The EMBO Journal vol.13 no.8 pp.1990-1997, 1994

A sequence related to a DNA recognition element is essential for the inhibition by nucleotides of proton transport through the mitochondrial uncoupling protein

F.Bouillaud[1], I.Arechaga[2], P.X.Petit[3], S.Raimbault, C.Levi-Meyrueis, L.Casteilla, M.Laurent[4], E.Rial[2] and D.Ricquier

Centre de Recherches sur l'Endocrinologie Moléculaire et le Développement, CNRS, 9 rue Jules Hetzel, 92190 Meudon, France, [2]Centro de Investigaciones Biologicas, CSIC, Velazquez 144, 28006, Madrid, Spain, [3]Centre de Génétique Moléculaire, CNRS, Avenue de la terrasse, 91190 Gif sur Yvette, France and [4]Service d'Imagerie Cellulaire, CNRS, Bâtiment 441, Université Paris-Sud, 91405 Orsay, France

[1]Corresponding author

Communicated by W.Neupert

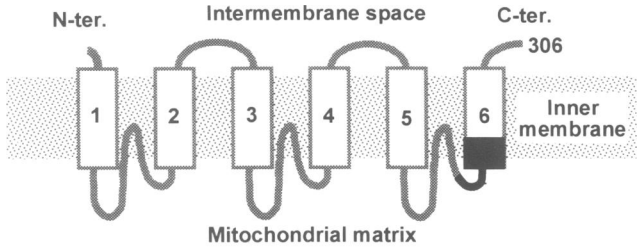
The uncoupling protein (UCP) is uniquely expressed in brown adipose tissue, which is a thermogenic organ of mammals. The UCP uncouples mitochondrial respiration from ATP production by introducing a proton conducting pathway through the mitochondrial inner membrane. The activity of the UCP is regulated: nucleotide binding to the UCP inhibits proton conductance whereas free fatty acids increase it. The similarities between the UCP, the ADP/ATP carrier and the DNA recognition element found in the DNA binding domain of the estrogen receptor suggested that these proteins could share common features in their respective interactions with free nucleotides or DNA, and thus defined a putative 'nucleotide recognition element' in the UCP. This article provides demonstration of the validity of this hypothesis. The putative nucleotide recognition element corresponding to the amino acids 261-269 of the UCP was gradually destroyed, and these mutant proteins were expressed in yeast. Flow cytometry, measuring the mitochondrial membrane potential *in vivo*, showed increased uncoupling activities of these mutant proteins, and was corroborated with studies with isolated mitochondria. The deletion of the three amino acids Phe267, Lys268 and Gly269, resulted in a mutant where proton leak could be activated by fatty acids but not inhibited by nucleotides.

Key words: DNA binding/flow cytometry/homology/recombinant expression/yeast

Introduction

The high thermogenic capacity of brown adipose tissue is due to the activity of the uncoupling protein (UCP), a unique protein located in the inner mitochondrial membrane. The UCP is a 33 kDa protein which allows proton re-entry into the mitrochondrial matrix. This proton flux bypasses the ATP synthase and frees brown adipocytes from the restraints of respiratory control, allowing them to develop a very high catabolic activity and to dissipate energy as heat (reviews in Klingenberg, 1990; Ricquier *et al.*, 1991; Nedergaard and Cannon, 1992). The activity of the UCP is regulated. Following binding of purine nucleotides (ATP, GTP, GDP, ADP) the proton transport of the UCP is inhibited, whereas free fatty acids increase it (Rial *et al.*, 1983); acylCoA may also affect its activity (Katiyar and Shrago, 1991). The UCP is also able to transport a variety of anions including chloride (Nicholls and Lindberg, 1973) and several synthetic compounds (Jezek and Garlid, 1991). The UCP sequence is related to those of other anion transporters of the mitochondrial inner membrane such as the ADP/ATP carrier (ACC), the phosphate carrier and the oxoglutarate carrier. These proteins are members of an evolutionary family of carriers of the inner mitochondrial membrane. These proteins of ~300 amino acids are thought to derive from triplication and divergence of an ancestor of 100 amino acids (reviewed in Walker, 1992). Observation of similarities between the regions 261-269 of the UCP, 263-271 of the AAC (Bouillaud *et al.*, 1986) and the DNA binding domain found in transcription factors of the steroid and thyroid hormone receptor superfamily (Evans, 1988) led to the proposal that these proteins share a homologous domain (Bouillaud *et al.*, 1992). This domain contains amino acids determining the DNA sequence specificity of the nuclear transcription factors (Berg, 1989; Schwabe and Rhodes, 1991 and references therein) and is considered as a DNA recognition element. An attractive hypothesis would be that this domain is used in the mitochondrial carrier to recognize free nucleotides and is a 'nucleotide recognition element'. The use of ATP derivatives for covalent labelling of the AAC (Dalbon *et al.*, 1988) and of the UCP (Mayinger and Klingenberg, 1992; Winkler and Klingenberg, 1992) identified amino acids close to this region; however, proof of the functional importance of residues 261-269 of the UCP in the nucleotide inhibition of proton transport was missing. In the present report three mutants of this putative nucleotide binding domain in the UCP, obtained by site directed mutagenesis, are presented. In the first mutant (UCPΔ9) a complete deletion of this homologous domain (amino acids 261-269) was achieved. The second mutant (UCPΔ3) corresponds to a deletion of three amino acids (267-269). The third mutant (UCP*F/Y) is a single amino acid substitution (Figure 1).

