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Contribution to the Identification and Analysis of the Mitochondrial Uncoupling Proteins

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This review is primarily focused on the contribution of our laboratory to study of the mitochondrial uncoupling UCPs. The initial stage was the description of a 32-kDa membranous protein specifically induced in brown adipose tissue mitochondria of cold-adapted rats. This protein was then shown by others to be responsible for brown fat thermogenesis and was referred to as the uncoupling protein-UCP (recently renamed UCP1). cDNA and genomic clones of UCP1 were isolated and used to investigate the topology and functional organization of the protein in the membrane and the mechanisms of control of UCP1 gene transcription. Orientation of the transmembrane fragments was proposed and specific amino acid residues involved in the inhibition of UCP1 by purine nucleotides were identified in recombinant yeast. A potent enhancer mediating the response of the UCP1 gene to retinoids and controlling the specific transcription in brown adipocytes was identified using transgenic mice. More recently, we identified UCP2, an UCP homolog widely expressed in human and rodent tissues we also collaborated to characterize the plant UCP. Although the biochemical activities and physiological roles of the novel UCPs are not well understood, these recent data stimulate research on mitochondrial carriers, mitochondrial bioenergetics, and energy expenditure.

KEY WORDS: Mitochondria; uncoupling; UCP; thermogenesis; brown adipose tissue; fatty acid oxidation; mitochondrial carrier; membrane; transport.

Our laboratory has been working on thermogenic brown adipocytes for many years. It led us to contribute to the discovery of the first uncoupling protein (UCP, now referred to as UCP1), unique to brown fat mitochondria. We analyzed the functional organization of this protein, the specific transcription of the UCP1 gene in brown adipocytes, and its molecular regulation by catecholamines and retinoids. More recently, we identified UCP2 as an UCP homolog which is expressed in most tissues (Fleury *et al.*, 1997) and also were the first to characterize a plant UCP (Laloi *et al.*, 1997). We also identified BMCP1, another mitochondrial carrier predominantly expressed in brain which shows an uncoupling activity when it is expressed in yeast (Sanchis *et al.*, 1998). Since this review was primarily written to cover our contribution to the study of these mitochondrial uncoupling proteins, the reference to work of others will be limited. Several reviews on these UCPs were published over the past years (Nicholls and Locke, 1984; Klingenberg, 1990, Klaus *et al.*, 1991b; Ricquier *et al.*, 1991; Nedergaard and Cannon, 1992; Himms-Hagen and Ricquier, 1997; Ricquier and Bouillaud, 1997; Silva and Rabelo, 1997; Boss *et al.*, 1998; Jezek and Garlid, 1998).

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PHYSIOLOGICAL AND BIOCHEMICAL BASIS OF RESPIRATION UNCOUPLING—CONTRIBUTION TO UCP1 DISCOVERY

BAT and Regulatory Thermogenesis

In mammals, birth, exposure to the cold, or arousal from hibernation, are physiological situations in which an elevated thermogenesis is required. Although most organs contribute to thermogenesis, it was demonstrated that the brown adipose tissue (BAT) was a major site of adaptive or regulatory thermogenesis in rodents (reviews in Nicholls and Locke, 1984; Nedergaard and Cannon, 1992; Himms-Hagen and Ricquier, 1997). BAT is composed of characteristic fat deposits present in particular anatomical areas in newborns of most species and in rodents through their whole life-span. However, this tissue is scarce in large size adult mammals. BAT is a thermogenic organ of which the activity is controlled by the central nervous system and sympathetic fibers, which directly innervate the brown adipocytes. BAT is an important contributor to nonshivering thermogenesis induced by a cold environment and also participates in diet-induced thermogenesis (review in Himms-Hagen and Ricquier, 1997). Acute exposure of rodents to the cold activates norepinephrine release at the surface of brown adipocytes, which immediately activates thermogenesis. Activation of brown adipocytes is followed by an immediate increase in blood flow through the organ. Prolonged exposure of rats to the cold activates BAT growth, which also is under the control of catecholamines. Analysis of the thermogenic mechanism in brown adipocytes led Lindberg's group and Smith's group to propose that a natural uncoupling of respiration was present in these cells; several researchers then observed that this uncoupling mechanism was activated by free fatty acids and inhibited by purine nucleotides (reviews in Nicholls and Locke, 1984; Nedergaard and Cannon, 1992). In particular, David Nicholls and his collaborators reported that the brown fat mitochondria were characterized by a unique high proton conductance inhibitable by GDP and activable by fatty acids.

