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Genomic Organization and Regulation by Dietary Fat of the Uncoupling Protein 3 and 2 Genes

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Uncoupling protein-1 (UCP1) dissipates the transmitochondrial proton gradient as heat. UCP2 and UCP3 are two recently discovered homologues that also have uncoupling activity and thus presumably have a role in energy homeostasis. We now report the genomic structure of murine UCP3 (7 exons) and UCP2 (8 exons). UCP3 is ~8 kilobases upstream of UCP2. An UCP3 variant mRNA, UCP3S, was also found and characterized. The effect of a high fat diet (45% versus 10%) on UCP3 and UCP2 mRNA levels was measured. Eating the 45% fat diet for eight weeks caused greater weight gain in AKR and C57BL/6J mice than in the obesity-resistant A/J mice. The high fat diet increased muscle UCP3 expression twofold in C57BL/6J animals. UCP2 expression increased slightly on the 45% fat diet in white adipose of AKR mice, but not in A/J or C57BL/6J mice. In skeletal muscle, UCP2 expression showed little variation with diet. Thus, UCP2 and UCP3 expression levels change in response to diet-induced obesity, but the changes are modest and depend on the tissue and genotype. The data suggest that it is not a reduction in UCP2 or UCP3 expression that causes obesity in the susceptible mice.

Key Words: **thermogenesis; diet induced obesity; uncoupling proteins.**

Uncoupling protein 1 (UCP1) is a member of the mitochondrial transporter family that dissipates the transmitochondrial proton gradient as heat rather than via ATP synthesis. Recently, two new genes, UCP2 and UCP3 were identified based on their similarity to UCP1 [1–5]. These new UCPs reduce the mitochondrial potential when overexpressed in vitro and presumably have uncoupling activity [1, 2, 5]. The UCPs have distinct tissue distributions. UCP1 is expressed exclusively in brown fat, while UCP2 is widely distributed and UCP3 is found in muscle and brown fat and, to a lesser extent, in heart and white fat.

In addition to their distinct tissue expression patterns, the three UCP genes show different responses to hormonal, metabolic, and environmental stimuli. Environmental cold increases UCP1 expression, via increased sympathetic stimulation, particularly β3-adrenergic, of the brown adipose tissue (reviewed in [6, 7]). UCP1-deficient mice are cold intolerant [8]. UCP2 can be regulated by inflammation, leptin, and peroxisome proliferator-activated receptor-γ (PPARγ) agonists [9–11]. Regulation of UCP3 is the most complicated, often with different regulation in muscle and brown adipose tissue. In muscle, UCP3 expression is increased by fasting, free fatty acids, exercise, and thyroid hormone, but not strikingly by cold or PPARγ agonists [5, 12–17]. The varied patterns of UCP expression suggests that different mechanisms regulate each UCP and that the different UCPs may have different functions.

Both UCP3 and UCP2 map to human chromosome 11q13 [1, 5, 18]. This region and the syntenic region in the mouse genome have been linked to variation in obesity, resting energy expenditure, and hyperinsulinemia [19–23]. Thus UCP3 and UCP2 are candidate genes for susceptibility to obesity and diabetes. We now report the genomic organization of murine UCP3 and UCP2 as a step towards understanding their regulation. We also examined the expression of UCP3 and UCP2 in mice prone (C56BL/6J and AKR) or resistant (A/J) to obesity induced by a high fat diet.

MATERIALS AND METHODS

Cloning murine UCP3 cDNAs. Standard cloning procedures were followed [24]. First-strand cDNA was made by reverse transcription from mouse muscle polyA(+) mRNA (Clontech). Next, PCR was performed using degenerate primers p610 5′-GG(AGCT)GC-(AGCT)ATGGC(AGCT)GT(AGCT)AC(AGCT)TG-3′ and p609 5′-GG(AGCT)CC(CT)TC(CT)TG(AGCT)GC(AGCT)ACCAT-3′ based on the translated human UCP3 sequence. The amplified fragment was cloned into the SmaI site of pBluescriptIIKS+ (Stratagene). Rapid

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Abbreviations used: UCP, uncoupling protein (prefixes: r, rat; m, mouse; h, human); PPARγ, peroxisome proliferator-activated receptor-γ.