Following the initial isolation of a CHO cell line permanently expressing the UCP (Casteilla *et al.*, 1990), we tried to isolate CHO cell lines expressing the UCPΔ9 or the UCPΔ3. In fact, although the UCPΔ9 and the UCPΔ3 synthesis and incorporation into CHO cell mitochondria were obtained upon transient transfection of CHO cells, several attempts to isolate stable CHO cell lines expressing these proteins failed repeatedly, and precluded any further analysis of the activity of these mutated UCPs (L.Casteilla and C.Levi-Meyrueis, unpublished observations). A simple explanation would be a lethal effect of these proteins, due to destruction of the nucleotide binding site of the UCP, that resulted in uncontrolled mitochondrial uncoupling. A shift




```
SIMILARITIES:

Consensus sequence:     EG--AFFK(R/G)
                  ppp     bp b   bq
                          **  *****
Estrogen Rec.     YHYGVWSCEGCKAFFKRSIQGHNDYM
                          ** :*****        :
AAC               CWRKIAKDEGPKAFFKGAWSNVLRGM
                  :       **: *****     ::
UCP +             CAMTMYTKEGPAAFFKGFAPSFLRLG
                   :  ::  **  ** ** ::   :
PCP               AAPKMYKEEGLKAFYKGVAPLWMRQI

MUTANTS OF THE UCP:
                        260       270
UCP*F/Y           CAMTMYTKEGPAAFYKGFAPSFLRLG
                              |       |
UCPΔ3             CAMTMYTKEGPAAF___FAPSFLRLG
                              |       |
UCPΔ9             CAMTMYTK_________FAPSFLRLG
```

Fig. 1. Sequences of the 'homologous domain'. Top: the mutated region is blackened on the representation of the structural model of the UCP; bottom: the sequences composing the 'homologous domain' are aligned: estrogen rec, human estrogen receptor (NBRF mnemonic: QRHUE, residues 195–220), the two cysteine residues participate with the first zinc finger of the DNA binding domain; above this sequence 'b' indicates an amino acid interacting with DNA base pairs, 'p' with phosphate groups, and 'q' with both, in the DNA–protein crystals (Schwabe *et al.*, 1993). AAC, beef heart ADP/ATP carrier (mnemonic NBRF XWBO, residues 256–281); UCP^+, rat UCP (mnemonic NBRF A26294 residues 253–278); PCP, phosphate carrier protein (mnemonic NBRF A29453, residues 203–228). Identities with the consensus sequence shown on top are highlighted by stars, whereas amino acids identical only in two proteins are indicated by two dots. The sequences of the mutant proteins UCP*F/Y, UCPΔ3 and UCPΔ9 are indicated, the mutation is underlined.

to a yeast expression system offered several advantages: the vector used could be tightly repressed and strongly induced, important quantities of cells could be grown for a low price, and the possibility of this organism being able to rely only on glycolysis for energy supply could permit the production of a totally unregulated mitochondrial uncoupler. The expression of the wild type UCP in yeast has already been reported by different groups (Murdza-Inglis *et al.*, 1991; Bathgate *et al.*, 1992; Arechaga *et al.*, 1993). With the help of flow cytometry and confocal microscopy, the influence of these mutations on the mitochondrial activity of yeast *in vivo* are described and are corroborated with studies with isolated mitochondria. The mutant UCPΔ3 demonstrates that Phe267, Lys268 and Gly269, are essential for the inhibition by nucleotides of the proton transport in the UCP.

Results

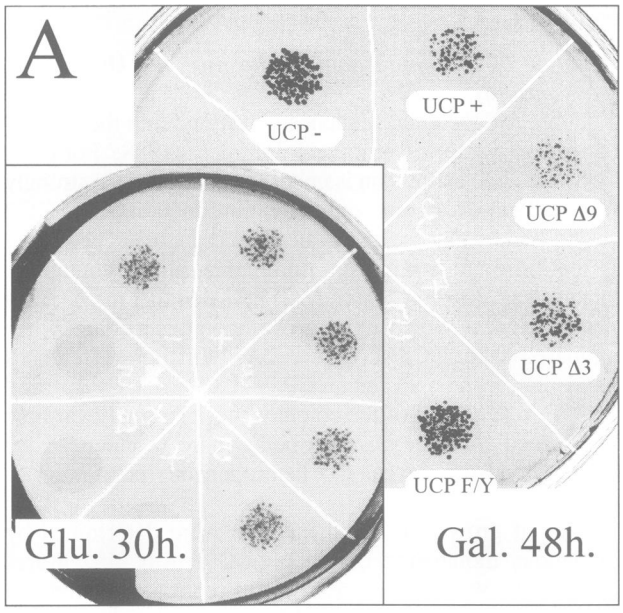
Influence of the expression of the mutated UCPs on yeast growth

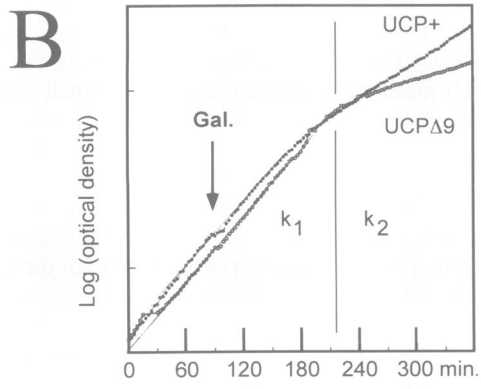
When the UCP coding sequence is inserted into the pYeDP expression vector (Cullin and Pompon, 1988; Pompon, 1988) the UCP expression is under the control of the strongly regulated gal-cyc promoter, and the production of the UCP is repressed by glucose and induced by galactose. The plating of the different strains on minimal medium containing galactose as a carbon source (SG) revealed that the UCPΔ9 mutant had severe difficulties growing on this medium when compared with UCP^+ (Figure 2A). Smaller differences could be seen between UCP^-, UCP^+, UCPΔ3 and UCP*F/Y. The size of the colonies was extremely variable on SG medium, this variation being more pronounced for UCPΔ9, and this revealed a heterogeneous population of yeasts.

Rates of growth in liquid medium were determined by continuous monitoring of the optical density of cultures (Figure 2B). When the expression was repressed (in minimal medium with 2% lactate and 0.1% glucose) the growth rates were similar for all strains. Two hours after the induction of expression by addition of 0.4% galactose the generation time of the UCPΔ9 strain increased from 2 h 20 min to 8 h; in contrast galactose induction had only a small effect for the UCP^+ strain. The effect of the expression of different mutated UCPs was estimated by the ratio between the generation times before and after induction. This ratio varied from 1.4 (UCP^+) to 3.5 (UCPΔ9); intermediate values, 1.7 and 1.8, were found for UCP*F/Y and UCPΔ3 respectively (Figure 2B). The slowing down of the growth of the UCPΔ9 strain after galactose addition was not sustained, and after several hours the growth rate recovered subnormal values (data not shown). This phenomenon was associated with the irreversible loss of expression of the protein UCPΔ9.

