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Functional Characterization of a *Drosophila* Mitochondrial Uncoupling Protein

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Sequence alignment of conserved signature motifs predicts the existence of the uncoupling protein 5 (UCP5)/brain mitochondrial carrier protein (BMCP1) homologue in *Drosophila melanogaster* (Hanak P. and Jezek P. (2001). *FEBS Lett.* **495**, pp. 137–141.). Here we demonstrate the functional characterization of the *Drosophila melanogaster* UCP5 protein (*Dm*UCP5) in the heterologous yeast system, the first insect UCP reported to date. We show that physiological levels of *Dm*UCP5 expression are responsible for an increase in state 4 respiration rates and a decrease in mitochondrial membrane potential. Furthermore, similar to UCP1, UCP2, and UCP3, the uncoupling activity of *Dm*UCP5 is augmented by fatty acids and inhibited by the purine nucleotide GDP. Thus, *Dm*UCP5 shares the mechanisms known to regulate the UCPs characterized to date. A lack of growth inhibition observed in *Dm*UCP5 expressing yeast is consistent with the notion that physiological uncoupling has a minimal effect on cell growth. Finally, semiquantitative RT-PCR analysis shows a distinctive pattern of *Dm*UCP5 expression predominantly localized in the adult head, similar to the expression pattern of its mammalian homologues. The conserved regulation of the expression of this gene from mammals to fruit flies suggests a role for UCP5 in the brain.

KEY WORDS: Mitochondria; uncoupling protein; respiration; *Drosophila melanogaster*.

INTRODUCTION

Located in the inner membrane of mitochondria, uncoupling proteins (UCP) form a family of mitochondrial anion transporters which allow protons to leak into the matrix thus dissipating the proton gradient generated during electron transport chain, and uncoupling respiration from ATP production (Stuart *et al.*, 2001a). In addition to its effect on energy metabolism, mitochondrial uncoupling is thought to alleviate reactive oxygen species (ROS) generation by allowing a more oxidized ubisemiquinone pool thereby reducing direct electron transfer to O_2 and decreasing O_2^- production (Brand, 2000).

The brown adipose tissue (BAT)-specific UCP1, the first uncoupling protein identified in this family allows heat generation in newborns and small mammals arousing from hibernation (Lowell and Flier, 1997; Nicholls and Locke, 1984). Indeed, mice deficient in UCP1 are cold sensitive (Enerback *et al.*, 1997). The role of UCP1 in controlling body weight is less clear and may depend upon the temperature of the environment (Liu *et al.*, 2003). Besides UCP1, four additional family members including UCP2, UCP3, UCP4, and UCP5/brain mitochondrial carrier protein 1 (BMCP1) have been identified in mammals (Fleury *et al.*, 1997; Gimeno *et al.*, 1997; Mao *et al.*, 1999; Sanchis *et al.*, 1998; Solanes *et al.*, 1997; Yu *et al.*, 2000). To date, UCP-like proteins have been found in all four eukaryotic kingdoms including animals, plants, fungi, and protists (Jarmuszkiewicz *et al.*, 2000). While the involvement of UCP1 in adaptive thermogenesis has been well established, physiological functions of UCP2 and UCP3 are only beginning to be revealed. The function of UCP4

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Key to abbreviation: BAT, brown adipose tissue; COX, cytochrome *c* oxidase; UCP, uncoupling protein; BMCP1, brain mitochondrial carrier protein 1; ROS, reactive oxygen species; *Dm*UCP5, *Drosophila melanogaster* uncoupling protein 5.

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and UCP5/BMCP1, both of which are predominantly expressed in the brain in mammals, is virtually unknown (Hanak and Jezek, 2001).

UCP2, with 59% sequence identity to UCP1 is widely expressed in mammalian tissues with predominant expression in skeletal muscle in human (Fleury *et al.*, 1997). Mice lacking UCP2 have a normal response to cold exposure but are resistant to parasitic infection, presumably due to elevated levels of ROS measured in their macrophages (Arsenijevic *et al.*, 2000). Additionally, UCP2 has been shown to negatively regulate insulin secretion and is implicated in diabetes (Fleury *et al.*, 1997; Zhang *et al.*, 2001). Moderate overexpression of UCP2 and UCP3 has been shown to reduce fat mass in transgenic mice (Horvath *et al.*, 2003c). Recent studies suggest that human UCP2 may protect the brain of mice from noxious insults. Expression of human UCP2 in mouse hippocampus increased neuronal survival after experimental epileptic seizures (Diano *et al.*, 2003). The neuroprotective role of human UCP2 was also demonstrated independently by Mattiasson *et al.* that mice expressing human UCP2 are more resistant to experimentally induced brain damage (Mattiasson *et al.*, 2003). These authors also demonstrated that in cultured cortical neurons expressing human UCP2, an inhibition of caspase-3 activity was observed when challenged with oxygen deprivation (Mattiasson *et al.*, 2003).

A third member, UCP3, sharing 56% sequence identity to UCP1 is expressed primarily in skeletal muscle and BAT (Boss *et al.*, 1997; Solanes *et al.*, 1997; Vidal-Puig *et al.*, 1997). Studies on UCP3 knock-out mice have demonstrated its role in reducing ROS production but not in affecting overall energy metabolism (Vidal-Puig *et al.*, 2000). However, transgenic mice overexpressing UCP3 in skeletal muscle have a reduction in adipose tissue mass, are lean compared to the wild-type littermates, and show an increased glucose clearance rate (Clapham *et al.*, 2000). The physiological role of UCP3 in energy expenditure and metabolism remains unclear. Two more recently identified UCPs, UCP4 and UCP5/BMCP1 sharing less homology to UCPs 1–3 (~30% overall identity) appear to be predominantly expressed in the brain (Mao *et al.*, 1999; Sanchis *et al.*, 1998; Yu *et al.*, 2000). The physiological function of UCP4 is virtually unknown whereas a reduction of ROS accumulation has been shown in UCP5 expressing neuronal cells *in vitro*, implying a potential role in neurodegenerative disorders involving oxidative damage (Kim-Han *et al.*, 2001).