Identification of UCP1—the Proton Pathway Responsible for Uncoupling and Thermogenesis in Brown Fat Mitochondria

The main characteristics of the thermogenic proton pathway of brown fat mitochondria are activation by fatty acids and inhibition by GDP, GTP, ADP or ATP, uniqueness to brown fat mitochondria, induction during prolonged exposure of rats to the cold, or downregulation in developing guinea pigs. Using photoaffinity labeling of hamster brown fat mitochondria with radioactive azido-ATP, David Nicholls identified a 32-kDa protein as the putative uncoupler (Heaton *et al.*, 1978). Before the publication of these data, Ricquier and Kader (1976) had described a 32-kDa protein that was present in abundance in membranes of rat brown fat mitochondria; this protein was absent in liver mitochondria, induced during exposure of rats to the cold for several days, and downregulated when cold-exposed rats were readapted to room temperature. Following these two original studies and other studies (Desautels *et al.*, 1978), a number of physiological and biochemical analysis were carried out to further characterize the protein. It was observed that the sympathetic innervation of BAT was necessary to induce the 32-kDa protein in rats (Mory *et al.*, 1982) and that pheochromocytoma tumors induced BAT growth as well as the 32-kD component in humans or rats (Ricquier *et al.*, 1982b, 1983a; Bouillaud *et al.*, 1983). In agreement with the decreased number of GDP binding sites in BAT mitochondria of *ob/ob* mice, originally reported by Desautels and Himms-Hagen (1978), a decreased level of this 32-kD protein was measured in BAT of obese rats (Seydoux *et al.*, 1982), whereas insulin was shown to stimulate the synthesis of the protein (Seydoux *et al.*, 1984). For several years, the assay of GDP binding to isolated brown fat mitochondria was used to monitor the level or activity of UCP1 (Nicholls and Locke, 1984; Nedergaard and Cannon, 1992; Himms-Hagen and Ricquier, 1997). The major stages of the analysis of this protein, referred to as uncoupling protein or UCP (Lin and Klingenberg, 1980) and recently renamed UCP1 (Fleury *et al.*, 1997), were the purification (Lin and Klingenberg, 1980; Ricquier *et al.*, 1982a), cell-free synthesis experiments demonstrating that UCP1 was not synthesized as a larger molecular weight precursor (Ricquier *et al.*, 1983b), the observation of an increased level of UCP mRNA in BAT of cold-exposed rats (Bouillaud *et al.*, 1984), and the demonstration of a rapid activation of UCP1 gene transcription by catecholamines (Ricquier *et al.*, 1986). The amino acid sequencing of the purified protein (Aquila *et al.*, 1985) and the first cloning as a rat cDNA identified the amino acid sequence of UCP1 (Bouillaud *et al.*, 1985, 1986). The proton-translocating activity of the UCP was then reconstituted in liposomes (Klingenberg, 1990). Isolation of UCP1

antibodies by several groups (B. Cannon and P. Trayhurn, our group, see review in Nedergaard and Cannon, 1992), as well as the cloning of cDNAs by others (L. Kozak and K. Freeman, see review in Boss *et al.*, 1998), confirmed the specific expression of UCP1 in BAT. Finally, the targeted disruption of the mouse UCP1 gene in Kozak's laboratory demonstrated its importance in maintaining body temperature in cold environment (Enerbäck *et al.*, 1997).

ANALYSIS OF UCP1 TOPOLOGY AND FUNCTIONAL ORGANIZATION

Purine nucleotide binding measurement to isolated UCP1 and hydrodynamic studies in Klingenberg's laboratory demonstrated that UCP1 was a dimer with one nucleotide binding site per dimer (Klingenberg, 1990). In our laboratory, experiments were set up to approach the topology of UCP1 as well as to identify amino acid residues involved in UCP1 activity or its regulation by ligands.

Analysis of UCP1 Topology

A structural model of the folding of the mitochondrial carriers was derived from the original observation that the sequence of the adenine nucleotide translocator showed internal similarities suggesting a triplicated structure. This was confirmed when the amino acid sequence of UCP1 was determined (Aquila *et al.*, 1985; Bouillaud *et al.*, 1986), and later when the amino acid sequences of other mitochondrial carrier proteins, such as the phosphate carrier, the oxoglutarate carrier, and the citrate carrier, were elucidated. Approaching the structure of UCP1 is a difficult challenge since most structural biological methods tend to damage membrane proteins. Therefore, only indirect and poorly resolutive biochemical methods are usable. To examine the topology of a membranous protein, nonpermeant probes are needed. Permeation properties of chemical reagents are not very reliable and, therefore, macromolecules like proteases or antibodies are preferred. In comparison with proteases, antibodies offer a much better site specifity. However, to draw conclusions from experiments testing the accessibility of an antigenic recognition site, it is necessary to locate it with sufficient precision in the amino acid sequence. One possibility is to raise antibodies against a peptide reproducing a subsequence of the protein. We used another strategy based on the production of fusion proteins in *E. coli* (Miroux *et al.*, 1992, 1993). An expression library corresponding to a number of short fragments of UCP1 was made. The screening of this library using polyclonal antibodies against UCP1 allowed the determination of several antigenic sites in UCP1. Specific antibodies corresponding to the different epitopes were purified and analyzed using mitoplasts or sonicated mitoplasts. Fortunately, the antigenic sites were distributed over the entire protein sequence, which allowed a relatively complete topological study of the UCP1 (Fig. 1). These experimental data were in good agreement with the computerized model predicting six transmembranous alpha-helixes. Data obtained with other mitochondrial carriers also support this model.