In vivo analysis of mitochondrial activity

At the low concentration used (0.1 μM) the potential sensitive probe $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide) stains exclusively mitochondria (Koning *et al.*, 1993), and it has been used previously to study the effects of the expression in yeast of the T-urf13 gene encoding a plant uncoupling protein (Glab *et al.*, 1993). The same procedures were applied to yeast grown on galactose (Figure 3). In the cells expressing the wild type UCP (UCP^+ cells) a Gaussian curve with a high level of fluorescence was obtained. The curve was almost identical to that obtained with the control cells: UCP^- (Figure 3A). When an uncoupler (50 μM mClCCP) was added prior to $DiOC_6(3)$ addition, it collapsed the membrane potential and abolished $DiOC_6(3)$ accumulation in mitochondria; consequently the value of the green fluorescence was very low (Figure 3A). The expression of the UCPΔ9 partially mimicked the effects of the artificial uncoupler mClCCP since a significant amount of cells approached the zero level of fluorescence, whereas others maintained a subnormal value (Figure 3B). This heterogeneity in the yeast population was also observed with confocal microscopy, where both highly fluorescent and non-fluorescent cells could be observed in the UCPΔ9 strain grown on galactose (Figure 5, top); in contrast UCP^+ cells





		k_1	k_2	k_2/k_1
UCP+	(2)	189	255	1.35
UCP*F/Y	(1)	161	267	1.66
UCPΔ3	(1)	139	252	1.81
UCPΔ9	(3)	142	502	3.53

Fig. 2. Growth of the different strains. (**A**) Comparison of growth of the different strains on minimal medium with glucose for 30 h (Glu. 30 h) and on minimal medium with galactose for 48 h (Gal 48 h). (**B**) Growth of the yeast strain in liquid medium and influence of galactose induction; the optical density at 600 nm was automatically recorded every 3 min by circulation of the liquid culture in the cuvette of the spectrophotometer. A minimal medium with 2% lactate and 0.1% glucose was used; after 90 min of growth galactose (0.4% final) was added. The generation times before (k_1) and after (k_2) the induction of expression are shown in minutes. The number of experiments done is shown in parenthesis.

were homogeneously labelled (Figure 5, bottom). In flow cytometry the UCP*F/Y and UCPΔ3 strains exemplified situations intermediary between UCP^+ and UCPΔ9. The decreasing magnitude of fluorescence: UCP^+ > UCP*F/Y > UCPΔ3 > UCPΔ9 (Figure 3B) correlates with the size of the mutations. When the expression was repressed (minimal medium with 2% lactate and 0.1% glucose) the

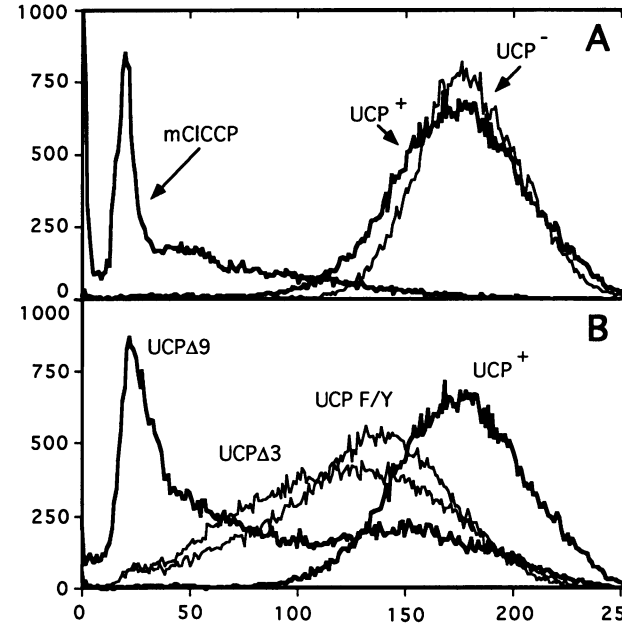


Fig. 3. Flow cytometry. The number of cells (*y* axis) relative to the value of the intensity of green fluorescence of the potential sensitive probe $DiOC_6(3)$ (*x* axis) is represented. The value of the fluorescence is represented on a logarithmic scale of 256 channels; a difference of 26 channels represents a doubling of the fluorescence intensity. (**A**) Comparison of UCP^+ and UCP^- grown in the presence of galactose; the effect of 50 μM mClCCP on the $DIOC_6(3)$ accumulation in cells is also shown. (**B**) Analysis of the different strains expressing the different mutants of the UCP; the type of UCP produced is indicated. The modal fluorescent channel was: 173 for UCP^+; 127 for UCP*F/Y; 120 for UCPΔ3; 86 for UCPΔ9. In this latter case the heterogeneous population is interpreted as a combination of 37% of the cells with a normal mitochondrial membrane potential (modal channel:173) and 63% of the cells with an extremely low fluorescence (modal channel: 46).

same high value of green fluorescence was found for all strains. This demonstrates that the differences observed were due to the expression of different UCPs (data not shown). Discrimination of single and budding cells according to forward angle light diffusion indicated that the ratio between dividing and non-dividing cells was 1.2 for UCP^-, 1 for UCP^+ strains, and that it was 0.6 with UCPΔ9 and UCPΔ3 strains, this lower value indicates a lower number of dividing cells.

A use of flow cytometry would be to sort UCP mutants according to their membrane potential. In order to test whether this would be applicable, a mixture of UCPΔ9 and UCP^+ strains grown in galactose was sorted according to the value of the $DiOC_6(3)$ fluorescence: cells with the highest fluorescence and with the lowest fluorescence were selected and plated on SD medium that repressed expression. In both cases the number of cells able to form colonies was approximately equal to half the number of objects selected by the cytometer. Individual colonies coming from the sorted cells were picked from the plates and the UCP insert was amplified by PCR. In this experiment the cells with a low mitochondrial potential contained the UCPΔ9 cDNA in eight cases out of 10, the UCP^+ cDNA in one, and probably a mixture of the two cDNAs in one (Figure 4). However, the unexpected result was that the cells selected for their high