Hanak and Jezek have shown by sequence homology that a predicted *Drosophila* protein, CG7314 (GadFly database) may be related to UCP5 (Hanak and Jezek, 2001). The high degree of conservation of all major protein domains including the presence of three unique UCP signatures and a purine nucleotide binding domain (PNBD) found in this predicted protein has led the authors to annotate CG7314 as the *Drosophila* UCP5/BMCP1 (*Dm*UCP5) (Hanak and Jezek, 2001). Given that the physiological functions of most of the UCPs are unclear, the identification of mitochondrial uncoupling like proteins in *Drosophila* would permit the powerful molecular genetic techniques available in *Drosophila* to be utilized to better understand the normal physiological roles of UCPs *in vivo*. A prerequisite for such studies is the biochemical–physiological characterization of these potential UCPs. In this report, we show the uncoupling activity of *Dm*UCP5 in the heterologous yeast system by demonstrating an increased respiration rate and a decrease in mitochondrial membrane potential as the result of *Dm*UCP5 expression. Moreover, the increased respiration rate can be modulated by lauric acid-dependent stimulation and GDP-dependent inhibition, two parameters validating the uncoupling activity. Little to no growth inhibition was seen in yeast cells expressing *Dm*UCP5. Finally, the expression profile of the endogenous *Dm*UCP5 shows predominant expression in the adult head, which mirrors the tissue distribution of the mammalian UCP5, and implies a potential role in the *Drosophila* brain.

EXPERIMENTAL PROCEDURES

Cloning and Expression of *Dm*UCP5 in Yeast

The full length *Dm*UCP5 cDNA fragment was RT-PCR amplified from RNA isolated from adult wild-type flies and a 24-nucleotide fragment (5′-GACTAC-AAGGACGACGACGACAAG-3′) encoding the FLAG epitope tag (DYKDDDDK) was fused in frame to the 3′ end of the *Dm*UCP5 coding sequence (Mao *et al.*, 1999). A sequence verified fusion fragment was then cloned into the GAL1 promoter driven expression vector pRS426 (gift of Dr. Ben-Mamoun, UCHC). Standard yeast transformation was carried out using a diploid strain BT4743 (gift of Dr. Ben-Mamoun, UCHC). Precultures of yeast transformed with either the vector alone or the *Dm*UCP5 construct were grown in minimal medium (DOB, Qbiogene) containing 2% raffinose as carbon source and necessary amino acids for uracil auxotrophic selection.

Isolation of Yeast Mitochondria

To prepare yeast cultures for mitochondrial isolation, overnight cultures grown in minimal medium containing 2% raffinose were induced with either 2% galactose or

2% glucose at $O.D._{600}$ of 0.8–1.0 and grown for 4 h at 30°C. Mitochondria were isolated following the procedure described previously (Stuart *et al.*, 2001c).

Mitochondrial Respiration with NADH as Substrate

Respiration of freshly isolated yeast mitochondria was determined in a Clark-type oxygen electrode at 30°C (Rank Brothers Ltd., UK). Mitochondria were resuspended in 150–200 μg/ml electrode buffer containing 3 mM NADH as substrate as described (Stuart *et al.*, 2001a). Oligomycin (1 μg/ml) was added to inhibit the ATP synthase to allow state 4 respiration. NADH, oligomycin, and GDP were dissolved in water and fatty acids laurate and palmitate were dissolved in ethanol and FCCP (carbonyl cyanide 4-trifluomethoxyphenylhydrazone) in methanol before adding to reactions. All chemicals were purchased from Sigma.

Measuring Mitochondrial Membrane Potential

Following 4 h of 2% galactose induction, 5 million yeast cells were washed in PBS and resuspended in minimal growth medium containing 10 μg/ml of JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanide iodide) (Molecular Probes, Oregon). Cells were protected from light and incubated at 30°C for 15–20 min and then washed twice in PBS and finally resuspended in 0.5 mL of PBS for flow cytometric analysis (Mao *et al.*, 1999). Flow cytometry was performed at excitation wavelength 488 nm and standard FL1 channel transmitting at 525 nm and FL2 channel transmitting at 590 nm (FACSCAN, Becton-Dickinson). A decrease in mitochondrial membrane potential is indicated by a fluorescence emission shift from red (590 nm) to green (525 nm). Yeast spheroplasts were prepared according to Averet *et al.*, (Averet *et al.*, 1998) and loaded with JC-1 in the presence of 1 μM FCCP to assess the mitochondrial membrane potential under the fully uncoupled state.

Yeast Growth Assays

Growth assays were performed by measuring both liquid culture proliferation and steady state colony formation. For liquid culture assays, overnight precultures were diluted into fresh minimal medium containing 2% galactose to $O.D._{600}$ of 0.2 (Harper *et al.*, 2002). Cell densities were measured during exponential phase of growth over 10 h and doubling times were calculated by least-squares regression. For colony assays, 1×10^2 cells were plated on either 2% glucose or 2% galactose containing agar plates and incubated at 30°C for 48–72 h (Sanchis *et al.*, 1998). The numbers of emerging colonies from triplicate plating of either vector expressing or *Dm*UCP5 expressing yeast were counted.

