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Research Article

Glutamine stimulates translation of uncoupling protein 2 mRNA

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Abstract. Uncoupling protein 2 (UCP2) belongs to a family of transporters/exchangers of the mitochondrial inner membrane. Using cell lines representing natural sites of UCP2 expression (macrophages, colonocytes, pancreatic beta cells), we show that UCP2 expression is stimulated by glutamine at physiological concentrations. This control is exerted at the translational level. We demonstrate that the upstream open reading frame (ORF1) in the 5' untranslated region (5'UTR) of the UCP2 mRNA is required for this stimulation to take place. Cloning of the 5' UTR of the UCP2 mRNA in front of a GFP cDNA resulted in a reporter gene with which GFP expression could be induced by glutamine. An effect of glutamine on translation of a given mRNA has not been identified before, and this is the first evidence for a link between UCP2 and glutamine, an amino acid oxidized by immune cells or intestinal epithelium and playing a role in the control of insulin secretion.

Keywords. Glutamine, mitochondria, UCP2, 5'untranslated region, translation, green fluorescent protein.

Introduction

The mitochondrial uncoupling proteins (UCPs) are transporters located in the mitochondrial inner membrane [1]. UCP1, is found in mammalian brown adipose tissue where it uncouples mitochondrial respiration from ATP synthesis; this leads to an increase in energy expenditure and thermogenesis [2, 3]. Since 1997, "new" UCPs have been discovered: UCP2 and UCP3 in mammals as well as related genes in other phyla including plants. These new UCPs are thought to be responsible for an inducible, mild uncoupling of mitochondria that may be able to prevent excessive mitochondrial ROS production. However, the physiological relevance of the proton transport activity of the new UCPs is still a matter of controversy [4–6].

An alternative strategy to evaluate the physiological relevance of the UCPs would be to determine triggers that induce UCP expression. In this respect we would like to emphasize here that the term "UCP2" would mean hereafter the protein UCP2, and whenever it will be referred to mRNA the term "UCP2 mRNA" will be used. Consequently, the term "UCP2 expression" used throughout the text will mean expression of the protein UCP2. Induction of UCP2 expression has been observed in several circumstances in mice, such as LPS injection or starvation, that have been associated with oxidative stress [7]. However, in our hands, treatments of cell lines known to promote oxidative stress failed to provide conclusive evidence for stimulation of UCP2 production (not shown). UCP2 is expressed in immune cells as well as in the gut, two sites of glutamine utilization as an energy substrate [8, 9]. An analysis of UCP2 expression in the mouse macrophage cell line RAW 264–7 revealed variations in UCP2 levels that correlated with the

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changes in the culture medium during the normal growth of this cell line. Glutamine is relatively unstable in the culture medium and therefore the hypothesis was that these variations in UCP2 expression level in fact reflected the glutamine level present in the medium. Therefore, the influence of glutamine on UCP2 expression was explored further.

Using several models of short-term variations in UCP2 expression, our previous studies have shown increased expression of UCP2 *in vivo* without change in UCP2 mRNA level [7]. Consequently, it was proposed that induction of UCP2 expression resulted from enhancement of translation of the UCP2 mRNA. The UCP2 mRNA has a long (370 nucleotides) 5' untranslated sequence (5'UTR) upstream of the UCP2 coding frame. In this 5'UTR there is a short 111-nucleotide open reading frame (ORF1). Using expression vectors, it was shown that shortening the 5'UTR and removal of the ORF1 enhance the production of UCP2 [7]. Recently, more detailed mutagenesis studies were performed [10] and it was hypothesized that, *in vivo*, ORF1 constitutively represses initiation of translation at the AUG codon of UCP2, whereas stimulation of UCP2 expression would be explained by the attenuation of this inhibitory role of the ORF1 [10].

We show here that glutamine specifically stimulates UCP2 mRNA translation, and we confirm the previously formulated hypothesis that mutations that abrogate the inhibitory role of ORF1 make the expression of UCP2 independent from the glutamine level.

Materials and methods

Media, chemicals and antibodies. Unless otherwise specified, anti-UCP2 antibody hUCP2-(2–606) [7] was used in this study. Aprotinin, bestatin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride (PMSF), bicinchoninic acid kit for protein determination (BCA1) and rabbit, mouse and goat horseradish peroxidase–-conjugated antibodies were from Sigma. RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), Ham's F12 medium, Earle's salt solution (EBSS), phosphate-buffered saline (PBS), fetal calf serum (FCS), penicillin, streptomycin, geneticin (G418), MEM amino acids (Invitrogen ref. 11130036 containing Arg, Cys, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, Val), glutamine, GlutaMAX I and TRIzol reagent were from Invitrogen. Cytochrome c and UCP2 (C-20) antibodies were from Santa Cruz Biotechnology (TEBU) and human mitochondrial porin antibody was from Molecular Probes.