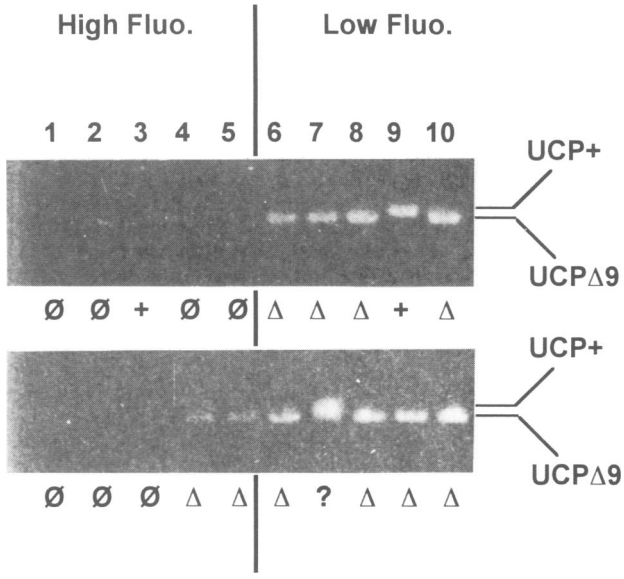


Fig. 4. Cell sorting. The products of amplification of the 3′ half of the UCP cDNA from colonies derived from single cells and sorted according to the value of green fluorescence were loaded on a 1.5% agarose gel. The type of UCP cDNA present in each lane is indicated by the symbols +, UCP^+; Δ, UCPΔ9; ∅, no cDNA detected; ?, mixture of both cDNAs.

value of $DiOC_6(3)$ fluorescence produced colonies that were ura^+ (they grew on minimal medium) but in which the amount of UCP cDNA could be decreased so drastically that it was not detected; moreover, when a UCP cDNA could be detected, it was either the UCP^+ or of the UCPΔ9 type (Figure 4). This suggests that the copy number of the expression vector could vary considerably from cell to cell. A high mitochondrial membrane potential is associated with a reduced number of copies of the expression vector in both UCPΔ9 and UCP^+ strains. This explains the behaviour of the UCPΔ9 strain when induction of expression occurred, since only those cells which did not express the deleterious UCPΔ9 gene could grow on minimal medium with galactose, thus invading the culture which progressively regained a normal growth rate.

Activity of the mutated UCP in isolated mitochondria

In a previous report on the mutagenesis of the UCP (Arechaga *et al.*, 1993) we described a protocol for yeast growth, induction of expression and preparation of mitochondria suitable for bioenergetic studies. This procedure included one step of growth under non-selective conditions. It could not be used with the UCPΔ9 and UCPΔ3 strains, as when galactose induction occurred, the selective pressure against cells expressing these mutant proteins was so high that they disappeared from the culture before there was any significant expression of the mutant proteins. Therefore the cultures used to prepare mitochondria had to be grown in the same conditions as those used for flow cytometry, i.e. overnight growth in galactose-containing minimal medium, which ensured maintenance of the selective pressure for ura^+ cells. Flow cytometry was used to check, before mitochondrial preparations, that the yeasts presented the expected modification of $DiOC_6(3)$ fluorescence. The amount of UCP present in the mitochondria was estimated

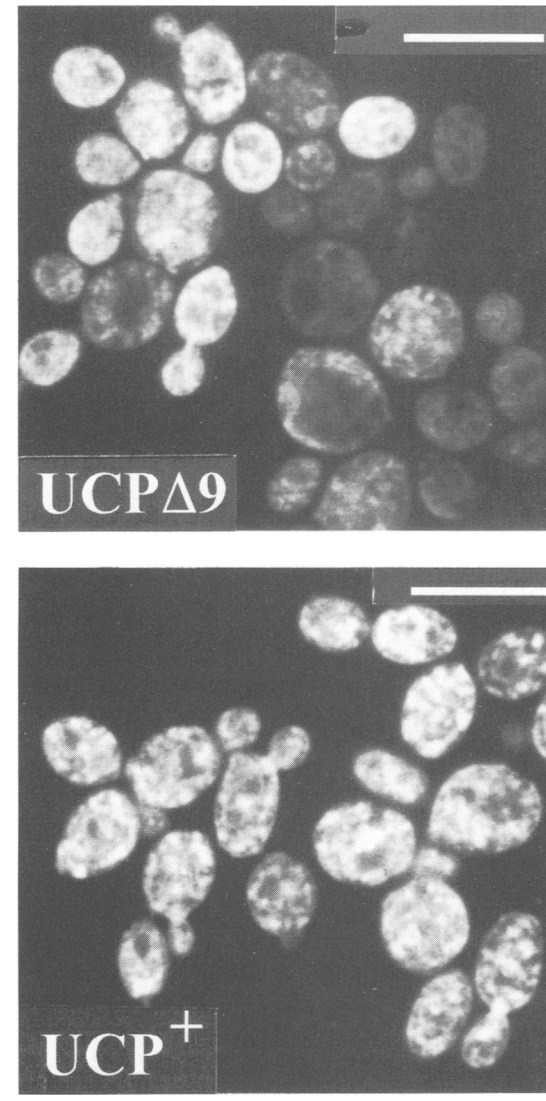


Fig. 5. Confocal microscopy picture of the UCP^+ and UCPΔ9 strains stained with $DiOC_6(3)$. Details are described in Materials and methods. Top view: UCPΔ9, bottom view: UCP^+. The bar on the picture represents 10 μm. The re-partition of the fluorescence is noteworthy as the patches observed fit in with the specific staining of mitochondria described previously (Koning *et al.*, 1993).

by Western blotting (Figure 6). High and similar amounts of UCP^+, UCP*F/Y and UCPΔ3 indicated a normal targeting of these proteins. The UCPΔ9 was present in much lower amounts in isolated mitochondria (Figure 6). This was probably not due to a defect in targeting, as the mitochondrial pellet was still the cellular fraction richest in UCPΔ9 (data not shown).