Recombinant Expression of Wild-Type or Mutant UCP1 in *Saccharomyces cerevisiae* and Search for Important Amino Acid Residues

Expression of rat UCP1 in *Xenopus* eggs (Klaus *et al.*, 1990) and CHO cells, (Casteilla *et al.*, 1990) demonstrated that in both systems the UCP1 was inserted in the mitochondria. In the case of CHO cells stably expressing UCP1, a regulated uncoupling of respiration of isolated mitochondria was observed. More recently, UCP1 was addressed to skeletal muscle of transgenic mice; the mitochondria isolated from

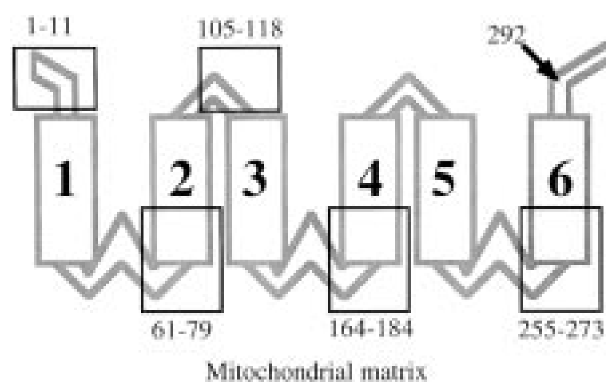

Fig. 1. Topology of rat UCP1 in the mitochondrial membrane. This picture is mostly based on identification of five antigenic sites in UCP1 by Miroux *et al.* (1992, 1993). The antigenic domains are indicated by boxes. Numbers above or below boxes refer to positions of amino acid residues. Rectangles 1 to 6 correspond to putative α-helices spanning the inner mitochondrial membrane. The arrow at position of amino acid residue 292 shows a tryptic cleavage site as demonstrated by Eckerskorn and Klingenberg (review in Klingenberg 1990).

skeletal muscles of these mice showed a marked uncoupling, activated by fatty acids and inhibited by GDP (Cassard-Doulcier, Fleury, Goubern, Ricquier, and Bouillaud, unpublished data). Most of our studies of structure/function relationship of UCP1 were made using expression in *S. cerevisiae* and were carried out in collaboration with Eduardo Rial (Arechaga *et al.*, 1993; Bouillaud *et al.*, 1994; Gonzalez-Barroso *et al.*, 1996, 1997).

Mitochondria prepared from recombinant yeast producing rat UCP1 were significantly different from mitochondria obtained from control yeast (transformed either with an empty expression vector or with a vector containing the UCP1 sequence in reverse orientation). First, the respiration of these mitochondria was less coupled than the respiration of control mitochondria; this was due to an elevated state 4. Second, it was possible to lower this state 4 respiration to the control value by the addition of millimolar concentrations of GDP. Third, these mitochondria were markedly more sensitive to the uncoupling induced by fatty acids than control mitochondria. Moreover, this fatty acid-induced uncoupling was abolished by addition of GDP (whereas such an effect was not observed with the higher concentrations of fatty acids able to uncouple control mitochondria). Therefore, introduction of UCP1 into yeast mitochondria made such mitochondria highly similar to mitochondria isolated from brown adipocytes, indicating that these recombinant yeast mitochondria could be used to examine the modifications of activity brought by mutations in the UCP1 sequence. Before discussing these experiments in more detail, some comments related to technical points or general aspects of UCP1 activity in yeast mitochondria can be made.

The whole 5′- and 3′-untranslated regions of the UCP1 cDNA cloned in yeast expression vector were removed in order to increase expression. In other respects, the use of an expression vector (pYeDP 1/8-10, gift of Denis Pompon, CNRS, Gif-sur-Yvette) that could be fully repressed, guaranteed that no selection against expressing cells occurred during preliminary phases of yeast culture prior to induction of expression in the culture used to prepare mitochondria. We also found that the use of diploid yeast strain favored the isolation of better mitochondria. Proteases inhibitors were used to avoid degradation of UCP1 during the preparation of yeast mitochondria. Finally, we observed a progressive softening of the bioenergetic properties of yeast mitochondria associated with long-term conservation of yeast in minimal medium at 4°C; retransformation of new yeast solved this problem.

Uncoupling of respiration by UCP1 is associated with reentry of protons into the matrix. There are several evidences supporting direct transport of protons by UCP1. However, it was also shown that UCP1 was able to transport other ions, such as chloride. The permeability of recombinant yeast mitochondria for protons or chloride was examined using osmotic swelling measurement. Using recombinant yeast, a proton or chloride permeability inhibitable by GDP was only observed in yeast mitochondria containing UCP1. During these experiments, it was observed that yeast mitochondria possess an endogenous uncoupling pathway referred to as YUP (yeast uncoupling pathway; Prieto *et al.*, 1992). YUP is responding to the ATP/ADP + P_i ratio and operates when ATP concentration is high and phosphate concentration is low. Therefore, YUP regulation differs from UCP1. We used millimolar phosphate concentration to prevent the activation of YUP in our experiments, with yeast expressing UCP1. Another contribution of the YUP discovery was that it stimulated our interest in the hypothetical existence of regulated uncoupling pathways in mitochondria, other than brown adipose tissue mitochondria.