Cell culture and treatments. The mouse macrophage cell line RAW 264–7 was obtained from the American Type Culture Collection (ATCC). Cells were routinely grown in RPMI 1640 medium containing 25 mM HEPES, 1 mM sodium pyruvate, supplemented with 10% heat-inactivated FCS, 2 mM glutamine or glutamax, 100 IU/ml penicillin and 50 µg/ml streptomycin. Raw 264–7 cells were seeded at a density of 1.7×10^4 cells /cm^2 and allowed to adhere for 24 h before changing the media. To test the difference in stability between glutamine and glutamax, cultures were processed for protein and mRNA 48 h later. The human colon carcinoma HT29 cell line was obtained from Dr M. Andriamihaja (INRA, Jouy en Josas). Before treatment, cells were cultured in DMEM-F12 medium supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin and 50 µg/ml streptomycin. The rat insulinoma cell line INS-1 was obtained from Dr. P. Maechler (University of Geneva) and maintained in RPMI 1640 medium containing 10 mM HEPES, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate, supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin and 50 µg/ml streptomycin. The stable transfected CHO-500 cells expressing UCP2 using the pcDNA3 expression vector were obtained using a method similar to that previously described with UCP1 [11] and maintained under G418 (50 µg/ml) selection pressure. The cells were grown in a humidified atmosphere of 5% CO_2. Media were changed every 2 days and cultures were serially passaged by trypsinization every 5–7 days.

To determine the role of glutamine, primary culture of macrophages, HT29, CHO-500 or INS-1 cells were transferred (after trypsinization) to EBSS for 3 h prior to addition of glutamine, unless otherwise indicated.

Protein synthesis rate in HT29 cells was estimated by determining the cycloheximide-sensitive incorporation of [L-U^{14}C]leucine into TCA precipitable material as in [12]. However, the incubation times in presence of the radiolabeled leucine were different (see below).

Isolation of mitochondria and preparation of total cellular extracts. To detect UCP2 in cell lines it was necessary to isolate mitochondria. Cells were harvested by scraping in EBSS, collected by centrifugation at 2000 *g* for 5 min, and washed in PBS. After 5-min centrifugation at 2000 *g*, the pellet obtained was resuspended in 10 mM Tris, pH 7.5, 250 mM sucrose (TS buffer) supplemented with 2 µg/ml DNase and the following protease inhibitors: 4 µg/ml aprotinin, 1 µg/ml pepstatin, 2 µg/ml leupeptin, 5 µg/ml bestatin and 0.1 mM PMSF. The cells were submitted to three cycles of 5-min freezing in liquid nitrogen followed by 10-min thawing at 37°C. Unbroken cells and nuclei were removed by centrifugation of the homogenate at 750 *g* for 10 min. The supernatant was centrifuged at 10 000 *g* for 20 min, and the mitochondrial pellet was suspended in 20 µl TS buffer.

This complex procedure was not necessary after transient transfection of CHO cells since the UCP2 level was high enough for cellular homogenates to be used. Cells harvested in PBS were collected by centrifugation and resuspended in lysis buffer (50 mM Tris pH 7.8, 150 mM NaCl, 1% Nonidet P-40) in the presence of the same protease inhibitor mix as above. After 10-min incubation at 37°C, insoluble material was eliminated by a 20-min centrifugation at 10000 *g*.

Detection of UCP2 and of UCP2 mRNA. Mitochondrial proteins (30 µg) were analyzed by Western blot using the anti-UCP2 antibody and the porin antibody following a procedure described previously [7]. Signals were detected and quantified with a CCD camera in the Gene Gnome apparatus (Syngene). The protein UCP2 signal was normalized using the corresponding porin value. For protein extracts, the same method was used but the protein UCP2 signal was normalized using the corresponding cytochrome c value.

Northern analysis of total RNA extracted with the TRIzol reagent from cell culture (20 µg) or transfected cells (5 µg) was carried out as described previously [7]. The UCP2 mRNA signal was quantified using a Packard instant imager (Packard Instrument Co., Meriden, CT), then normalized using the corresponding 18S rRNA.

Transient transfection. Approximately 7×10^5 cells were seeded per 60-mm dish and allowed to grow without antibiotics to 90% confluency before their transfection with 10 µl LipofectAMINE 2000 and 4 µg DNA in Opti-MEM medium. According to the manufacturer's instructions, the transfection mixture was replaced with complete culture medium after 5 h. After 23 h in culture, transfected cells were harvested for UCP2 mRNA and UCP2 protein quantitation.