The properties of isolated mitochondria were studied as previously described (Arechaga *et al.*, 1993). Yeast mitochondria devoid of UCP (UCP^- strain) did not respond to palmitic acid (UCP activator) or GDP (UCP inhibitor). On the other hand, when the wild type UCP was present (UCP^+), addition of palmitic acid induced a decrease in membrane potential and an increase in mitochondrial respiration, while subsequent addition of GDP restored the membrane potential and brought back the respiration rate to its initial value (Figure 7). This indicated the presence

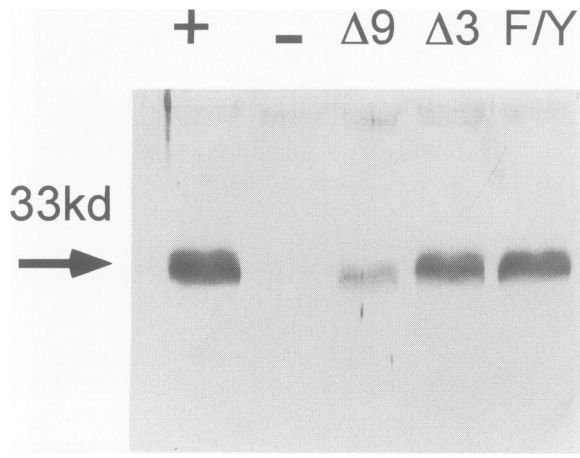


Fig. 6. Immunodetection of the mutated UCP in yeast mitochondria. Equal amounts of mitochondrial proteins were loaded on the gel; the proteins were transferred on a nitrocellulose membrane and the UCP protein was detected immunologically. The decreased intensity of the UCPΔ9 band could not be due to an altered immunological recognition of the mutant protein as it was also observed with sera known to recognize mainly sequences of the UCP located outside the mutated region (Miroux *et al.*, 1993).

of a fully active UCP, where a proton conducting pathway is opened in the presence of fatty acids and closed in the presence of nucleotides. Mitochondria isolated from UCP*F/Y strain were indistinguishable from mitochondria containing the wild type UCP (Figure 7).

Interestingly, mitochondria prepared from the UCPΔ3 strain were sensitive to low concentrations of palmitic acid, but this effect was not reversible by addition of GDP (Figure 7). This would be the modification of the UCP function expected, if the deleted amino acids played a crucial role in nucleotide binding.

The preparation of mitochondria from the UCPΔ9 strain grown in the presence of galactose occurred always with a lower yield; moreover for the same amount of proteins the respiratory activity was lower. Mitochondrial activity is enhanced in budding cells, which are also more sensitive to the enzymatic digestion of the cell wall necessary for mitochondrial preparation. Consequently mitochondria coming from budding cells are expected to be over-represented in our preparations. As stated earlier, a higher growth rate (i.e. active budding) is associated with loss of expression of the UCPΔ9 gene. Thus in our preparations there was probably a predominance of mitochondria devoid of the UCPΔ9 protein, and therefore it should not be very surprising that the mitochondria obtained from the culture of the UCPΔ9 strain looked like UCP^- mitochondria (Figure 7).

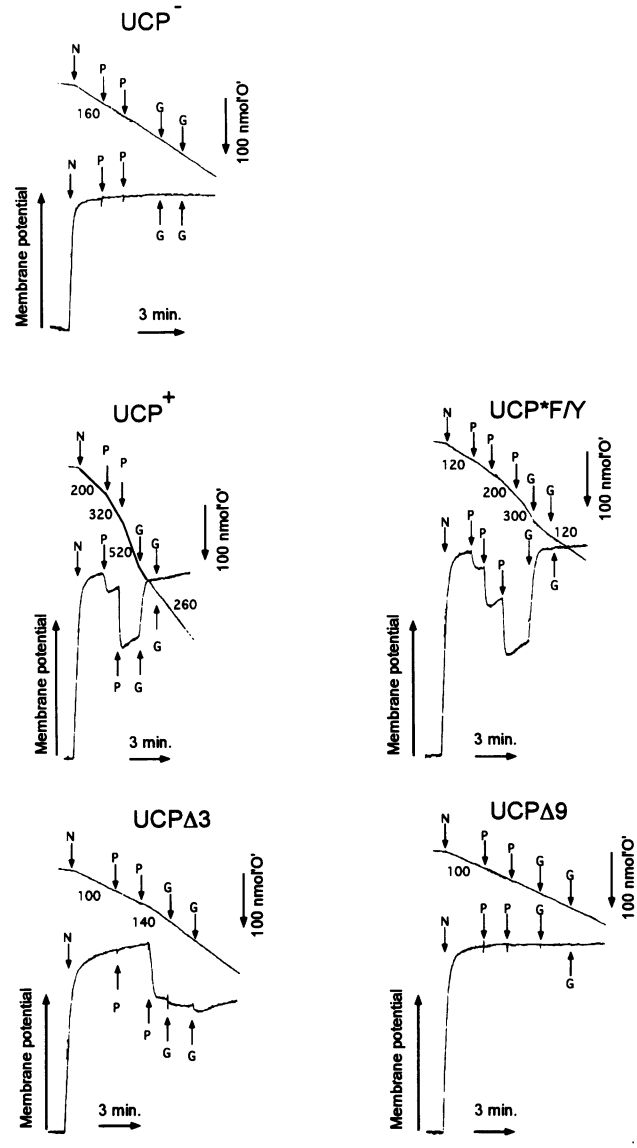


Fig. 7. Respiration and membrane potential of isolated mitochondria. The oxygen consumption was measured while the simultaneous changes in the fluorescence of the probe $DiSC_2(5)$ were used to estimate the membrane potential. Respiration rates are indicated in nmol 'O'/min/mg. The horizontal bar represents 3 min and the vertical bar 100 nmol of 'O'. Additions: N, 3 mM NADH; P, 64 μM palmitate; G, 1 mM GDP.

Discussion

The UCP and the ADP/ATP carrier (AAC) are related proteins that can both bind nucleotides; however, they show very different functional characteristics. The UCP can bind either adenine or guanine nucleoside di- or tri-phosphate; binding inhibits the proton transport activity of the protein. The AAC binds exclusively ATP or ADP; binding is followed by the translocation of the nucleotide across the mitochondrial inner membrane. Investigation of the residues involved in the binding of nucleotides, by using ATP azido derivatives, has led to the identification of 'labelling areas'. The first one is in the central part of the beef heart AAC: Lys162, Lys165 and Ile183 (Dalbon *et al.*, 1988), which was also found in the yeast AAC (Mayinger *et al.*, 1989); in contrast no labelling area has been identified in the central part of the UCP. A second labelling area is present in the C-terminal third: Val254 and Lys259 of the AAC (Dalbon *et al.*, 1988), and Cys253, Thr259 and Thr264 of the hamster UCP (Mayinger and Klingenberg, 1992; Winkler and Klingenberg, 1992). While these disparities may be related to the different functional characteristics of the two proteins, it should be pointed out that the second area is close to the region where the strongest homology between these