Endogenous *Dm*UCP5 Expression Analysis

To determine the expression pattern of *DmUCP5* during development, semiquantitative RT-PCR analysis was carried out (Radyuk *et al.*, 2003). Total RNA was isolated from wild-type Canton S embryos, larvae, pupae, and aged adult flies using TRIzol® Reagent (Invitrogen). Reverse transcription of 1 μg of total RNA was accomplished with oligo d(T) primers. Approximately 100 ng of first strand cDNA was used to amplify a 430 bp *Dm*UCP5 product with gene-specific primers (5′-ATACGAGGGCGTTCGTGG-3′ and 5′-GTACTTC TTTAGTTGTTCGTA-3′). Primers for coamplification of the rp49 gene were as previously reported (Radyuk *et al.*, 2003). To analyze tissue distribution of *DmUCP5* expression, heads and bodies of snap frozen adult flies were collected on ice after severing the head from the body in individual flies and subjected to RNA analysis.

Western Blot Analysis

To detect mitochondrial expression of FLAG tagged *Dm*UCP5 in transformed yeast, 200 μg of mitochondrial protein from isolated mitochondria (see above) was resolved on a 12% SDS-PAGE. Following transferring proteins onto a PVDF membrane, Western blot analysis with an anti-FLAG antibody at 1:1000 (Sigma) was performed (Harper *et al.*, 2002). The antibody against the yeast mitochondrial protein cytochrome *c* oxidase subunit III was used at 1:1000 (DA5, Molecular Probes) to demonstrate the purity of mitochondrial fractionation and copurification of this protein and the exogenously expressed *Dm*UCP5.

RESULTS

Sequence Alignment of Human and the Putative *Dm*UCP5 Proteins

The predicted sequence of the putative *Drosophila* UCP5 (CG7314, Gadfly database) protein is 53% identical to human UCP5, based on the sequence alignment

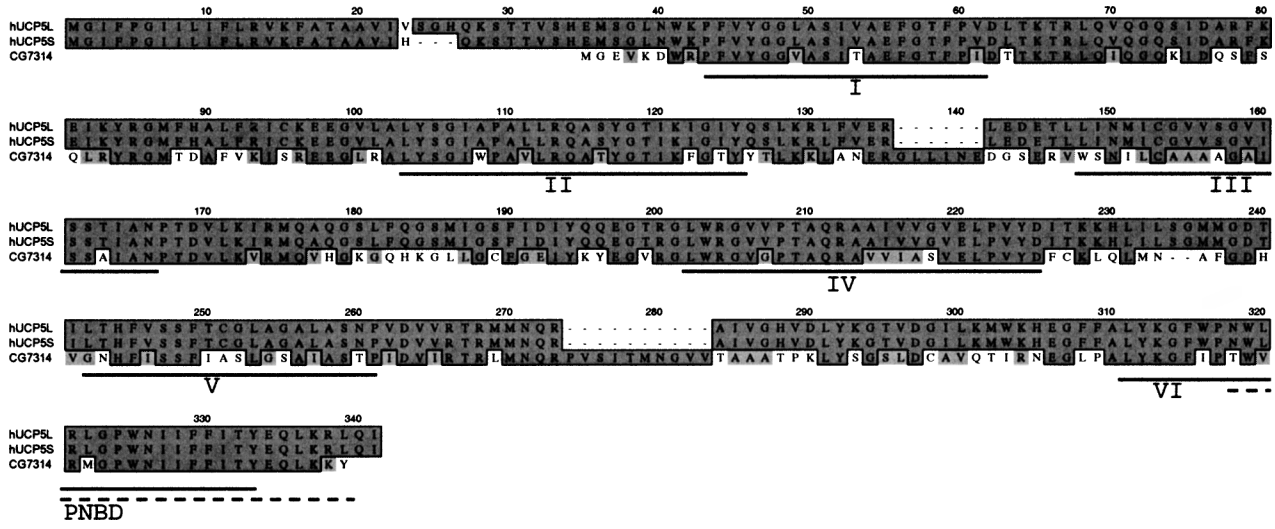

Fig. 1. Amino acid sequence comparison between *Drosophila* and human UCP5's. Predicted amino acid sequences for CG7314 (*Dm*UCP5) (GenBank™ accession # NM_140244) and two forms of hUCP5 [GenBank™ accession # AF155809 (L; long form), AF155811 (S; short form)] are shown (Yu *et al.*, 2000). The alignment was carried out with MacVector® ClustalW analysis software. The six putative transmembrane motifs (I–VI) and the purine nucleotide binding domain (PNBD) are underlined with the PNBD marked in dashed line (Hanak and Jezek, 2001; Jezek and Urbankova, 2000).

software MacVector® (Fig. 1) and (Hanak and Jezek, 2001).

Expression of *Dm*UCP5 Protein in Yeast

To assess the uncoupling characteristics of the *Dm*UCP5 protein, we utilized the yeast GAL1 promoter inducible system, an *in vitro* system widely used for determining physiological activities of several known and novel uncoupling proteins (Stuart *et al.*, 2001a,c). To allow detection of the *Dm*UCP5 protein in yeast, we fused the FLAG epitope tag in frame to the 3′ end of the *Dm*UCP5 coding sequence and cloned the fused fragment into the expression vector pRS426 (see Experimental Procedures). As shown in Fig. 2(A), using an anti-FLAG antibody, a 34 kDa protein band was detected in mitochondria isolated from *Dm*UCP5 transformed yeast after 4 h of galactose induction, but not in an identical culture induced with glucose. Mitochondria from yeast culture transformed with the pRS426 vector lacking the *Dm*UCP5 coding region also showed no expression of the 34 kDa protein band upon induction with galactose. We further confirmed the localization of the *Dm*UCP5 protein to the mitochondria by demonstrating that the *Dm*UCP5 protein copurified with the mitochondrial protein cytochrome *c* oxidase (COX) in the mitochondrial fraction, but not in the remainder of the intracellular organelles (Fig. 2(B)). These results are in agreement with previous findings that epitope tagging of the human UCP4 does not affect localization to mitochondria in tissue culture cells (Mao *et al.*, 1999).