The pTurboGFP-dest1 vector was obtained from Evrogen (www.evrogen.com). The TurboGFP-dest1 protein is a destabilized form of the GFP produced by fusing the PEST amino sequence encoded by the 422–461 region of the mouse ornithine decarboxylase gene to the C terminus of the TurboGFP [13]. To obtain the WT-GFP vector, the 5'UTR of the UCP2 mRNA was amplified by PCR using primers that

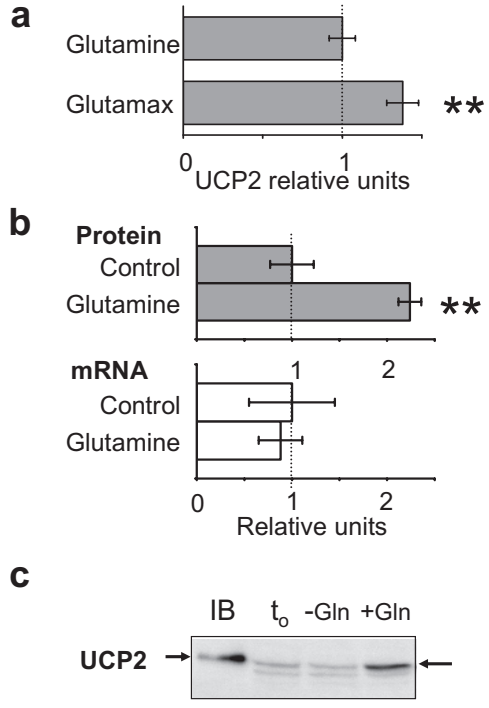

Figure 1. Glutamine induces uncoupling protein 2 (UCP2) expression in various cell types. (*a*) Macrophages: Raw 264–7 cells were cultured in RPMI medium as described in the Materials and methods supplemented with glutamine or with glutamax. UCP2 expression was evaluated by western blotting. The results are expressed as the mean ± SEM of 10 independent experiments. Statistical analysis was done using the Mann-Whitney test: ** $p<0.01$. (*b*) Colonocytes: HT29 cells grown in their medium were treated with 2 mM glutamine for 60 min. UCP2 levels and UCP2 mRNA levels were quantified as described in the Materials and methods. The values obtained with the untreated cells were considered as 1. The values represent the mean ± SEM of 12 independent determinations for UCP2 protein level (protein) and 5 for UCP2 mRNA (mRNA), ** $p<0.01$. (*c*) Insulinoma cell line INS-1. The arrows indicate the position of the UCP2 protein, distinguished from an immunoreactive band of a slightly lower molecular weight. This band is much less intense here [blot probed with the UCP2 (C-20) antibody] than when our own preparation of anti-UCP2 antibody was used. This C-20 antibody is directed against the C-terminal sequence of UCP2. IB: Inclusion bodies from UCP2-expressing bacteria. Glutamine was added (+Gln) or not (-Gln) for 2 h to the cells previously maintained for 3 h in Earle's salt solution (EBSS) (t_o). This image is representative of three independent experiments.

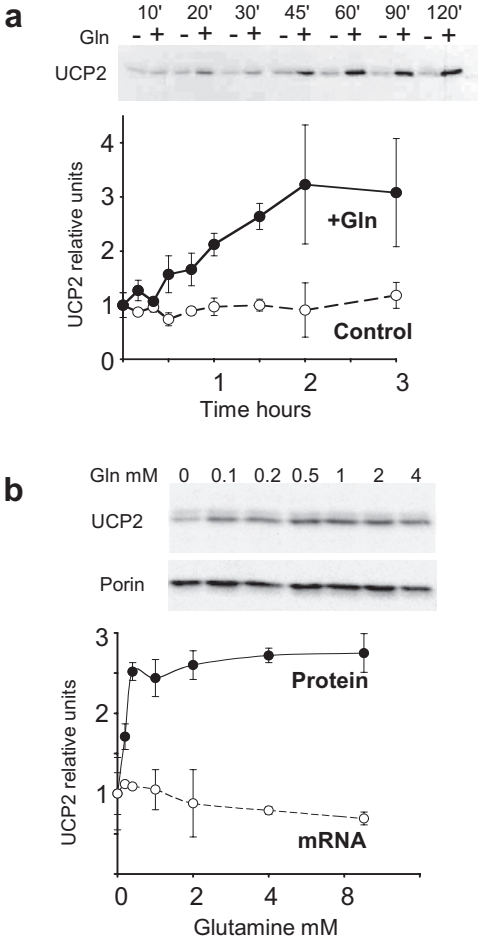

Figure 2. Time course and dose dependence. (*a*) Cells were first incubated for 2 h in the EBSS medium containing no amino acids, and glutamine was then added (2 mM final). UCP2 levels were quantified at time zero (before glutamine addition, normalized to 1), and 10, 20, 30, 45, 60, 90, 120 or 180 min after glutamine addition. Top: A representative Western blot probed with anti UCP2 antibody. Bottom: Quantitation, the values are expressed considering the mean value of the untreated cells as 1, and represent the mean ± SEM of three determinations. (*b*) Induction of UCP2 expression was performed following the method defined in (*a*). These "starved" HT29 cells were incubated with 0.2, 0.4, 1, 2, 4 or 10 mM glutamine. After 60 min of incubation, UCP2 protein or mRNA levels were determined as indicated in the Materials and methods. Top: A representative Western blot probed first with anti UCP2 antibody or with porin antibody. Bottom: Quantitation, the values are expressed as above and represent the mean ± SEM of two to three determinations.

created a *Sac*I site at the 5' end and a *Eco*RI site at the 3' end. This fragment was then introduced between the *Sac*I and *Eco*RI sites of the multiple cloning site (MCS) of the pTurboGFP-dest1 vector. Similarly, the ATG3/stop mutant of the 5'UTR of UCP2 was cloned in front of the TurboGFP-dest1 coding sequence.