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                                                E2 ↓ E3
mUCP3L  MVGLQPSEVP PTTVVKFLGA GTAACFADLL TFPLDTAKVR LQIQGENPGA -S...VQYRG VLGTILTMVR TEGPRSPYSG LVAGLHRQMS FASIRIGLYD 97
mUCP3S  ----Q--E-- --TV-N---- ---------L ---------- -------PGV -S...----- ---------- ----R---S- -----H---- ---------- 97
hUCP3L  ----K--D-- --MA-K---- ---------V ---------- -------QAV -TARL----- ---------- ----C---N- -----Q---- ---------- 100
hUCP3S  ----K--D-- --MA-K---- ---------V ---------- -------QAV -TARL----- ---------- ----C---N- -----Q---- ---------- 100

                E3 ↓ E4                                                                                 E4 ↓ E5
mUCP3L  SVKQFYTPKG ADHSSVAIRI LAGCTTGAMA VTCAQPTDVV KVRFQAMIRL G.TGGERKYR GTMDAYRTIA REEGVRGLWK GTWPNITRNA IVNCAEMVTY 196
mUCP3S  ----F----- T-H--VAI-- ---------- ---------- ------M-R- -.TGGE---R ---------- ---------- --W---T--- ---------- 196
hUCP3L  ----V----- A-N--LTT-- ---------- ---------- ------S-H- -PSRSD---S ---------- ---------- --L---M-- ---------- 200
hUCP3S  ----V----- A-N--LTT-- ---------- ---------- ------S-H- -PSRSD---S ---------- ---------- --L---M-- ---------- 200

                E5 ↓ E6                                                                                 E6 ↓ E7
mUCP3L  DIIKEKLLES HLFTDNFPCH FVSAFGAGFC ATVVASPVDV VKTRYMNAPL GRYRSPLHCM LKMVAQEGPT AFYKGFVPSF LRLGAWNVMM FVTYEQLKRA 296
mUCP3S  --------ES --F-GENG* 214
hUCP3L  --------DY --L-DNFP-- ---------- ---------- -------S-P -Q-----D-- ---------- ---------- ---GS----- ---------- 300
hUCP3S  --------DY --L-DNFP-- ---------- ---------- -------S-P -Q-----D-- ---------- -----* 275

mUCP3L  LMKVQMLRES PF* 308
hUCP3L  ---------- --* 312
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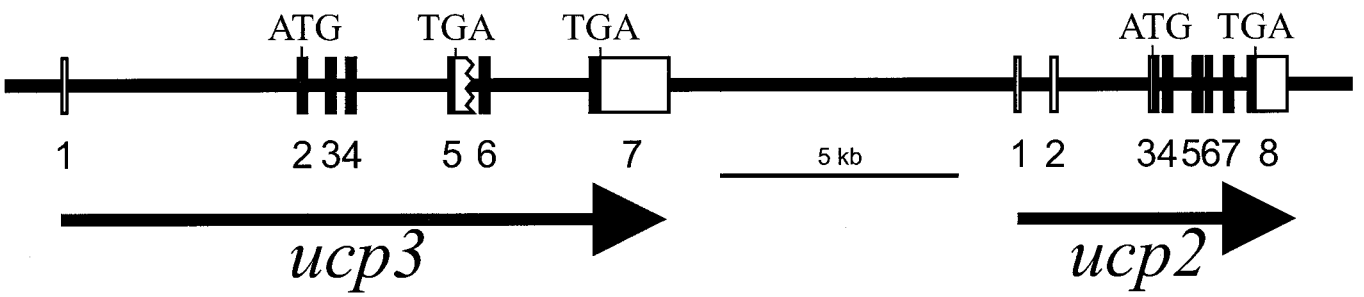

FIG. 1. Structure of the murine ucp3 and ucp2 genes. Top, exon numbers and boundaries are depicted above the deduced ucp3 protein sequence. The short form of murine ucp3 does not use the splice donor at the 3′ end of exon 5, while the short form of human ucp3 does not use the splice donor at the 3′ end of exon 6. GenBank Accession Nos. are mUCP3 AF053352, hUCP3 AF011449. Bottom, genomic organization of the ucp3/ucp2 region. Exons are indicated to scale and numbered below. Coding regions are shaded.

amplification of cDNA ends (RACE) was used to obtain the 5′- and 3′-cDNA ends. For the 5′-cDNA end, reverse-transcription used specific primer p613 5′-TCGGGTCTTTACCACATCCAC-3′ with mouse muscle polyA(+) mRNA and PCR used primer p614 5′-GTCGTAGGTCACCATCTCAGC and nested primer p615 5′-TTTCTTGTGATGTTGGGCCAA-3′ with anchor primers (Life Technologies). For the 3′-RACE, a dT18-M13F primer was used for reverse-transcription, then primer p616 5′-GATGGCGGTGACGTGCGCCCA-3′, and nested primer p692 5′-GGTGAAGGTCCGATTTCAAGC-3′ were used along with M13F. The PCR products were cloned and sequenced (dRhodamine terminator kit, Applied Biosystems).