two proteins is found: positions 261−269 of the UCP and 264−273 of the AAC (Bouillaud *et al.*, 1986). Neither the UCP nor the AAC displays the consensus sequence described in many ATP binding molecules and which contains a glycine-rich loop (Walker *et al.*, 1982; Milner-White *et al.*, 1991). This has been related to the fact that the UCP and AAC bind free nucleotides and not nucleotides complexed with magnesium ions (Mayinger and Klingenberg, 1992). On the other hand the similarities of these two proteins with the DNA binding domain of the superfamily of steroid and thyroid hormone receptors (Bouillaud *et al.*, 1992, and Figure 1) suggested an evolutionary relationship between nucleotide binding activity and DNA recognition, and pointed to this domain of the UCP as primary target for site directed mutagenesis experiments. Detailed structural data are available for the transcription factors: the amino acids (CEGCKAFFKRSI) form an α-helix (the DNA recognition element) lying in the major groove and contacting bases (Härd *et al.*, 1990; Luisi *et al.*, 1991; Schwabe *et al.*, 1993). The detailed description of the interaction of the estrogen receptor with DNA (Schwabe *et al.*, 1993) indicates that the amino acids: 1 (Glu), 2 (Gly) and 8 (Lys) of the consensus sequence (Figure 1) are in contact with the same AT pair on double stranded DNA. It is thus possible to consider that these amino acids interact with only one nucleotide in the UCP or AAC. As the membranous proteins withstand structural explorations, only limited information is available for the UCP and the ADP/ATP carrier: this sequence is predicted to participate in the last (sixth) transmembranous α-helix of these proteins and is facing the mitochondrial matrix (Miroux *et al.*, 1992).

*UCP*F/Y: mutation of Phe267 into Tyr*

The alignment of the sequences of the mitochondrial carriers showed that the two phenylalanine residues present in the consensus are conserved in the ADP/ATP carrier and the UCP, whereas in the closest sequence found in the phosphate carrier the second phenylalanine is replaced by a tyrosine (Figure 1). Because the phosphate carrier is unable to bind nucleotides, we thought that the replacement of Phe267 by a tyrosine residue would affect nucleotide binding to the UCP. While this limited modification did not qualitatively change the activity of UCP in isolated mitochondria, flow cytometry suggested that this mutation did increase its uncoupling activity: the $DiOC_6(3)$ fluorescence was consistently lower in this strain when compared with the UCP^+ strain. Determining the quantitative modifications of UCP function caused by this mutation with isolated mitochondria would be a cumbersome task.

The power of flow cytometry is highlighted by this mutant since it has revealed quantitative variations in the UCP activity where it is most relevant, i.e. *in vivo*, in the presence of all intracellular regulators.

UCPΔ3: deletion of amino acids 267−269

This deletion removed the last three amino acids of the homologous domain, but left the two contiguous phenylalanines; deletion of amino acids 268−270 would produce the same final sequence (Figure 1). Induction of the expression moderately affected the growth potency (Figure 2A). However, flow cytometry indicated a large decrease in the mitochondrial membrane potential. Studies with isolated mitochondria clearly demonstrated that UCPΔ3 is an active uncoupling protein activatable by fatty acids, but insensitive to nucleotides. The comparison of the UCP^+ and UCPΔ3 strains (Figure 3B) indicates also that nucleotide inhibition of the UCP activity does occur in yeast cells expressing the wild type UCP, and this explains why no phenotypic alteration was found in these cells. Whether the decrease in mitochondrial potential seen in the UCPΔ3 strain by cytometry was due to intrinsic activity of UCPΔ3 or to its activation by endogenous fatty acids is not clear, although the fact that a high mitochondrial potential is observed in isolated mitochondria (Figure 7) argues for the second hypothesis. All the data obtained with this single mutant, from yeast growth to isolated mitochondria, confirm the hypothesis that amino acids participating in the homologous sequence are essential for nucleotide inhibition of proton transport by the UCP.

UCPΔ9: deletion of amino acids 261−269

Deletion of the whole homologous domain resulted in a protein that was extremely deleterious to the yeast that contained it. Consequently only a low amount of it could be produced, and this hindered studies with isolated mitochondria. This is why the only data we have about the effect of the UCPΔ9 on mitochondria came from flow cytometry. There was considerable inhibition of the accumulation of the potential sensitive probe into mitochondria. This effect is close to that observed with 50 μM mClCCP (Figure 3). There is no doubt that UCPΔ9 is targeted to mitochondria. Thus the mutation either alters the UCP in such a way as to create a considerable ionic leak that makes the membrane potential drop, or since this is a relatively big deletion, it removes a significant part of the last transmembranous α-helix of the UCP, thus converting the UCP into a protein highly toxic for mitochondrial activity for another unknown reason.

A mixture of UCPΔ9 and UCP^+ strains was used to test the efficiency of the cell sorting procedure to select mutants of the UCP according to their mitochondrial membrane potential. At low membrane potential the procedure proved efficient, since most selected cells were UCPΔ9. At high membrane potential a surprising result was obtained: instead of the expected UCP^+ yeasts, cells nearly devoid of the expression vector were found. A careful examination of this result reveals that what was subject to selection was the expression vector itself and not the yeast cells. According to this, cell sorting will be able to select other mutant cDNAs of the UCP with increased uncoupling activities in the cellular context, and eventually should be able to select at high potential plasmids propagating inactive UCPs. Consequently the application of flow cytometry to select randomly mutated UCP cDNA is under development.

It must now be emphasized that the results obtained *in vivo* with these mutants [(i) the failure to isolate stable CHO cell lines, (ii) the effects on yeast growth, (iii) the gradation of the values of membrane potentials observed with flow cytometry] are in agreement with the hypothesis that these mutations gradually destroyed a domain of the UCP involved in the inhibition by cytosolic nucleotides of the uncoupling activity of the protein, and progressively converted it to an unregulated uncoupler. Studies with isolated mitochondria and the UCPΔ3 mutant brought the final demonstration of

this hypothesis. The *in vivo* results also illustrate the power of flow cytometry for the study and probably the selection of mutant proteins which have an influence on the mitochondrial membrane potential.