Results

Glutamine induction of UCP2 expression in different cellular models. The use of a stabilized form of glutamine, the dipeptide L-alanyl-L-glutamine (glutamax), in the culture medium resulted in an enhancement of the UCP2 expression in the macrophage cell line RAW 264–7 (Fig. 1a). Other cell types where UCP2 expression occurs were subsequently examined to generalize the effect observed in macrophages. In the human colonocyte cell line HT29, a short-term exposure to 2 mM glutamine increased the UCP2 expression without any increase in mRNA level (Fig. 1b). Stimulation of UCP2 expression by glutamine was also observed in the rat pancreatic beta cell line INS-1 (Fig. 1c). Glutamine induction of UCP2

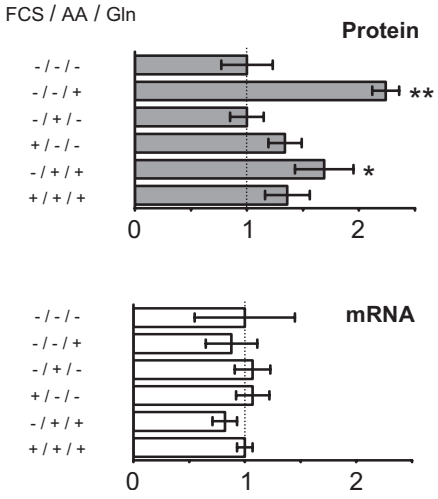

Figure 3. Interference with other components of the culture medium. At 3 h after the transfer of HT29 cells in EBSS, glutamine (2 mM), fetal calf serum (10%) or MEM amino acids were added to the medium. The cells were harvested 60 min later. UCP2 protein and UCP2 mRNA were determined as described in the Materials and methods. The values are expressed as the mean ± SEM of five to ten determinations for protein and four to eight for mRNA, * $p<0.05$; ** $p<0.01$.

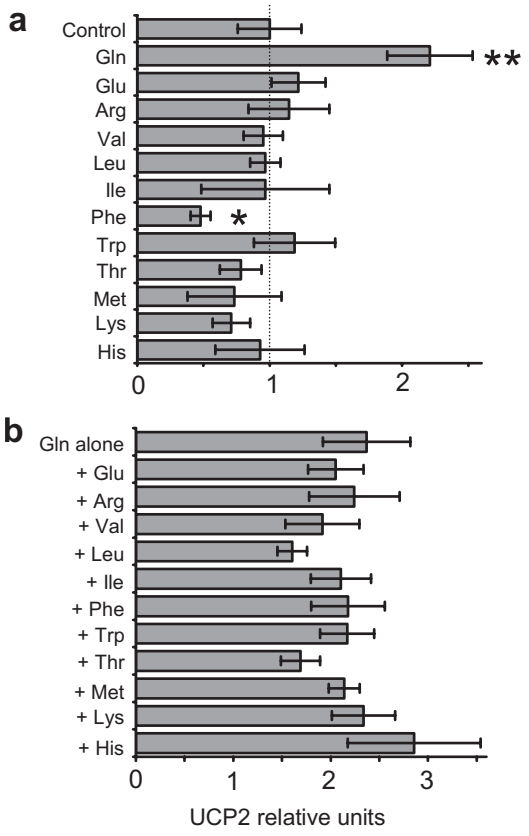

Figure 4. Specificity for glutamine. (*a*) UCP2 expression was examined in HT29 cells exposed to EBSS for 3 h, followed by the addition of 2 mM glutamine or 500 μM of the amino acid indicated on the left. Values are mean ± SEM of 5–11 determinations, * $p<0.05$, ** $p<0.01$ with control. (*b*) Interference of other amino acids with glutamine induction: UCP2 expression was examined in HT29 cells exposed to EBSS for 3 h, followed by the addition of 2 mM glutamine associated with 500 μM of the amino acid indicated on the left. Values are mean ± SEM of 5–11 determinations.

translation was therefore observed in three cell lines representing natural sites of UCP2 expression: macrophages, gut, and pancreatic beta cells.

Induction of UCP2 expression by glutamine was observed in primary culture of mouse macrophages (T. Nübel, personal communication). Therefore, this phenomenon is not restricted to immortalized cell lines.