The relative expression level of the mitochondrially localized *Dm*UCP5 protein was determined by comparing the intensity of the protein band to that of a known quantity of a purified FLAG-tagged protein, FLAG-BAP (Sigma). Densitometric analysis showed the protein expression level of *Dm*UCP5 to be approximately 25 ng of per mg of mitochondria (data not shown). This is a level comparable to or lower than what is detected for the endogenous UCP2 protein (31–313 ng/mg) in various mammalian tissues (Stuart *et al.*, 2001c) and significantly lower than the amount shown to cause artifactual uncoupling of yeast mitochondria by mouse UCP1 (Stuart *et al.*, 2001b). Thus, we have established an inducible, *in vitro* system likely to allow physiological measurements of the uncoupling activity of the *Dm*UCP5 protein.

Increased Respiration in Yeast Expressing the *Dm*UCP5 Protein

As a consequence of mitochondrial uncoupling, an increase in respiration and a decrease of mitochondrial membrane potential should be detected (Stuart *et al.*, 2001b). To characterize whether the putative *Dm*UCP5 protein possessed uncoupling activities, we first measured

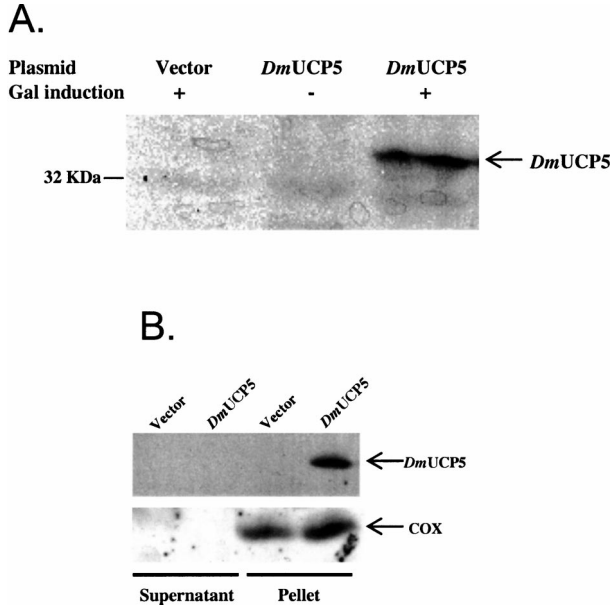

Fig. 2. Western blot analysis of galactose-induced expression of *Dm*UCP5 (A) and mitochondrial location of this protein (B). (A) Western blot analysis of mitochondrial protein of either vector alone or *Dm*UCP5 expressing yeast was performed. A protein product with the predicted size of 34 kDa recognized by the anti-FLAG antibody was only detected in *Dm*UCP5 expressing cells induced with 2% galactose (Gal) but not with 2% glucose (−). (B) Equal amounts of proteins from the mitochondrial fraction (pellet) and the supernatant after the last centrifugation of the mitochondrial preparations (see Experimental Procedures) was resolved on a 12% SDS-PAGE followed by Western blot analysis. The protein bands for *Dm*UCP5 and the subunit III of the mitochondrial protein cytochrome *c* oxidase (COX), respectively were detected to demonstrate the colocalization of the *Dm*UCP5 with the COX protein in the mitochondrial fraction. The anti-FLAG antibody was used at 1:1000 (top panel) (M2, UBI) for detection of FLAG-tagged *Dm*UCP5 whereas an antibody against the subunit III of yeast COX protein was used at 1:1000 (bottom panel) (DA5, Molecular Probes).