Different considerations determined the choice of mutations to be introduced in UCP1. First, following the observation of dramatic permeability changes in brown fat mitochondria treated with reagents of SH groups, the importance of cysteine residues of UCP1 was studied. In fact, it was concluded that none of the cysteine residues were essential to the activity of the UCP1 (Arechaga *et al.*, 1993). However, a quantitative change in sensitivity toward fatty acids was noticed when the C-terminal cysteine residue (Cys304) was mutated. Yeast mitochondria with the Cys304Gly UCP1 mutant were more sensitive to fatty acid uncoupling than mitochondria containing wild-type UCP1, suggesting that the C-terminal region of UCP1 participated in the regulation of UCP1 activity by fatty acids (Gonzalez-Barroso *et al.*, 1996). However, the basal uncoupling activity of this mutant (in absence of added fatty acid) was not different from the basal activity of wild-type UCP1, suggesting that UCP1 acted as an uncoupler even in absence of added fatty acid.

Another clue to the design of UCP1 mutants came from the observation of sequence similarities between UCP1 and other mitochondrial carriers. UCP1 belongs to a family of related proteins, which are transporters of the inner mitochondrial membrane (*vide supra*). When sequences of the adenine nucleotide translocator

and UCP1 were compared (Aquila *et al.*, 1985; Bouillaud *et al.*, 1986), the highest similarity was noticed in their C-terminal domains (Bouillaud *et al.*, 1986). The ADP/ATP carrier binds and transports ADP and ATP, whereas UCP1 binds, but does not transport, ADP, ATP, GDP, or GTP. It was proposed that this similar domain was implicated in the recognition and binding of nucleotides by both proteins (Bouillaud *et al.*, 1986). Then it was noticed that a similar domain was present in the DNA recognition element of the second zinc finger of several transcription factors (Bouillaud *et al.*, 1992). This reinforced our interest in this domain, and stimulated us to mutate or delete this domain in UCP1. Two mutants exhibiting remarkable properties were obtained (Bouillaud *et al.*, 1994). On the one hand, the deletion of amino acid residues 267–269 suppressed the inhibition by nucleotide of the fatty-acid-activated proton transport of UCP1. On the other one hand, the deletion of this entire domain (amino acid residues 261–269) generated a mutant that was extremely deleterious to yeast growth. It was concluded that this deletion converted UCP1 into a pore allowing permeation of molecules up to 1000 D (Gonzalez-Barroso *et al.* 1997). Moreover, several attempts to isolate mammalian cells producing these mutated UCP1 invariably failed, suggesting that these UCP1 mutants had deleterious effects on mammalian cells.

As mentionned above, some UCP1 mutants were extremely deleterious for the growth of yeast, whereas expression of a significant amount of the wild-type UCP1 was well tolerated by yeast growing on galactose. A good correlation was found between the effect on growth rates and the uncoupling potency of several mutants of the UCP1 in presence or absence of fatty acids. The uptake of carbocyanine probe DiOC(6)3 by yeast is related to the mitochondrial membrane potential, but also depends on the mitochondrial volume. Dramatic changes in the uptake of the DiOC(6)3 probe were recorded when certain mutants were expressed. A correlation between the effect on growth rate, the extent of labeling of yeast by fluorescent DiOC(6)3 probe, and the level of uncoupling in isolated mitochondria was observed (Fleury *et al.*, unpublished data). All together, these observations indicate that alterations of the yeast phenotype faithfully reflect the intensity of the uncoupling effect of UCP1 mutants. These data suggest that it should be possible to set up procedures of screening of random mutants based on these phenotypical modifications in order to sort out mutants of interest.

Speculations on the Activity of the UCP1

Studies and interpretation of UCP1 activity in terms of mechanism of transport have led to a vigorous debate between several laboratories. A first question is whether fatty acids are absolutely required for uncoupling activity of UCP1. The authors of this review think they are not (Gonzalez-Barroso *et al.*, 1998). The second question deals with the mechanism operating when fatty acids are present. Although there is presently no published proof for that, the authors of this review are prone to accept the idea that UCP1 works according to two different modes of transport, depending on whether these activators are present or not.

ANALYSIS OF UCP1 GENE ORGANIZATION AND TRANSCRIPTIONAL REGULATION

The transcription of the UCP1 gene which is restricted to brown adipocytes motivated us and others to try to identify the corresponding molecular mechanisms. Following the isolation of the UCP1 gene, the search for regulatory elements was undertaken.

UCP1 Gene Organization

Rat (Bouillaud *et al.* 1988) and human (Cassard *et al.*, 1990) UCP1 genomic clones were isolated and sequenced; the mouse gene was isolated by Kozak *et al.* (1988). Sequencing of genomic clones and comparison to cDNAs revealed that the UCP1 gene is composed of six exons and has a very similar organization in the three animal species (Fig. 2). Moreover, it was observed that each exon was encoding a putative transmembrane domain of the protein. The extremities of the transcription unit of the rat UCP1 gene were identifed and 4.5 kb of DNA upstream of the transcriptional start site were sequenced (Bouillaud *et al.*, 1988). More recently, 6.3 kb of DNA in the 5′-side of the human UCP1 gene were sequenced (Cassard-Doulcier *et al.*, unpublished data).