Induction by glutamine is fast, specific and involves translation. These studies were performed in the HT29 cell line. Little or no delay in induction was observed (Fig. 2a) and the maximum level was attained within 2 h. Induction reached a stable maximum value at 0.4–1 mM glutamine in the medium (Fig. 2b). Interference with other components of the culture medium was also evaluated (Fig. 3): Addition of glutamine alone resulted in the largest induction factor in UCP2 expression. Addition of the essential amino acids present in the normal medium produced no induction. A mixture of other non-essential amino acids had no effect (data not shown). Adding the fetal calf serum alone seemed to cause low induction ($P = 0.07$). It is likely that some glutamine present in the serum, not dialyzed before use, explains this. Adding glutamine and essential amino acids together seemed rather to reduce the induction in UCP2 expression. Finally in a complete medium (+/+/+ in figure 3) the increase in UCP2 expression did not reach anymore statistical significance ($P = 0.06$). It could be compared to the situation shown in figure 1a where UCP2 expression was studied in macrophages grown in a complete medium. A detailed study used each single essential amino acid, plus arginine and glutamate (Fig. 4). Except for glutamine, none of the single amino acids used increased UCP2 expression, but phenylalanine significantly decreased UCP2 expression (Fig. 4a). No significant decrease/increase in glutamine induction was observed when another amino acid was added (Fig. 4b). Surprisingly, phenylalanine had no effect. This suggests the interesting possibility of a different regulation by this amino acid. However, a consequence of this is that glutamine and phenylalanine effects could not be studied simultaneously. To exclude the possibility that the increase in UCP2 merely reflected a general increase in protein synthesis rate, the effect of glutamine addition on protein synthesis was evaluated in two different experiments (Fig. 5). HT29 cells were incubated as before: *i.e.*, 3 h in EBSS before addition (or not) of 2 mM glutamine

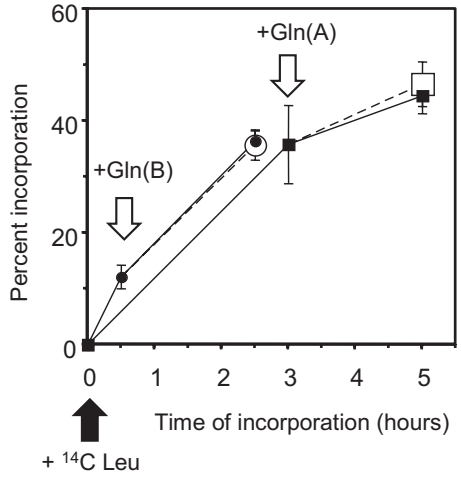

Figure 5. [^{14}C]Leucine incorporation into total cellular proteins. Experiment A (squares): HT29 cells were incubated in EBSS containing [^{14}C]leucine. After 3 h, incorporation was measured and glutamine (2 mM final) was added (empty symbol) or not (black symbol). Values given are the mean ± SEM of three measurements. Experiment B (circles): The experimental protocol was identical except that leucine was added later: 2.5 h after the beginning of the experiment (30 min before the optional glutamine addition). Therefore, the time of incorporation was shorter. The use of symbols is similar as in experiment A.

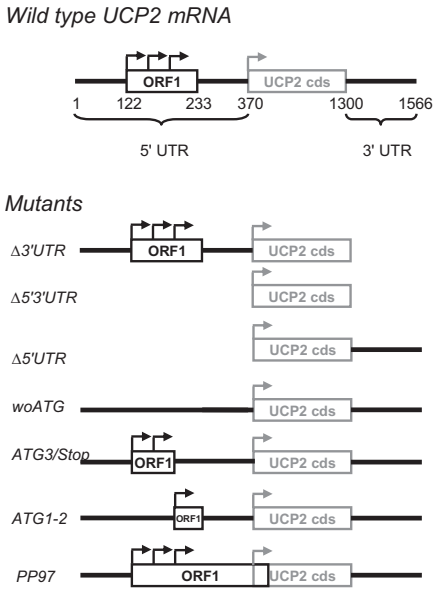

Figure 6. Mutants of the 5' untranslated region (5'UTR). The structure of the wild-type UCP2mRNA is shown on the top (the poly A tail is not indicated). For clarity, the scale is not exact but numbers indicate the positions (in nucleotides from 5' end) of the relevant limits. It corresponds to the UCP2 cDNA sequence introduced in the wild-type (wt) expression vector. The open reading frames (ORF) are shown as boxes: ORF1 and the UCP2 coding sequence (UCP2cds). The ATG codons are shown as arrows, all (three) ATGs of the ORF1 are indicated, whereas only the first ATG of the UCP2 is shown. The modifications introduced in the UCP2mRNA sequence in the expression vectors used in this study are shown below (Mutants). In the PP97 mutant the two ORFs overlap and are not in frame.

followed by a further 2 h of incubation. Protein synthesis rate was evaluated by detection of the cycloheximide-sensitive ^{14}C leucine incorporation in TCA precipitable material before glutamine addition (3 h) and at the end of the experiment (5 h). These two experiments indicated that in the conditions used the addition of glutamine had no effect on the overall protein synthesis rate in HT29 cells.