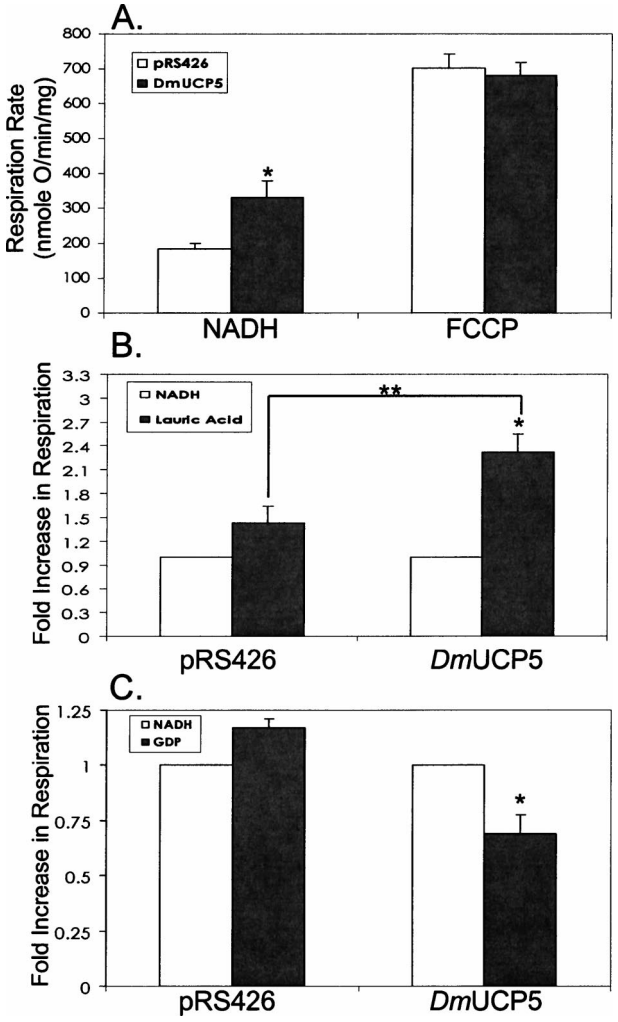

Fig. 3. Mitochondrial respiration of *Dm*UCP5 expressing yeast. (A) An increased mitochondrial respiration rate as the result of *Dm*UCP5 protein expression. Using NADH (3 mM) as substrate, the mitochondrial respiration rate was measured with 150–200 μg of mitochondrial protein from either *Dm*UCP5 or the pRS426 vector containing yeast ($^{*}p = 0.0128$, $n = 7$). The respiration rate for fully uncoupled mitochondria in the presence of the chemical uncoupler FCCP (1 μM) is shown for both *Dm*UCP5 and the pRS426 vector containing yeast. (B) Effects of lauric acid on the *Dm*UCP5 uncoupling activity. The respiration rate with NADH as substrate for either the pRS426 vector alone or *Dm*UCP5 expressing yeast mitochondria was arbitrarily set as 1 (open bars), to illustrate the relative fold increases in respiration rates in the presence of 100 μM lauric acid (filled bars) ($^{*}p = 0.02$, $n = 5$). (C) The *DmUCP5* uncoupling activity is purine nucleotide sensitive. The inhibitory effect of the purine nucleotide GDP (0.5 mM) in mitochondrial respiration was measured (filled bars) relative to the respiration rate with NADH as substrate (open bars). The addition of GDP resulted in a significant decrease in respiration rates affecting only *Dm*UCP5 expressing yeast but not vector controls ($^{*}p = 0.0072$, $n = 3$). All values are presented as mean ± SEM. Statistically significant differences are indicated by * and ** based on Student's *t* test.

respiration of isolated yeast mitochondria expressing the *Dm*UCP5 protein using NADH as substrate (Stuart *et al.*, 2001c). As shown in Fig. 3(A), following 4 h of 2% galactose induction, an increased, oligomycin-insensitive respiration rate was detected in *Dm*UCP5 expressing mitochondria as compared to control mitochondria. The respiratory control ratio value (the ratio between fully uncoupled respiration rate in the presence of FCCP and the respiration rate without FCCP) for the *Dm*UCP5 and the control vector expressing mitochondria is 3.175 ± 0.196 ($n = 8$) and 4.58 ± 0.358 ($n = 7$), respectively (mean ± SEM, $p = 0.0034$, Student's *t* test). These measurements demonstrate that yeast expressing *Dm*UCP5 at a level of approximately 25 ng of protein per mg of mitochondria are in a less coupled state of mitochondrial respiration than controls.

The Respiration Rate of Yeast Expressing *Dm*UCP5 is Stimulated by Fatty Acids and Inhibited by the Purine Nucleotide GDP

Although the precise mechanism whereby mammalian uncoupling proteins (UCPs 1–3) allow proton leak into the matrix is currently under intense investigation, it is clear that the activity of these proteins is stimulated by fatty acids and inhibited by purine nucleotides (Klingenberg and Echtay, 2001). As shown in Fig. 3(B), in the presence of the fatty acid, laurate, *Dm*UCP5 expressing mitochondria, but not control mitochondria containing only vector, show a 2.3-fold increase in respiration rates. Similar effects were observed when another fatty acid, palmitate was used (data not shown) (Stuart *et al.*, 2001c). Importantly, the specificity of the *Dm*UCP5 uncoupling activity was confirmed by demonstrating that a lower respiration rate was obtained as a result of the purine nucleotide, GDP inhibition (Fig. 3(C)). To further control our assay conditions, in all respiration experiments described here, yeast expressing the mouse UCP1 protein was included. The mouse UCP1 respiration rates in response to fatty acids and GDP were consistent with the reported results under our assay conditions (data not shown) (Stuart *et al.*, 2001b). Taken together, we have demonstrated in respiration experiments that *Dm*UCP5 can function as a *bona fide* uncoupling protein.

Decreased Mitochondrial Membrane Potential in Yeast Expressing *Dm*UCP5

To measure the effect of *Dm*UCP5 protein on mitochondrial membrane potential, we performed flow cytometric analysis on whole yeast loaded with the fluorescent, mitochondrial membrane specific sensor, JC-1 (Mao *et al.*, 1999). Following 4 h of 2% galactose induction, a decrease in mitochondrial membrane potential was detected in *Dm*UCP5 expressing yeast as compared to vector controls (Fig. 4). This finding is consistent with the increased respiration as the result of *Dm*UCP5 expression mentioned above. The presence of the chemical uncoupler FCCP resulted in a dramatic reduction of mitochondrial membrane potential reflecting the fully uncoupled state of mitochondria.

Effects of *Dm*UCP5 Expression on Yeast Growth

Mammalian UCPs, when expressed in yeast, have been shown to retard growth. However, this phenotype may vary depending upon the level of expression. For example, high levels of UCP expression in yeast cause a severe growth retardation and exhibit artifactual, purine nucleotide-insensitive uncoupling (Harper *et al.*, 2002; Stuart *et al.*, 2001b). Given our results demonstrating uncoupling of yeast mitochondria by *Dm*UCP5 protein at expression levels of only 25 ng/mg of mitochondrial protein, it is of interest to determine the effect of this level of *Dm*UCP5 expression on growth. We performed growth assays measuring both liquid culture proliferation during exponential phase of growth and steady state colony formation to assess the effect of the *Dm*UCP5 on yeast growth. As shown in Fig. 5(A), although there is a tendency toward an increase in doubling time in the yeast expressing *Dm*UCP5 under exponential phase growth, this difference is not statistically significant (3.19 ± 0.24 vs. 3.56 ± 0.17, $p = 0.13$; Student's t test). No growth inhibition was seen in the colony formation assay (Fig. 5(B)). These results suggest that induced mitochondrial uncoupling at a physiological level does not cause a significant detrimental effect on cell growth.