Genomic cloning. A mouse UCP3 probe (nt 1 to 720 in GenBank Accession AF053352) was used to screen a 129 mouse genomic bacterial artificial chromosome library arrayed on filters (Genome Systems, St. Louis, MO). Hybridization was conducted at 65°C in Rapid-Hyb buffer (Amersham) with washing (0.5 × SSC/1% SDS) at 65°C for 15 minutes twice. Positive clones (numbers 17481, 17482) were analyzed by restriction mapping or subcloned into pBluescriptIIKS+. Plasmids containing exons were identified by hybridization and then sequenced.

Dietary manipulation and mRNA quantitation. Male A/J, AKR, and C56BL/6J mice (Jackson Laboratory) were housed in groups of 5 at ~22°C on a 12 hour light/dark cycle. At 5 weeks of age (5 days after arrival) the mice were begun on ad libitum feeding with a low fat diet (10 kcal% as fat; D12450, Research Diets, Inc., New Brunswick, NJ) or a high fat diet (45 kcal% as fat; D12451). Food intake in the first 2 weeks was determined. Intake was converted from grams to kcal using 3.85 and 4.73 kcal/g for the 10% and 45% fat diets, respectively, as determined by the manufacturer. Mice were euthanized with CO_2 and tissues were immediately frozen in liquid nitrogen. RNA was isolated from epididymal fat pads and quadriceps muscle and Northern analysis was performed as described [5]. The blots contained 15 μg total RNA per lane and were sequentially probed with mUCP3, mUCP2 [5], rUCP1 [5], and β-actin (nucleotides 1260-1279, of GenBank accession J00691). RNA levels were quantitated by Phosphorimager and statistical significance was determined using Student's t-test. RNA loading was checked using either ethidium bromide staining or the β-actin signal.

RESULTS

Genomic structure and organization of mUCP3 and mUCP2. We cloned and sequenced the murine UCP3 cDNA from muscle using degenerate PCR, 5′-RACE, and 3′-RACE (see Methods). The predominant cDNA, named mUCP3L, corresponds to the long form of human UCP3. mUCP3L encodes a 308-amino acid protein and is 88% identical to human UCP3. Our cDNA sequence confirms those independently reported in GenBank (AB008216, AB010742, and AF032902).

The genomic structure of murine UCP3 has not been reported previously. We isolated two genomic clones (see Methods) and used restriction mapping and Southern analysis to demonstrate that the clones over-

lapped. In both clones, UCP3 was located ~8 kb upstream of UCP2, with the two genes being transcribed from the same strand. mUCP3 and mUCP2 contain 7 and 8 exons, respectively (Fig. 1, Table 1).

An UCP3 mRNA variant. During characterization of the UCP3 mRNA using 3′-RACE, a new variant mUCP3 transcript was found and named mUCP3S. In mUCP3S, exon 5 is followed by sequence from intron 5, suggesting that the splice donor is not used in these transcripts. Inclusion of the intron results in a predicted protein truncated to 214 amino acids, missing two putative transmembrane helices (Fig. 1). mUCP3S mRNA was much less abundant than mUCP3L, as determined using both Northern blotting and RT-PCR (data not shown). Humans have a different hUCP3S isoform, resulting from use of a polyadenylation site in intron 6, thus encoding a protein missing only one transmembrane region. No murine homologues of the short human form were present in the 38 muscle 3′-RACE clones analyzed, suggesting that this form is not present in mice.

Regulation of UCP3 and UCP2 mRNA levels by dietary fat. To examine the role of UCPs in diet-induced obesity, we measured UCP mRNA levels in mice on low or high fat diets. Since genetic background influences susceptibility to obesity, three different strains of mice were used: AKR and C57BL/6J which are susceptible to diet-induced obesity, and A/J which is more resistant [25]. After 8 weeks on a 45% fat diet the AKR and C57BL/6J mice showed weight gains of 79% and 91% of initial body weight, while the A/J mice gained only 59%. Over this time, the weight gain was less in mice on a 10% fat diet (47%, 52%, and 36%, in the AKR, C57BL/6J, and A/J mice, respectively). The mice eating the high fat diet took in more calories than those on the low fat diet (16.8 vs. 14.6 kcal/d/mouse in AKR; 12.8 vs. 11.5 in C57BL/6J; and 14.6 vs. 12.9 in A/J).