Identification of *cis*-Regulatory Elements

In order to identify *cis*-elements regulating the transcription of the UCP1 gene, the 4.5 kb-long DNA upstream of the transcriptional start site of the rat

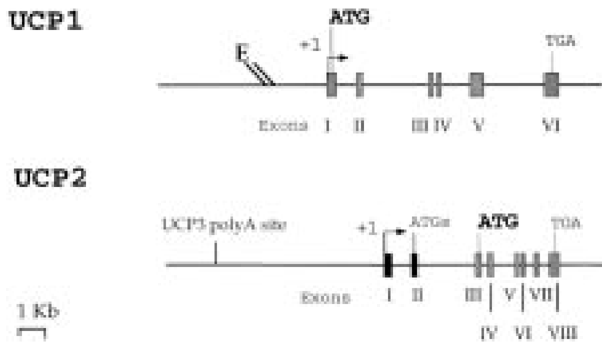

Fig. 2. Organization and comparison of UCP1 and UCP2 gene. In both genes, the coding sequence is distributed over six exons (hatched boxes); the UCP2 gene contains two 5′ untranslated exons (black boxes). Transcriptional start sites (+1) are indicated, as well as ATG initiation codons and TGA termination codons. E identifies a potent 211-bp enhancer around position −2.4 kb in UCP1 gene, (see Fig. 3). Data are from Bouillaud *et al.*, 1998 (rat UCP1 gene) and Pecqueur *et al.*, 1999 (mouse UCP2 gene). The UCP2 gene is 8.2 (Surwit *et al.*, 1998; Pecqueur *et al.*, 1999) or 7 kb (Pecqueur *et al.*, 1999) downstream of the end of the UCP3 gene on mouse chromosome 7 or human chromosome 11, respectively.

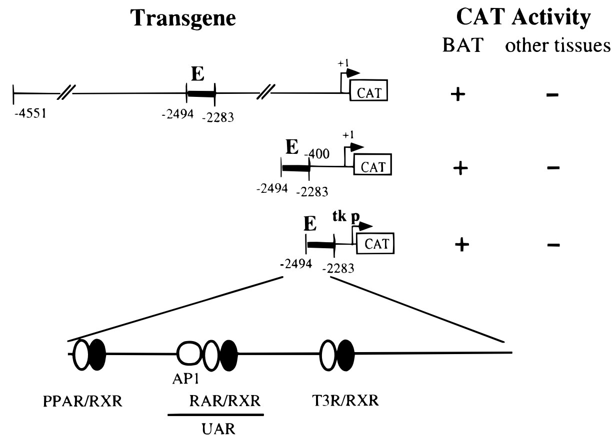

Fig. 3. Characterization of a potent enhancer controlling and regulating the specific expression of the rat UCP1 gene in brown adipose tissue. Three types of UCP1 transgenes in front of the CAT gene used as a reporter gene were introduced in transgenic mice. A long construct made of 4551 bp of the 5′ flanking region of the rat gene induced CAT uniquely in BAT of transgenic mice (Cassard-Doulcier *et al.*, 1993). In the same study, a 211-bp enhancer E, bp −2494 to −2283, relative to bp +1 taken as transcriptional start site) was identified using deleted contructs transfected in brown adipocytes. A shorter construct made of the 211-bp enhancer in front of bp−400 tp + 111 suggested a role for this enhancer in the tissue-specific transcription in transgenic mice (Larose *et al.*, 1996). This role was established using a transgene solely containing the enhancer in front of *Herpes simplex* thymidine kinase promoter (tk p) (Cassard-Doulcier *et al.*, 1998). The rat UCP1 gene enhancer has a complex organization and is composed of response elements for thyroid hormones (Silva and Rabelo, 1997), rexinoids (Sears *et al.*, 1996), and retinoids (Cassard-Doulcier *et al.*, 1994; Alvarez *et al.*, 1995; Larose *et al.*, 1996; Rabelo *et al.*, 1996). UAR (UCP gene-activating region) is composed of a binding site for RXR (retinoid X receptor)/ RAR (retinoid acid receptor) preceded by an AP1 site able to *in vitro* bind proteins of the Jun and Fos family (Larose *et al.*, 1996). PPAR, peroxisome proliferator-activated receptor.

gene was inserted in front of the chloramphenicol-acetyltransferase (CAT) gene and used as a transgene to create transgenic mice. Lines of mice with this transgene inserted in their DNA showed a specific and cold-induced expression of the CAT gene in their BAT (Cassard-Doulcier *et al.* 1993). This experiment demonstrated that the essential elements controlling UCP1 gene transcription were located in its 5′ region. A search for the exact regulatory regions was undertaken using truncations or deletions of the 4.5-kb DNA present in the CAT expression vector and transfected in cultured brown adipocytes (Klaus *et al.*, 1991a). A potent enhancer of the 211 bp located between positions bp-2494 and -2283 relative to the transcriptional start site was identified (Cassard-Doulcier *et al.*, 1993). A similar enhancer was identified in the mouse gene (L. Kozak, review in Silva and Rabelo 1997). Using transgenic mice, Cassard-Doulcier *et al.* (1998) reported that this enhancer alone was able to drive a reporter gene specifically to BAT and was controlling the specific expression of the UCP1 gene (see Fig. 3). Actually, this enhancer has a very complex organization and is made of several response elements for thyroid hormones, retinoids or rexinoids as well as binding sites for AP1, ets1, peroxisome-proliferation-activated-receptor-γ (or PPAR-γ) and NF1 (Cassard-Doulcier *et al.*, 1994; Alvarez *et al.* 1995; Larose *et al.*, 1996, Rabelo *et al.*, 1996; Sears *et al.*, 1996, review in Silva and Rabelo, 1997). These elements were characterized through analysis of *in vitro* interactions between DNA and transcriptional factors, as well as measuring effect of a number of mutations introduced in the enhancer fused to the CAT gene (see Fig. 3). The specific and regulated transcription of the UCP1 gene in brown adipocytes seems to result from interactions between several nuclear factors binding to an essential enhancer.