Mutations in the uORF abolish glutamine induction of UCP2 expression. Previous studies [7, 10] have used different expression vectors for UCP2 (Fig. 6). UCP2 mRNA translation was induced by glutamine in a stable transformant of the CHO cell line obtained after transfection with the wild-type UCP2 expression vector (CHO-500). The cells were incubated in saline medium with glucose (EBSS) to which glutamine was added immediately or after 3 h (Fig. 7a). Incubation in the EBSS medium resulted in a progressive decrease in the amount of UCP2 present (empty circles). The decay observed here is consistent with the observation that UCP2 has a short half-life [14]. Glutamine alone is sufficient to prevent this decrease (gray) or to induce expression afterwards (black). This experiment shows that the *cis*-acting elements contained within the UCP2 mRNA sequence are interacting with *trans*-acting factors (belonging to translation machinery), which are probably all ubiquitous since they exist in CHO cells normally not expressing UCP2. This allowed the use of mutants of the UCP2 expression vector [7, 10] whose characteristics are summarized in Figure 6.

Stable transformants obtained with deletion mutants of the UCP2 cDNA showed that the 5'UTR of the UCP2 mRNA is necessary to observe a stimulation of UCP2 production by glutamine (Fig. 7b). A protocol was designed to study the glutamine induction of UCP2 after transient transfection of CHO cells with UCP2 expression vectors (Fig. 8a). To improve the reproducibility of the results, 6 h after transfection, cells were incubated in the EBSS medium either deprived of glutamine (control) or supplemented with 2 mM glutamine. In these experiments the wild-type UCP2 expression vector was compared to the ATG3/stop mutant over an 18-h period of glutamine induction. A significant increase due to glutamine addition was observable with the wild-type expression vector (Fig. 8a circles). Induction appeared to peak 2 h after glutamine addition, which was consistent with our previous observations. No effect of glutamine was observed with the ATG3/stop mutant (Fig. 8a squares). This lack of stimulation was observed with three mutants of the 5'UTR ATG3/stop, woATG and Δ5'UTR (Fig. 8b). The stimulation was significantly reduced in the other mutants PP97 and

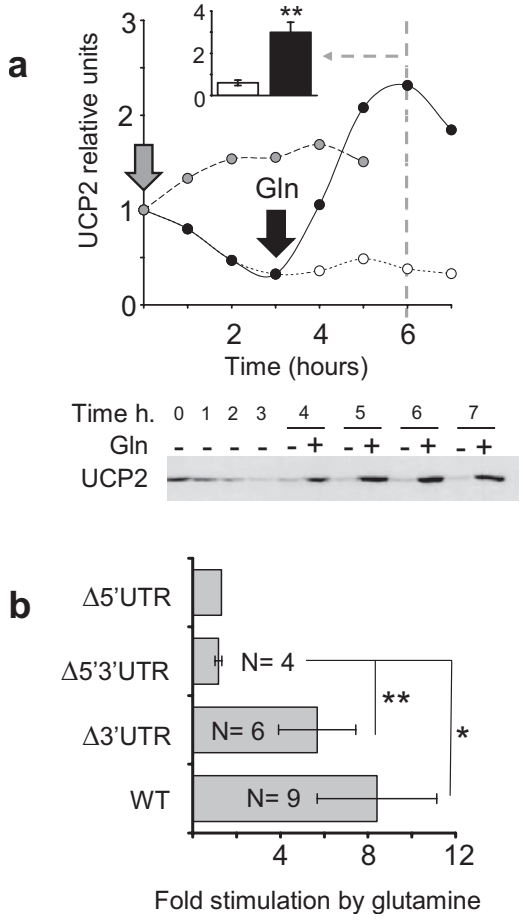

Figure 7. Glutamine stimulation of UCP2 expression in stably transfected CHO cells. (*a*) Use of the CHO-500 UCP2-expressing clone: cells were transferred to EBSS, a medium without amino acid. Glutamine (Gln) was added immediately (gray dots), never (white dots), or after 3 h of "starvation" (black dots). Glutamine addition is represented as a gray or black arrow. The curves represent the average of two experiments. Below is shown a representative Western blot (addition of glutamine at 3 h) probed with the anti-UCP2 antibody. Inset: UCP2 expression at the time indicated by the dotted gray line, EBSS alone for 6 h (empty histogram) or 3 h after glutamine addition (black histogram). The values are expressed as the mean ± SEM of four determinations. (*b*) Stable transformants were obtained after transfection with the wild-type UCP2 expression plasmid (WT), or expression vectors where the 3'UTR or 5'UTR or both UTRs of the UCP2 mRNA have been deleted. Clones expressing measurable level of UCP2 were studied in one experiment similarly as for the inset in "*a*". N refers to the number of independent clones and not to the number of experiments. Consistently, no statistical analysis is provided with the Δ5'UTR construct (only one clone could be studied), * $p<0.05$; ** $p<0.01$.