The high fat diet increased the UCP3 mRNA levels 2-fold in C57BL/6J mice after 8 weeks of treatment (Fig. 2). A smaller increase was seen in the AKR strain, while no effect was observed in the A/J mice. Thus there is a difference between the three strains in their response to the high fat diet.

In contrast to UCP3, UCP2 expression in skeletal muscle showed little variation with diet (Fig. 2). Neither the AKR nor A/J strains showed a significant effect of diet. Only the C57BL/6J mice on the 45% fat diet showed slightly increased UCP2 mRNA levels.

UCP2 expression levels were also determined in epididymal white adipose tissue (Fig. 3). In the A/J and C57BL/6J mice there was no significant difference between the low and high fat diets. In contrast, there was a relatively small, but significant increase in the UCP2 mRNA levels in the AKR mice.

In brown adipose tissue, no increase in either UCP3 or UCP2 mRNA in the mice of any of the three genotypes fed the high fat diet was observed (data not shown).

TABLE 1

Exon–Intron Organization of the mUCP3 and UCP2 Genes

Exon No.	Exon size (bp)	5′ Splice donor	Intron size (kb)	3′ Splice acceptor
		UCP3		
1	~70	GCACAGgtaaga	7.0	ctgcagCTTCCT
2	219	CTGCAGgtgagt	0.5	ccccagATCCAA
3	202	CGGACCgtgagt	0.19	ccccagACTCCA
4	201	GGAAAGgtaacc	1.2	taacagGGACTT
5	102	TTACTGgtgaga	0.6	ctacagACAACT
5S	>220			
6	181	CAAAGGgtaagc	2.2	cctcagATTTGT
7	~1700			
		UCP2		
1	~40	GGTCCGgtgagc	0.75	ccacagGACACA
2	162	CTCCAGgtaagg	2.5	gcctagAACTCC
3	223	CTGCAGgtgagg	0.15	tttcagATCCAA
4	211	CAGAGCgtgagt	0.8	ccacagATGCAG
5	195	GGAAAGgtgtgt	0.08	ctacagGGACTT
6	102	TGACAGgtgtgt	0.29	tggcagATGACC
7	180	CAAGGGgtgagc	0.32	ctctagGTTCAT
8	~380			

DISCUSSION

Structure of UCP3 and UCP2. We report a new, minor mUCP3 mRNA, mUCP3S, which is predicted to encode a protein truncated by 31%. The human UCP3s mRNA previously reported would produce a protein missing 12% at its C-terminus [18]. The functional significance of the species-specific splicing is not clear, nor is it known if the shortened UCP3 proteins are stable in the cell. The missing C-terminal region has been implicated in purine nucleotide inhibition of uncoupling activity in the related protein, UCP1 [26–28].

We also report the genomic organization of murine UCP3. The introns are in the same positions as in human UCP3 [18, 29, 30] (YH, DWG, and MLR, unpublished observations). Our murine UCP2 genomic structure agrees with that recently reported [31]. The organization of UCP1, UCP2, and UCP3 is conserved, with each containing six coding exons interrupted by introns in homologous places. There is variation in the number of 5′ noncoding exons, with UCP1 having none, UCP2 having two, and UCP3 having one.

UCP3 has been mapped to human chromosome 11q13 and mouse chromosome 7, the same chromosomal regions to which UCP2 maps. We now show that UCP3 and UCP2 are very close to each other, only ~8 kb apart in the mouse genome, and are transcribed in the same direction. Since these genes are adjacent, it will be particularly important to demonstrate that any