DISCOVERY OF UCP2 AND A UCP FAMILY

The specificity of the thermogenic mechanism present in brown adipocyte and the identification of UCP, a component unique to BAT, probably prevented researchers from postulating the existence of UCP homologs in other tissues. Actually, three different types of data were suggesting the presence of UCP homologs:

(1) Over the past years, using UCP1 cDNAs, or antibodies against UCP1, we occasionally observed some hybridization to RNA or proteins extracted from tissues other than BAT. Although they were not explained, these data suggested the existence of other mitochondrial carriers related to UCP.

(2) It was known that the coupling of respiration to ADP phosphorylation in liver or skeletal muscles mitochondria was far below 100%. Brand measured proton leaks in liver or skeletal muscle mitochondria and proposed that such leaks could explain the partial coupling of respiration and account for 20% of whole body oxygen consumption (Brand *et al.*, 1994). These data motivated us to speculate that, besides brown fat mitochondria, which possess a unique proton translocator identified as UCP1, the mitochondria of other tissues might contain proton carriers, possibly related to UCP1.

(3) The recent complete sequencing of the genome of *S. cerevisiae* yeast revealed the putative existence of 34 mitochondrial carriers, suggesting that much more mitochondrial carriers than expected were existing in mammals.

From UCP1 to UCP2 and a Family of UCPs

The screening of a cDNA library of mouse skeletal muscle led us to clone cDNAs containing an open reading frame of a putative protein 59% identical to UCP1 and producing a 1.7-kb mRNA expressed in most tissues. A clone corresponding to the whole coding sequence was expressed in yeast: it inhibited growth rate and decreased the mitochondrial membrane potential and lowered the respiratory control ratio, showing that this protein was able to uncouple the respiration and was a UCP homolog, referred to as UCP2 (Fleury *et al.*, 1997). Similar data were obtained in Tartaglia's laboratory (Gimeno *et al.*, 1997). Starting from an EST present in GenBank, Warden and Seldin (Davis University) cloned a human UCP2 cDNA and observed that the corresponding gene mapped to mouse chromosome 7 or human chromosome 11 in a region previously linked to obesity and insulinemia (Fleury *et al.*, 1997). Interestingly, it was observed by Collins and Surwit (Duke University), that strains of mice genetically resistant to obesity induced by hyperlipidic diet, were overexpressing UCP2 mRNA in their white adipose tissue and upregulated the level of UCP2 mRNA when they were fed a hyperlipidic diet (Fleury *et al.*, 1997; Surwit *et al.*, 1998). Taken together, these studies led us to propose that UCP2 was promoting oxidation of substrates and energy expenditure (Fleury *et al.*, 1997). Then, cDNAs encoding UCP3, another homolog of UCP1 and UCP2 dominantly expressed in skeletal muscle (and in BAT of mouse), were described by other groups (Boss *et al.*, 1997; Gong *et al.*, 1997, Vidal-Puig *et al.*, 1997). These studies revealed the existence of new mitochondrial carriers and demonstrated that several mitochondrial transporters sharing 60–70% identity exist and form a subfamily of carriers related to the UCPs inside the family of mitochondrial carriers.

GENETIC, BIOCHEMICAL, AND PHYSIOLOGICAL STUDIES OF UCP2

UCP2 Gene: Structure, Vicinity to UCP3 Gene, Linkage, and Association Studies

Mouse (Surwit *et al.*, 1998; Yamada *et al.*, 1998; Pecqueur *et al.*, 1999) and human (Argyropoulos *et al.*, 1998; Tu *et al.*, 1998; Pecqueur *et al.*, 1999) UCP2 genes were cloned and sequenced. As shown in Fig. 2, both UCP1 and UCP2 coding sequences are distributed over six exons. UCP1 and UCP2 coding exons can be aligned and share domains. In addition to the six coding exons, mouse and human UCP2 genes contain two untranslated exons located on the 5′-side of the gene, which do not exist in front of the UCP1 gene. UCP2 exon 2 contains an open reading frame potentially encoding a 36-amino acid peptide; we observed that the presence of this open reading frame did not prevent the synthesis of UCP2 in an *in vitro* synthesis system (Pecqueur *et al.*, 1999). Solanes *et al.* (1997) and Gong *et al.* (1997) reported that the UCP3 and UCP2 genes were located on the same chromosome and that UCP3 gene was within 75 to 100 kb of the UCP2 gene. We analyzed mouse (chromosome 7) and human (chromosome 11) genomic clones encompassing the two genes and observed that in both species, the UCP2 gene was downstream of the UCP3 gene, the distance between the two genes being 8.2 or 7 kb in mouse or man, respectively (Surwit *et al.*, 1998; Pecqueur *et al.*, 1999). These data indicate that the two genes are adjacent and strongly suggest that one gene derived from the duplication of the other one.