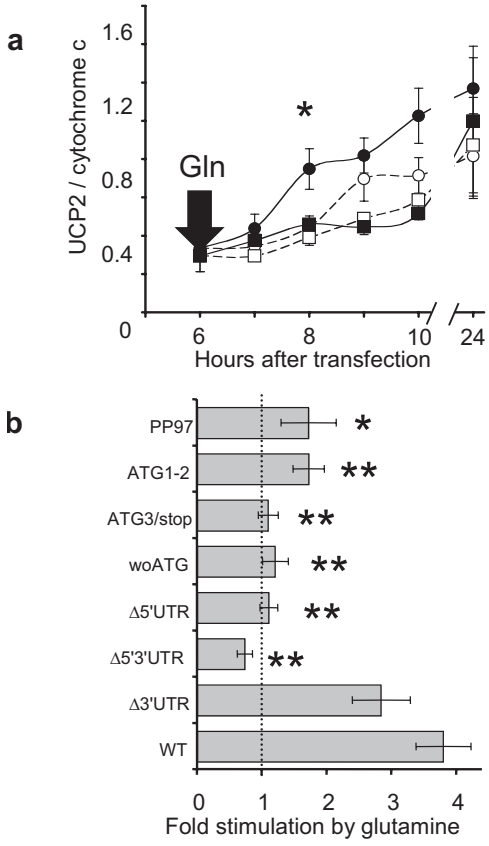

Figure 8. Glutamine stimulation of UCP2 expression in transiently transfected CHO cells. (*a*) Time course of induction in transient transfection. Cells were transfected with the plasmid at t=0.5 h after transfection, and the medium was replaced by EBSS; 1 h later (6 h after transfection) glutamine was added to one dish, the other was kept as control. UCP2 expression was evaluated subsequently every hour from 6 to 10 h and after 24 h. Filled symbols: UCP2 expression in presence of added glutamine; empty symbols: control in absence of glutamine; circles: wild-type UCP2 expression vector, squares: ATG3/stop mutant. (*b*) A protocol similar to that used in (*a*) was used with wild-type or mutated expression vectors and the induction of UCP2 (measured by the ratio: +Gln/control) 2 h after glutamine induction was determined, histogram shows mean values ± SEM, * $p<0.05$; ** $p<0.01$ compared with the wild-type expression vector (WT).

ATG1–2. All the UCP2 deletion constructs presented in Figure 7b were included in these experiments, and, although absolute values of stimulation were different between experiments (compare Figs 7b and 8b), the conclusions are identical to those obtained with stable transformants.

Our previous study used green fluorescent protein (GFP) as a reporter gene to evaluate the effect of the 5'UTR of UCP2 mRNA on the translation of the ORF located downstream [10]. For this study, a reporter gene product with a short half-life, comparable to that of UCP2 [14] was more suitable. Therefore, we replaced the GFP used previously by a destabilized form of this protein (see the Materials and methods section). Experiments made to evaluate the half-life of this "destabilized GFP" gave values of roughly 3 h in our experimental system (data not shown). Three constructs were studied (Fig. 9): (i) the original expression vector for destabilized GFP as a control "GFP", (ii) the same plasmid in which the 5'UTR of the UCP2mRNA has been introduced in front of the GFP sequence "WT-GFP", and (iii) the same construct but with the mutated form ATG3/stop of the UCP2 5'UTR "ATG3/stop-GFP". This reporter

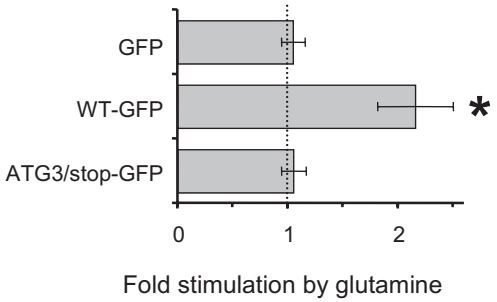

Figure 9. The 5'UTR of the UCP2 mRNA confers glutamine sensitivity to the green fluorescent protein (GFP) reporter gene mRNA. CHO cells were transfected with three different constructs made with a short-lived GFP reporter gene (see Materials and methods). GFP: pTurboGFP-dest1 expression vector; WT-GFP: the 5'UTR of the UCP2 mRNA was inserted in front of the GFP coding sequence in the pTurboGFP-dest1 expression vector; ATG3/stop-GFP: the same as before except that the mutated form ATG3/stop of the 5'UTR of the UCP2 mRNA was used. The measurement of the stimulation factor in the presence of glutamine was performed essentially as before (Fig. 8), * $p<0.05$.

gene approach confirmed that the 5'UTR of the UCP2mRNA confers glutamine sensitivity (compare GFP with WT-GFP). As expected, the response to glutamine of the GFP reporter gene was lost when the ATG3/stop mutated form of the UCP2 mRNA 5'UTR was used instead of the wild type.

