyrosine at the equivalent position (Tyr_{270}) and may thus be less susceptible to the inhibitory effects of nucleotides. Furthermore, the extreme COOH-terminus of UCP, a region that has been implicated in regulation of UCP activity by fatty acids (39), is less well conserved between UCPH and UCP, possibly resulting in differences in regulation. Given the importance of posttranslational regulation for the biological function of UCP (4,5), it will be important to examine how UCPH activity is regulated by small molecules.

To further evaluate the potential role for UCPH in thermogenesis and body weight regulation, we assessed the tissue distribution of the UCPH transcript in mice and humans. On poly-A^+ Northern blots, low levels of the UCPH transcript can be detected in a large number of tissues in both mice and humans. Interestingly, the highest levels of expression are observed in the white adipose tissue in both mice and humans. In addition, human UCPH mRNA is also highly expressed in muscle relative to many other tissues. The differences in expression observed between mice and humans may be partly due to the relative importance of these tissues to the overall thermogenesis of the organism: in mice, the majority of regulated thermogenesis is thought to be mediated via the brown fat with other tissues playing a more minor role (1,2,24), while in adult humans, the majority of regulated thermogenesis is thought to take place in muscle and, to a lesser extent, in white fat (14,20–22). Thus, the presence of high levels of UCPH transcript in human muscle and white fat is consistent with a role for UCPH in mediating thermogenesis in humans.

A tantalizing suggestion for a role of UCPH in body weight homeostasis comes from the observation that the UCPH mRNA is regulated in the context of obesity. In *ob/ob* and *db/db* mice, UCPH mRNA was found to be upregulated approximately fivefold. Because increased UCPH expression would be expected to decrease rather than increase body weight, the obesity of *ob/ob* and *db/db* mice cannot be mediated by defective UCPH expression. We propose that the upregulation of UCPH expression is a compensatory change in which UCPH induction is attempting to act against powerful genetically induced obesity.

Our data suggest that UCPH is an excellent candidate for a molecular mediator of human thermogenesis and body weight regulation. Future studies should be directed toward evaluating the importance of UCPH in mammalian thermogenesis and body weight control through mouse knockout and transgenic studies. In addition, an understanding of the regulation of the UCPH transcript and activity is critical in evaluating its role in normal physiology as well as in developing methods to increase the effective activity of this molecule for potential therapeutic applications.

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