The existence of a UCP3/UCP2 locus is important for genetic studies. Bouchard *et al.* (1997) have tested the possibility that the UCP2 gene may be involved in regulation of energy balance or in energy expendi-

ture and investigated potential genetic linkage relationships between microsatellite markers close to the UCP2 gene location with resting metabolic rate. Using a linkage analysis strategy based on sibling, avuncular, grandparental and cousin pairs, they reported a strong evidence of linkage between the D11S911 marker and resting metabolic rate. Since the UCP3 gene is adjacent to the UCP2 gene, it is actually impossible to determine which gene (maybe both genes) contributes to resting metabolic rate. Several groups conducted association studies based on polymorphism of human UCP2 exons 4 and 8. Otabe *et al.* (1998) did not measure any association with morbid obesity in a french cohort, whereas Walder *et al.* (1998) calculated a significant association with metabolic rate during sleep in Pima Indians. Previously, several studies had concluded an association between human UCP1 gene polymorphism and fat gain (Oppert *et al.*, 1994), body weight gain (Clément *et al.*, 1996), or resistance to caloric restriction (Fumeron *et al.*, 1996).

Biochemical Activity of UCP2

The sequence similarities between UCP1 and the other UCPs suggest, but do not demonstrate, that UCP2 or UCP3 have a true uncoupling activity. The original characterization of UCP2 expressed in yeast was in favor of an uncoupling activity of UCP2 (Fleury *et al.*, 1997; Gimeno *et al.*, 1997). We recently confirmed that UCP2 expressed in yeast decreased the respiratory control ratio and lowered the mitochondrial membrane potential in conditions where the state-4 respiration increased; moreover, certain activators of UCP2 and UCP1, distinct from fatty acids, were identified (Rial *et al.*, 1999). These data support an uncoupling activity of UCP2. We did not detect neither an activation of UCP2 by fatty acids, which are the physiological activators of UCP1, or its inhibition by nucleotides, which maintain UCP1 in a nonactive state. Therefore, it seems that the regulation of UCP2 activity markedly differs from the regulation of UCP1. An important stage is the purification of UCP2 and the measurement of its proton transport activity in reconstituted liposomes (Jaburek *et al.*, 1999).

Physiological Roles of UCP2

The genetic linkage of the UCP3/UCP2 region to resting metabolic rate in man (Bouchard *et al.*, 1997) and the genetic association of UCP2 gene polymorphism to sleeping metabolic rate (Walder *et al.*, 1998), are in favor of a role for these UCPs in energy expenditure, as was suggested from the apparent uncoupling activity recorded in recombinant yeasts. Barbe *et al.* (1998) reported that the level of UCP2 mRNA in white adipose tissue of humans was strongly correlated to basal energy expenditure. Moreover, thyroid hormones, which are known to have a marked thermogenic effect, promote UCP2 (and UCP3) mRNA expression in tissues (Gong *et el.*, 1997; Lanni *et al.*, 1997; Masaki *et al.*, 1987). Leptin, a cytokine which is produced by adipose cells and activates energy expenditure and thermogenesis, has a positive effect on UCP2 gene expression when it is chronically infused in brain (Cusin *et al.*, 1998). On the contrary, the upregulation of UCP2 mRNA in skeletal muscle and adipose tissue of humans (Millet *et al.*, 1997) or rodents (Boss *et al.*, 1998) during starvation does not fit with a primary thermogenic function of UCP2. In other respects, the analysis of the tissue distribution of UCP2 did not provide very much information about the function of UCP2, since UCP2 mRNA seemed to be present in all cell types, although it is expressed at very high levels in macrophages (Fleury *et al.*, 1997), Kupffer cells (Larrouy *et al.*, 1997), and gut cells (Richard, Erlanson-Albertsson and Ricquier, unpublished data). UCP2 mRNA level is increased in obese humans (Millet *et al.*, 1997) or genetically obese mice (Gimeno *et al.*, 1997), or in rodents fed a hyperlipidic diet (Fleury *et al.*, 1997; Matsuda *et al.*, 1997; Surwit *et al.*, 1998), and also in diabetic rats (Kageyama *et al.*, 1999). Fatty acids and retinoids increase UCP2 mRNA expression in adipocytes (Aubert *et al.*, 1997; Camirand and Silva, see review in Boss *et al.*, 1998). All together, these data suggest that UCP2 expression is modulated according to the level of circulating fatty acids and fluxes of fatty acids toward tissues (review in Boss *et al.*, 1998). A possible role of UCP2 in transport of fatty acids across the inner mitochondrial membrane should be investigated. It is expected that analysis of UCP2^{-}/$^{-}$ mice will help in elucidating the biological role(s) of UCP2.