The sequence 261–269 is important for the nucleotide inhibition of UCP activity and its counterpart in transcription factors constitutes a DNA recognition element. It is tempting to conclude that this could be related to universal rules that govern the interactions between proteins and nucleotides. The first hypothesis would be that this domain derives from a common ancestor (homology); alternatively functional convergence could have led to the observed similarity. None of these hypotheses weakens the interest of this observation, which introduces a link at the level of the structure–function relationship between DNA specific recognition by soluble transcription factors and nucleotide specificity of inner mitochondrial membrane transporters.

Materials and methods

Construction of the expression vectors

The expression vector pYeDP-UCP$^+$ coding for the wild type rat UCP and the control plasmid pYeDP-UCP$^-$ were described previously (Arechaga *et al.*, 1993). For mutagenesis the rat UCP cDNA was inserted at the *Eco*RI site of M13mp18. The resulting recombinant phage called MUC$^+$ produced single stranded DNA consisting of anti-coding strand of the UCP; it was used as matrix for synthesis of the complementary strand with a mutated oligonucleotide as primer. The 30mer '563': ACC ATG TAC ACC AAG TTT GCG CCT TCT TTT encoding TMYTKFAPSF generated a deletion of amino acids 261–269 in the UCP. The 30mer '564' GGA CCG GCA GCC TTT TTT GCG CCT TCT TTT encoding GPAAFFAPSF generated a deletion of amino acids 267–269. Phage DNA was transferred onto a membrane (Hybond N, Amersham, UK) and the same oligonucleotides were used as probes to identify clones containing the appropriate mutations. The pSELECT mutagenesis system (Promega, Madison, WI, USA) was used to replace phenylalanine 267 by a tyrosine in the third mutant. For each mutant the coding frame was amplified and inserted in the pYeDP vector (Cullin and Pompon, 1988; Pompon, 1988) as previously described (Arechaga *et al.*, 1993), and the resulting plasmids were called pYeDP-UCPΔ9-261-269, pYeDP-UCPΔ3-267-269 and pYeDP-UCP*F/Y-267. The sequence of the UCP coding frame in the three pYeDP vectors was analysed to check for unwanted mutations. In this article, the terms UCP$^-$, UCP$^+$, UCPΔ9, UCPΔ3 and UCP*F/Y are used (Figure 1).

Expression in yeast and detection of the UCP activity

The diploid yeast strain W303 (**a**/α; *ade2-10; his3-11-15; leu2-3,112; ura3-1; can1-100; trp1-Δ1*) was transformed by electroporation, and yeast clones containing the expression vector were selected for uracil autotrophy on plates with SD minimal medium (2% glucose, 0.67% yeast nitrogen base, 0.1% casamino acids, 20 mg/l tryptophan, 40 mg/l adenine, 2% agar). Expression of the UCP under the control of the gal-cyc promoter (Cullin and Pompon, 1988; Pompon, 1988) was induced by galactose in the absence of glucose. For mitochondrial preparations or flow cytometry analysis yeast strains were grown overnight in liquid SG medium (2% galactose, 0.67% yeast nitrogen base, 0.1% casamino acids, 20 mg/l tryptophan, 40 mg/l adenine). Mitochondrial preparation, measurement of the mitochondrial respiration and of the membrane potential with the $DiSC_2(5)$ fluorescent probe have been described elsewhere (Arechaga *et al.*, 1993). The amount of the UCP expressed in yeast mitochondria was determined by Western blotting of mitochondrial proteins as previously described (Fernandez *et al.*, 1987; Casteilla *et al.*, 1990).

Flow cytometry

Flow cytometric analyses were performed with an EPICS V flow cytometer (Coulter Electronics) equipped with an argon laser (Spectra Physics 2025-05). A standard 76 mm nozzle was used in association with a confocal optic system to improve light scatter resolution. Room temperature water was used as sheath fluid. For the cyanine dye $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodine, Molecular Probes, Eugene, OR), the excitation was 488 nm at 200 mW and emission filters were 515 nm LP interference, 515 nm LP absorbance, 525 nm LP absorbance and a 560 nm SP interference for yellow/green fluorescence. The count rate was automatically set at 1500–2000 objects/s. Yeast cell viability was assayed using 1.5 μM propidium iodide; for this dye a 590 nm dichroic filter and a 610 nm LP absorbance filter were added. All dyes were prepared in ethanol at 100 μM. $DiOC_6(3)$ was added at 0.1 μM to about 1×10^6 yeast cells in order to avoid toxic effects on the respiratory chain activity or on mitochondrial F_0F_1-ATPase function (Mai and Allison, 1983), and the cells were incubated 30 min at 25°C prior to flow cytometric analysis. According to the values of forward scatter, side scatter and red fluorescence, debris, aggregates and dead cells could be recognized and were not considered in the analysis. The histograms were taken on 5×10^4 cells. For the expression of results, we used the pulse integral of fluorescence on a three-order logarithmic scale (Petit *et al.*, 1990; Glab *et al.*, 1993), for which an increment of 26 channels (of 256 channels) represents a doubling of fluorescence intensity.

For the cell sorting experiment, cultures of UCPΔ9 and UCP$^+$ yeast were mixed, equivalent amount of cells grown in SG medium were introduced. This mixture was subjected to cell sorting according to the level of $DiOC_6(3)$ fluorescence; 50 000 cells were selected corresponding to extreme values (the highest 1%, or lowest 1%) of green fluorescence. Sorted cells were diluted in SD medium in order to repress expression, then plated on SD plates on which colonies formed (25 000 viable cells were present in both cases). The DNA extraction from individual colonies was done with glass beads. Enzymatic amplification of the UCP cDNA with the oligonucleotide OL3 (GTGAAGGTCAGAATGCAAGC) and the anti-sense oligonucleotide OL6 (CAAGAATTCTATGTGGTGCAGTCCACTGT) generated a 524 bp and a 497 bp fragment respectively for UCP$^+$ and for UCPΔ9 coding sequences. This difference in size could be detected by electrophoresis through a 1.5% agarose gel.