The Expression of *Dm*UCP5 is Developmentally Regulated and May Be Brain Specific

In order to begin to understand the physiological role of *Dm*UCP5 in its native environment, the fly, we examined the expression pattern of this gene during development and in adult *Drosophila*. Semiquantitative RT-PCR analysis showed that *DmUCP5* is expressed throughout development, and expression increases significantly in adult life (Fig. 6(A)). Given that the mammalian counterpart of *DmUCP5* is predominantly brain specific, we compared RNA isolated from the heads and the bodies of adult flies and found that a higher level of *DmUCP5* expression was associated with adult heads. The head of adult flies is predominantly made up of the brain, suggesting the possibility that similar to human and rodent *UCP5*, *DmUCP5* may be concentrated in the brain (Fig. 6(B)) (Sanchis *et al.*, 1998).

DISCUSSION

While the involvement of the "classic" UCP1 in BAT-specific thermogenesis and perhaps regulation of body weight has been established (Diehl and Hoek, 1999; Jezek, 2002), UCP2 remains the only other known UCP whose biological functions are better understood. The fact that UCP2 is involved in diverse cellular functions including regulation of insulin secretion, dopamine release, neuroprotection, and immunity further underscores

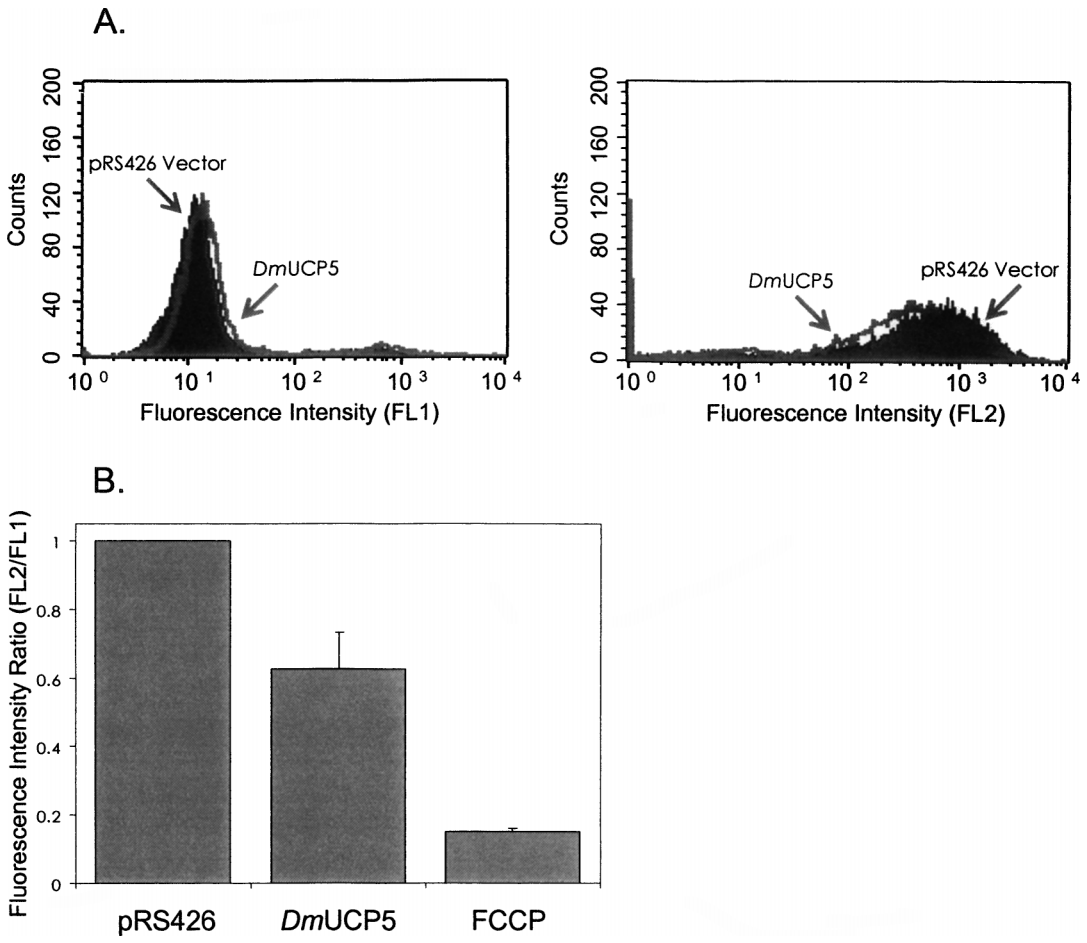

Fig. 4. A decrease of mitochondrial membrane potential in *Dm*UCP5 expressing yeast. Using flow cytometry the shift of fluorescent emission of JC-1, from wavelength 590 nm (FL2) to 525 nm (FL1), indicative of a decrease in mitochondrial membrane potential was measured and presented as FL2/FL1 ratios. (A) An increase in fluorescent intensity in the FL1 channel and a decrease in fluorescent intensity in the FL2 channel detected in *Dm*UCP5 expressing yeast is shown. (B) A decrease in FL2/FL1 ratios is seen in *Dm*UCP5 expressing yeast. The FL2/FL1 ratio for the pRS426 vector controls was arbitrarily set as 1 to reflect the decrease in mitochondrial membrane potential as the result of *Dm*UCP5 expression. Statistically significant differences between vector control (pRS426) and *Dm*UCP5 expressing yeast in FL2/FL1 ratios are shown ($^* p = 0.0001, n = 9$).

the importance and complexity of mitochondrial uncoupling (Horvath *et al.*, 2003a,b,c; Jezek, 2002; Yamada *et al.*, 2003). To begin to investigate the biological function of "novel" UCP family members, we sought to characterize the uncoupling activity of the *Drosophila* UCP5/BMCP1 homologue in order to establish an *in vivo Drosophila* model system for studying this endogenous UCP. The well-characterized *Drosophila* genetic system should complement molecular approaches for a better understanding of the biological consequences of mitochondrial uncoupling.