BMCP1 AND stUCP

The characterization of UCP2 and UCP3 suggested that these proteins have a true uncoupling activity. They may be considered as candidates for explaining the proton leaks present in most mitochon-

dria. However, this hypothesis remains to be demonstrated. The existence of other UCP homolog or of other carriers having the ability to translocate protons is an open issue. This is a difficult question since it has been proposed that other mitochondrial carriers, such as the adenine nucleotide translocator, can mediate proton transport upon addition of fatty acids. We recently identified BMCP1, another mitochondrial carrier showing an uncoupling activity in recombinant yeast (Sanchis *et al.*, 1998) and also collaborated to the first characterization of a plant UCP (Laloi *et al.*1997).

BMCP1, A New Mitochondrial Carrier Expressed in Brain

In collaboration with C. Warden (Davis University) and others, a search for other UCPs was undertaken. We cloned a new mitochondrial carrier referred to as BMCP1 of which the mRNA was predominantly observed in brain (Sanchis *et. al.*, 1998). The amino acid sequence of BMCP1 predicts that it represents a mitochondrial carrier with a triplicated structure. This predicted amino acid sequence is moderately homologous to sequence of the UCPs and its level of identity toward UCP2 is 38%, whereas the three UCPs share 60–70% identity. The transport activity of BMCP1 is unknown, and its weak sequence similarity to the UCPs suggested it was not a mitochondrial uncoupling protein. However, when it was expressed in yeast, BMCP1 markedly inhibited cell growth and the mitochondrial potential. To further investigate the properties of BMCP1, yeast spheroplasts were prepared. A detailed measurement of the force/flux relationship between mitochondrial membrane potential and state-4 respiratory rate was made in collaboration with Goubern (Jouy-en-Josas). These experiments established that BMCP1 increased a proton leak in the mitochondrial membrane of yeast and had an uncoupling activity, at least when it was expressed in yeast (Sanchis *et al.*, 1998). It remains to analyze the activity of BMCP1 in mammalian cells and, in particular, in braincells.

stUCP, A Plant Cold-Induced Uncoupling Protein

Laloi *et al.* (1997) cloned a cDNA encoding a peptide related to the UCPs from a potato flower library. This cDNA encoded a 306 amino acid sequence (referred to as stUCP; see Table I), which is 44 and 47% identical to human UCP1 and UCP2, respectively. When the stUCP cDNA was expressed in yeast, a marked decrease in the uptake of a membrane-potential-sensitive probe was observed, suggesting an uncoupling effect. The stUCP gene is expressed as a 1.5-kb mRNA at low level in leaves and tubers, and high level in stems, fruit, roots, and flowers. Surprisingly, a strong induction of stUCPmRNA was observed in leaves of potato plants kept at 4°C for 1, 2, or 3 days (Laloi *et al.* 1997), similarly to what is known for UCP1 in BAT of rodents exposed to the cold (Table I). These data suggested that stUCP and respiration uncoupling was important for plant thermogenesis.

CONCLUSIONS AND PERSPECTIVES

Studies of UCP1 have firmly established that it is a mitochondrial carrier with a true uncoupling activity, with a major physiological role in thermogenesis and the control of body temperature. The identification of UCP2 and UCP3, which are significantly related to UCP1, revealed that a larger number of mitochondrial carriers than expected exists. A consequence of the identification of plant UCPs is that the uncoupling proteins are more widespread than previously believed and that animal and plant UCPs have evolved by diversification from a common ancestral gene. These discoveries will certainly contribute to renewed research on mitochondria. Studies of these newly identified components indicate that they have a metabolic role and contribute to bioenergetics of mitochondria, although their exact functions are not well known. Further analysis of the uncoupling activity of UCP2 and UCP3 will be required. It is unknown yet if UCP2 and UCP3 are only proton transporters and if they work as uniport or symport (Jaburek *et al.*, 1999). A major challenge will be to determine the structure of the UCPs. It is expected that analysis of UCP2 and UCP3 knockout mice, which is under progress in several laboratories, will contribute to identify the exact physiological roles of these genes. It is unknown if the major roles of UCP2 and UCP3 is related to energy expenditure or to something else. Finally, further analysis of the uncoupling proteins may be of interest regarding new strategies of treatment of metabolic diseases (obesity, diabetes) or inflammation and fever.

Table I.

	UCP1[a]	UCP2[a]	stUCP[a]
Expression	BAT	Mammalian tissues	Plant organs
Identity (%)	100	59	44
Amino acids	306	309	306
Signatures of mitochondrial carriers	Yes	Yes	Yes
Triplicated structure	Yes	Yes	Yes
mRNA (kb)	1.5	1.7	1.5
Induction by cold	Yes	Yes/no[b]	Yes
Function(s)	Thermogenesis	Unknown[c]	Thermogenesis

[a] Data refer to mouse UCP1 and mouse UCP2; stUCP data are from Laloi *et al.* (1997).
[b] Fleury *et al.* (1997) did not measure a significant induction of UCP2 mRNA in mice exposed to the cold whereas Carmona *et al.* (1998) and Boss *et al.* (review in Boss *et al.*, 1998) reported an upregulation by cold in mice or rats.
[c] The uncoupling activity of UCP2 suggests that it can activate substrate oxidation. UCP2 activity and UCP2 genetic locus are related to energy expenditure although thermogenesis may not be the primary function of UCP2. Further experiments are required to establish the exact physiological role(s) of the mammalian UCP2 and the plant stUCP.

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