Confocal microscopy

Yeast cells stained with 0.05 μM $DiOC_6(3)$ were examined with a MRC-600 confocal microscope (Bio-Rad) equipped with a 25 mW argon-ion laser and two detector channels. Excitation was performed using the 488 nm line from the argon-ion laser. Using an appropriate combination of filters, fluorescence emission was divided into yellow/green (515–540 nm) and red (600 nm and above) components, which were directed through different confocal apertures to the two separate photomultipliers. The red fluorescence of the propidium iodide was used to eliminate the dead cells from the picture taken from the yellow/green channel. Confocal sections were made at increments of 0.3 μm. Maximum brightness projections (Laurent *et al.*, 1992) of parts of the complete 3-D data set of optical sections were performed.

Acknowledgements

The authors wish to thank Pilar Zaragoza for mitochondrial preparations, Pedro Lastres (Madrid) and Dr Spencer Brown (Gif-sur-Yvette) for help with flow cytometry experiments, and Drs Philippe Djian and Odette Champigny for critical reading of the manuscript. This work was supported by the Centre National de la Recherche Scientifique, the Direction des Recherches, Etudes et Techniques (90-109), the Spanish Plan Nacional de I+D (BIO93-0544-C03-02), and by the PICASSO integrated action programme, between the Spanish Ministry of Education and Science and the French Foreign Affairs Ministry. I.A. was recipient of a fellowship from the Basque Government.

References

Arechaga,I., Raimbault,S., Prieto,S., Levi-Meyrueis,C., Zaragoza,P., Miroux,B., Ricquier,D., Bouillaud,F. and Rial,E. (1993) *Biochem. J.*, **296**, 693–700.

Bathgate,B., Freebairn,E.M., Greenland,A.J. and Reid,G.A. (1992) *Mol. Microbiol.*, **6**, 363–370.

Berg,J.M. (1989) *Cell*, **57**, 1065–1068.

Bouillaud,F., Weissenbach,J. and Ricquier,D. (1986) *J. Biol. Chem.*, **261**, 1487–1490.

Bouillaud,F., Casteilla,L. and Ricquier,D. (1992) *Mol. Biol. Evol.*, **9**, 970–975.

Casteilla,L., Blondel,O., Klaus,S., Raimbault,S., Diolez,P., Moreau,F., Bouillaud,F. and Ricquier,D. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 5124–5128.

Cullin,C. and Pompon,D. (1988) *Gene*, **65**, 203–217.

Dalbon,P., Brandolin,G., Boulay,F., Hoppe,J. and Vignais,P.V. (1988) *Biochemistry*, **27**, 5141–5149.
Evans,R.M. (1988) *Science*, **240**, 889–894.
Fernandez,M., Nicholls,D.G. and Rial,E. (1987) *Eur. J. Biochem.*, **164**, 675–680.
Glab,N., Petit,P.X. and Slonimski,P.P. (1993) *Mol. Gen. Genet.*, **236**, 299–308.
Härd,P., Kellenbach,E., Boelens,R., Maler,B.A., Dahlman,K., Freedman,L.P., Carlstedt-Duke,J., Yamamoto,K.R., Gustafsson,J.-Ü. and Kaptein,R. (1990) *Science*, **249**, 157–159.
Jezek,P. and Garlid,K.D. (1991) *J. Biol. Chem.*, **265**, 19303–19311.
Katiyar,S.S. and Shrago,E. (1991) *Biochem. Biophys. Res. Commun.*, **175**, 1104–1111.
Klingenberg,M. (1990) *Trends Biochem. Sci.*, **15**, 108–112.
Koning,A.J., Lum,P.Y., Williams,J.M. and Wright,R. (1993) *Cell Motil. Cytoskel.*, **25**, 111–128.
Laurent,M., Johannin,G., Le Guyader,H. and Fleury,A. (1992) *Biol. Cell*, **76**, 113–124.
Luisi,B.F., Xu,W.X., Otwinoski,Z. Freedman,L.P., Yamamoto,K.R. and Sigler,P.B. (1991) *Nature*, **352**, 497–505.
Mai,M. and Allison,W.S. (1983) *Arch. Biochem. Biophys.*, **221**, 467–476.
Mayinger,P. and Klingenberg,M. (1992) *Biochemistry*, **31**, 10536–10543.
Mayinger,P., Winkler,E. and Klingenberg,M. (1989) *FEBS Lett.*, **244**, 421–426.
Milner-White,E.J., Coggins,J.R. and Anton,I.E. (1991) *J. Mol. Biol.*, **221**, 751–754.
Miroux,B., Casteilla,L., Klaus,S., Raimbault,S., Grandin,S., Clement,J.M., Ricquier,D. and Bouillaud,F. (1992) *J. Biol. Chem.*, **267**, 13603–13609.
Miroux,B., Frossard,V., Raimbault,S., Ricquier,D. and Bouillaud,F. (1993) *EMBO J.*, **12**, 3739–3745.
Murdza-Inglis,D.L., Patel,H.V., Freeman,K.B., Jezek,P., Orosz,D.E. and Garlid,K.D. (1991) *J. Biol. Chem.*, **260**, 11871–11875.
Nedergaard,J. and Cannon,B. (1992) In Ernster,L. (ed.), *Molecular Mechanisms in Bioenergetics—New Comprehensive Biochemistry*. Elsevier, Amsterdam, The Netherlands, Vol. 23, pp. 385–420.
Nicholls,D.G. and Lindberg,O. (1973) *Eur. J. Biochem.*, **37**, 523–530.
Petit,P.X., O'Connor,J.E., Grunwald,D. and Brown,S.C. (1990) *Eur. J. Biochem.*, **194**, 389–397.
Pompon,D. (1988) *Eur. J. Biochem.*, **177**, 285–293.
Rial,E., Poustie,E. and Nicholls,D.G. (1983) *Eur. J. Biochem.*, **137**, 197–203.
Ricquier,D., Casteilla,L. and Bouillaud,F. (1991) *FASEB J.*, **5**, 2237–2242.
Schwabe,J.W.R. and Rhodes,D. (1991) *Trends Biochem. Sci.*, **16**, 291–296.
Schwabe,J.W.R., Chapman,L., Finch,J.T. and Rhodes,D. (1993) *Cell*, **75**, 567–578.
Walker,J.E. (1992) *Curr. Opin. Struct. Biol.*, **2**, 519–526.
Walker,J.E., Saraste,M., Runswick,M.J. and Gay,N.J. (1982) *EMBO J.*, **1**, 945–951.
Winkler,E. and Klingenberg,M. (1992) *Eur. J. Biochem.*, **203**, 295–304.

Received on January 27, 1994