Using yeast to express one of the putative UCP-like proteins from *Drosophila* we have shown that the *Dm*UCP5 protein possesses three main properties of mitochondrial uncoupling proteins: (i) an increase in state 4 respiration rate with a concomitant decrease in mitochondrial membrane potential, (ii) a fatty acid-stimulated uncoupling activity, and (iii) a purine nucleotide-inhibited uncoupling activity. Fulfillment of these criteria thus qualifies the *Dm*UCP5 protein as a functional uncoupling protein, adding *Drosophila melanogaster* to an already large group of species from all four kingdoms of the eukaryotic world that express characterized endogenous mitochondrial uncoupling proteins (Jezek, 2002). Although yeast is widely used as a heterologous system for studying known and novel uncoupling proteins, it has been demonstrated that dramatic overexpression of UCPs can result in artifactual uncoupling and thus caution needs to

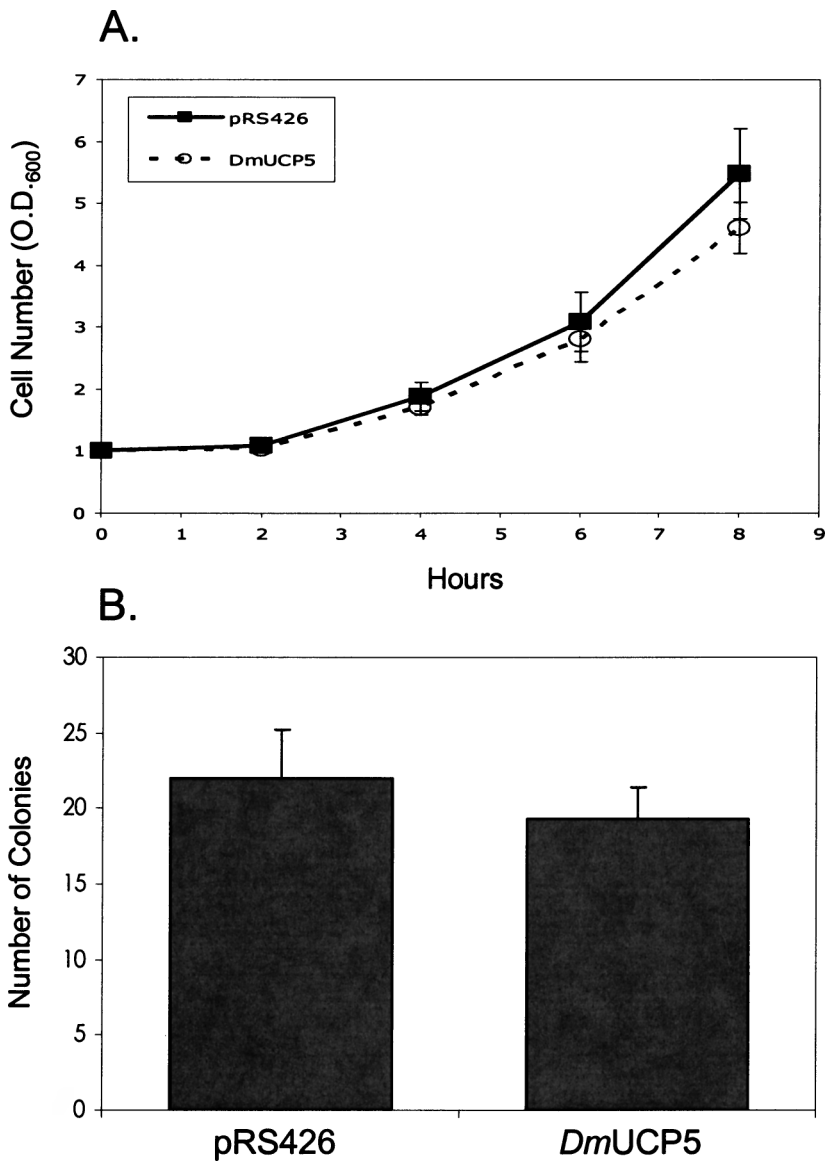

Fig. 5. *Dm*UCP5 expression does not retard growth. No statistically significant differences in cell proliferation were observed during the exponential growth phase (A) and colony assays (B) as the result of *Dm*UCP5 expression. (A) The growth curves during exponential phase of proliferation were monitored by spectrophotometric readings at $O.D._{600}$ and presented as mean ± SEM from four independent experiments (Student's *t* test, $p = 0.13$). (B) Yeast cells transformed and selected with either vector alone, pRS426 or *Dm*UCP5 were plated on minimal agar plates containing 2% galactose and incubated at 30°C for 72 h. Colonies emerging from each plate were counted. Each bar represents a mean ± SEM of quadruplicate plating experiments. Similar results were observed in two independent experiments.

be taken when using such a system for characterization of novel UCPs (Stuart *et al.*, 2001a,b). To address these concerns, we first determined the expression level of the FLAG-tagged *Dm*UCP5 following galactose induction to be comparable to or lower than what is detected for the endogenous UCP2 protein (31–313 ng/mg) in various mammalian tissues (Stuart *et al.*, 2001c). It is also lower than the amount of UCP1 expressed in yeast mitochondria shown to induce artifactual uncoupling (Stuart *et al.*, 2001b). At this level of expression, it is unlikely that *Dm*UCP5 protein would overload the mitochondria and damage the integrity of the inner membrane in a nonspecific manner. In fact we