Discussion

Induction of UCP2 and physiology of glutamine. To our knowledge this is the first report in which glutamine is identified as a signaling molecule promoting translation of a given mRNA. The full induction of UCP2 expression occurred in the 0–1 mM range (Fig. 2b), a concentration consistent with the approximately 0.5 mM glutamine in plasma [15]. Consequently, this regulation of UCP2 expression is likely to take place *in vivo*. In Figure 7a, both an increase and a decrease of UCP2 expression are observed according to glutamine concentration. It suggests that UCP2 expression level acutely reflects the glutamine concentration in the surrounding medium. The hypothesis that glutamine specifically acts as a regulator of UCP2 levels is supported by the fact that none of the amino acids tested could substitute for glutamine. Although it is not an essential amino acid, glutamine availability is of importance for cells because of its metabolic roles [16]: it is a precursor for many other molecules, and it is a substrate for mitochondrial ATP synthesis, which is preferred to glucose by lymphocytes or macrophages [17]. Accordingly, efforts have been made to provide conclusive evidence that glutamine indeed stimulates, in a specific way, the translation of the coding sequence situated downstream the wild-type UCP2 mRNA 5'UTR (Figs 6–9) and to rule out the possibility that the effect of glutamine on UCP2 production could be the result of a general effect of glutamine on cellular metabolism (Fig. 5) as observed in other conditions [18].

Glutamine induction and the 5'UTR of the UCP2 mRNA. The present results are consistent with the hypothesis that induction of UCP2 expression in the presence of glutamine operates through the release of inhibition caused by the presence of an upstream ORF1 in front of the UCP2 coding frame [10]. Accordingly, in most mutants of the 5'UTR, the loss of stimulation by glutamine can be attributed to the fact that mutagenesis diminished the ORF1 inhibitory power (woATG, Δ5'UTR, ATG3/stop) [10]. In contrast, this is not the case with the PP97 mutant, which shows both a decreased production of UCP2 in the absence of stimulation [10] and a decreased stimulation factor (Fig. 8b). This provides further information about how the stimulation by glutamine is likely to occur. Initiation at the ATG of the UCP2 coding sequence (UCP2cds) could result from two different mechanisms: (i) ribosomal subunits fail to initiate at ORF1 [10], or (ii) after translation of the ORF1, ribosomes remain bound to UCP2 mRNA and are available for a second round of initiation (re-initiation). In the PP97 mutant, the stop codon of the ORF1 has been moved into the UCP2cds. Therefore, in this mutant re-initiation is extremely unlikely as it would imply that when translation of the ORF1 is terminated the ribosome will move backward before resuming scanning. Consequently, the UCP2 produced by this mutant originates essentially from ribosomes that ignored the ATGs of the ORF1. The lack of stimulation observed with PP97 indicates that termination of translation of the ORF1 in front of UCP2cds plays a role in the stimulation by glutamine and therefore suggests a re-initiation step. This is similar to the situation of GCN4 in yeast, where intracellular amino acid concentration influences re-initiation events [19]. There are other examples of translational control implicating ORF in the 5'UTR of an mRNA: arg-2/CPA1 expression repressed by arginine [20, 21], and ornithine decarboxylase (ODC) or S-adenosylmethionine decarboxylase (AdoMetDC) expression repressed by polyamines [22]. In contrast, the present study provides an example of positive regulation by a single amino acid. Molecular events underlying this mechanism remain to be elucidated.

Relevance to UCP2 activity. Our experiments showed induction factors close to four- and as high as eightfold (Figs 7b, 8b). However, when the endogenous gene was studied, glutamine increased the expression level

of the UCP2 by a factor two or more but lower than three (Figs 1–4). These data are consistent with the induction of UCP2 expression seen *in vivo* [23]. Could such relatively modest variations be of physiological relevance? Predominant hypotheses concerning new UCPs (UCP2, UCP3) suggest that these proteins acts as partial uncouplers of oxidative phosphorylation. "Partial" means that the uncoupling activity (explained by the proton conductance of the UCP) remains of limited importance in comparison with the proton pumping activity of the mitochondrial respiratory chain (Mitchell's theory). Under these circumstances, UCP abundance limits the maximal extent of this partial uncoupling and therefore any variation in UCP2 abundance would have a directly proportional impact on the amount of metabolic energy that could be wasted through the UCP2 proton pathway. As mentioned previously, the physiological relevance of the proton transport of the new UCPs is still a matter of discussion. The present study suggests that UCP2 could ensure the transport of solutes related to glutamine metabolism through the mitochondrial inner membrane. In this latter case, the extent to which the UCP2 abundance controls glutamine metabolism remains to be determined. At this point it should be recalled that glutamine metabolism also takes place in organs that do not express significant amount of UCP2 (liver, kidney) [7]. Therefore, there is no strict correlation between the presence of UCP2 and glutamine metabolism. Future studies will aim to unravel whether UCP2, if it is expressed, is of any importance for the metabolism of glutamine.

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