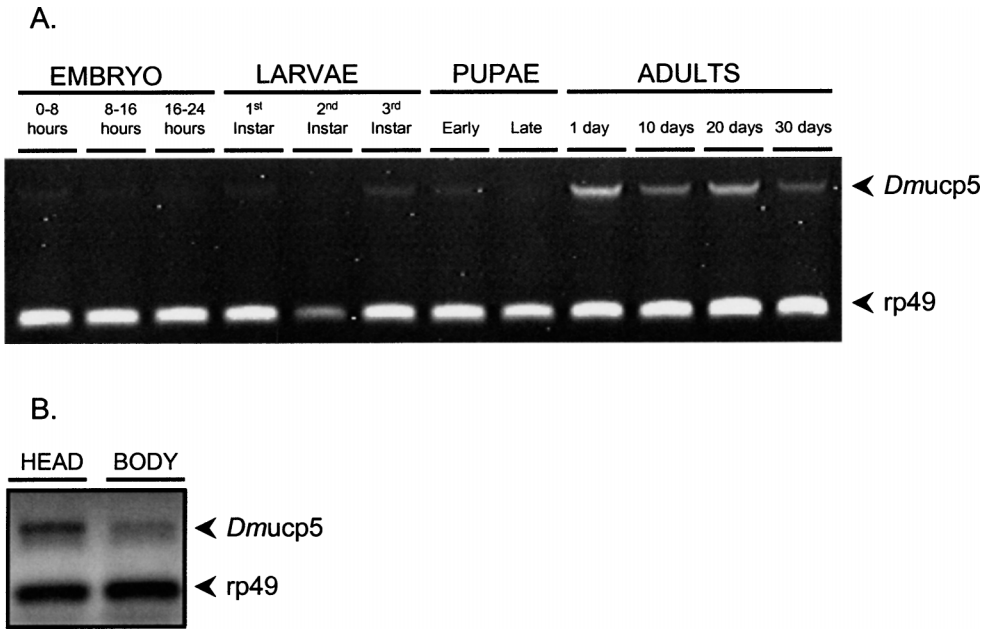

Fig. 6. Semiquantitative RT-PCR analysis of the *DmUCP5* expression. (A) Developmental profile of *DmUCP5* expression relative to the ubiquitous expression of the ribosomal protein gene *rp49*. (B) Enriched expression of *DmUCP5* in the adult head.

have obtained high levels of expression of a *Drosophila* homolog of the oxoglutarate carrier (∼500 ng/mg of mitochondrial protein) and only at this level of expression are we able to observe a mild decrease in membrane potential, suggestive of a nonspecific uncoupling effect (data not shown).

Two lines of regulatory control have been reported to fine-tune the activity of UCPs. First, fatty acids have been shown to activate UCPs characterized to date from all four eukaryotic kingdoms (Garlid *et al.*, 2001; Zackova *et al.*, 2003; Kim-Han *et al.*, 2001; Hourton-Cabassa *et al.*, 2002). In our studies we show stimulation of the *Dm*UCP5 uncoupling activity in the presence of two fatty acids tested. Thus insect UCPs also appear to conserve this regulatory mechanism. The second regulatory mechanism modulating the uncoupling activity of our insect UCP is through purine nucleotides. Sensitivity to purine nucleotides are thus conserved in mammalian, insects, fungal and protist UCPs but not in plants (Hourton-Cabassa *et al.*, 2002). This suggests that the ancestral UCP may have been sensitive to purines, but this mode of regulation may have been lost in plants.

Despite measurable uncoupling activity we found a lack of a significant growth slowdown in the exponential growth phase or in the colony growth assay of yeast expressing *Dm*UCP5. This is consistent with the mild to no growth retardation seen in UCPs1–3 expressing yeast at physiological levels (Harper *et al.*, 2002; Stuart *et al.*, 2001b,c). A marked growth inhibition in yeast colony assays with expression of mouse BMCP1 has been reported (Sanchis *et al.*, 1998). However, the level of BMCP1 expression in these studies was not determined and therefore may have been at a supraphysiological level, a level of expression shown to cause severe growth defects in yeast expressing UCPs1–3 (Stuart *et al.*, 2001c).

It has recently been suggested that mitochondrial uncoupling may protect cells, particularly neurons, against various toxic insults. An increase in mitochondrial uncoupling, resulting from expression of human UCP2 in a neuronal cell line or in hippocampal neurons in mice was shown to trigger a protective, antiapoptotic effect in response to various cellular insults (Diano *et al.*, 2003). Moreover, it has been shown that a controlled decrease of mitochondrial membrane potential in neuronal cells, a function that could be accomplished by an uncoupling protein, is beneficial to alleviating harmful Ca^{+2} influx during excitotoxic insults (Budd and Nicholls, 1996; Castilho *et al.*, 1998). Consistent with the notion that the *DmUCP5* gene may be predominantly expressed in the brain is the recent report that this gene was identified in a screen for dynamically expressed genes during the onset of neural lineages in *Drosophila* embryos (Brody *et al.*, 2002). Although further detailed analysis is required to determine the tissue distribution of the *DmUCP5* transcript in adult

flies, our findings of an enrichment of this transcript in the head suggests that similar to mammalian UCP5, *Dm*UCP5 may be predominantly expressed in nervous system tissue. The characterization of *Dm*UCP5 as a *bona fide* mitochondrial uncoupling protein provides the opportunity of employing the powerful molecular and genetic techniques of *Drosophila melanogaster* to further understand the normal physiological role of uncoupling proteins.

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