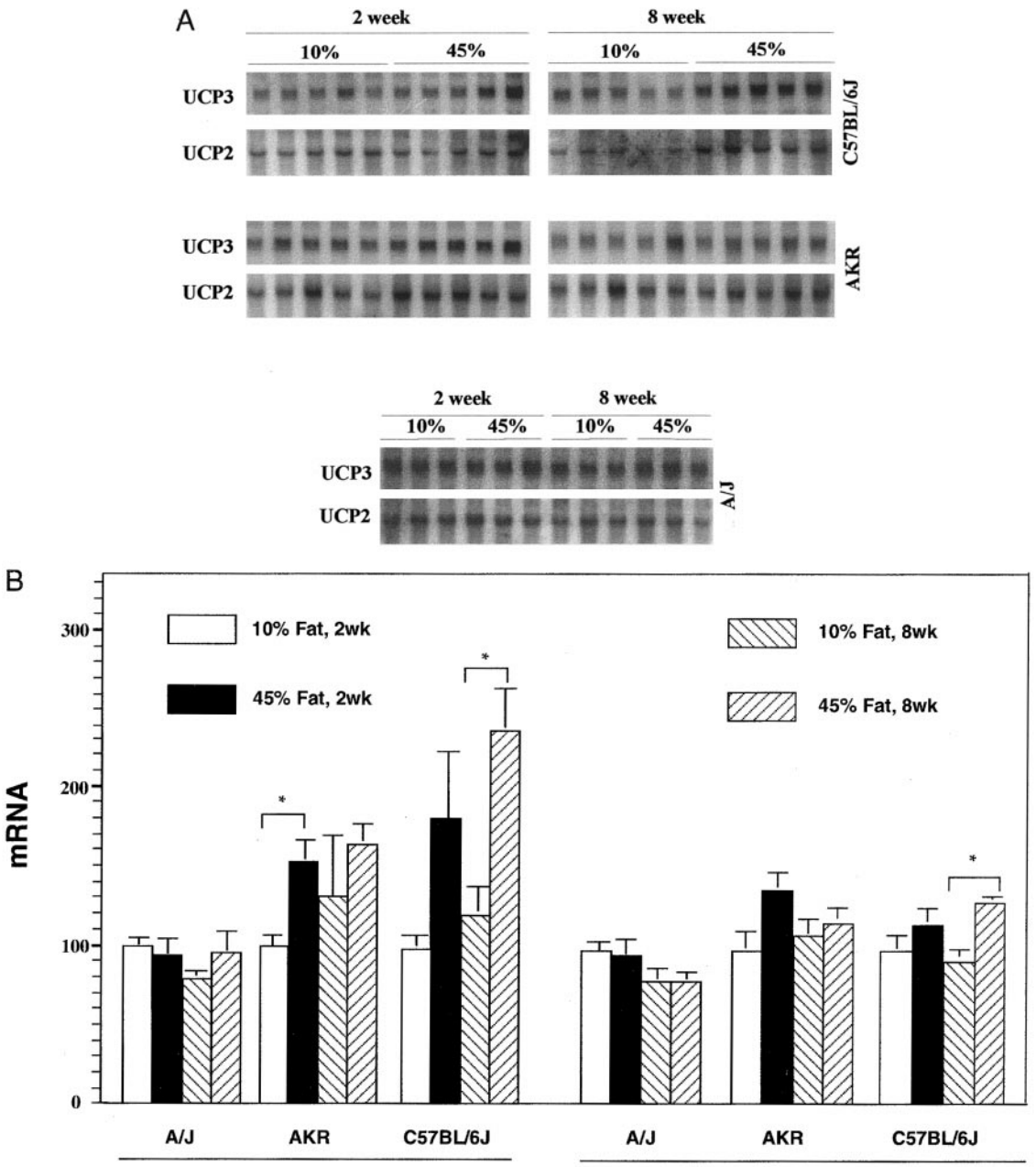

FIG. 2. A. UCP3 and UCP2 mRNA levels in skeletal muscle. Each lane contains RNA from an individual mouse fed a 10 or 45% fat diet for 2 or 8 weeks, as indicated. Blots were probed successively for UCP3, UCP2, and β-actin. B. Quantitation of UCP3 and UCP2 mRNA levels. Northern blots were quantitated using a Phosphorimager. Data are presented normalized to the 10% fat, 2-week samples within each strain. Data are mean, SEM. Significant differences ($P < 0.05$) between the low and high fat groups are indicated by an asterisk.

polymorphisms/mutations implicated in genetic studies be examined at the protein function level. The genomic organization also suggests that a local duplication produced the current UCP3 and UCP2 genes. The relatively low nucleotide identity between UCP3 and UCP2 implies that gene conversion or recombination between these genes is unlikely.

UCPs and diet. A role for adipose UCP2 in resistance to dietary obesity was hypothesized by Fleury et al. and expanded upon by Surwit et al. [1, 32]. They proposed that early induction of adipose UCP2 mRNA occurred in obesity-resistant strains, presumably mediating increased energy expenditure and delaying the onset of obesity. Supporting data included the observations that epididymal white adipose UCP2 mRNA levels were elevated 2.7-fold in obesity-resistant A/J mice fed a high fat diet for up to 4 weeks, but were relatively unchanged in obesity-prone C57BL/6J fat

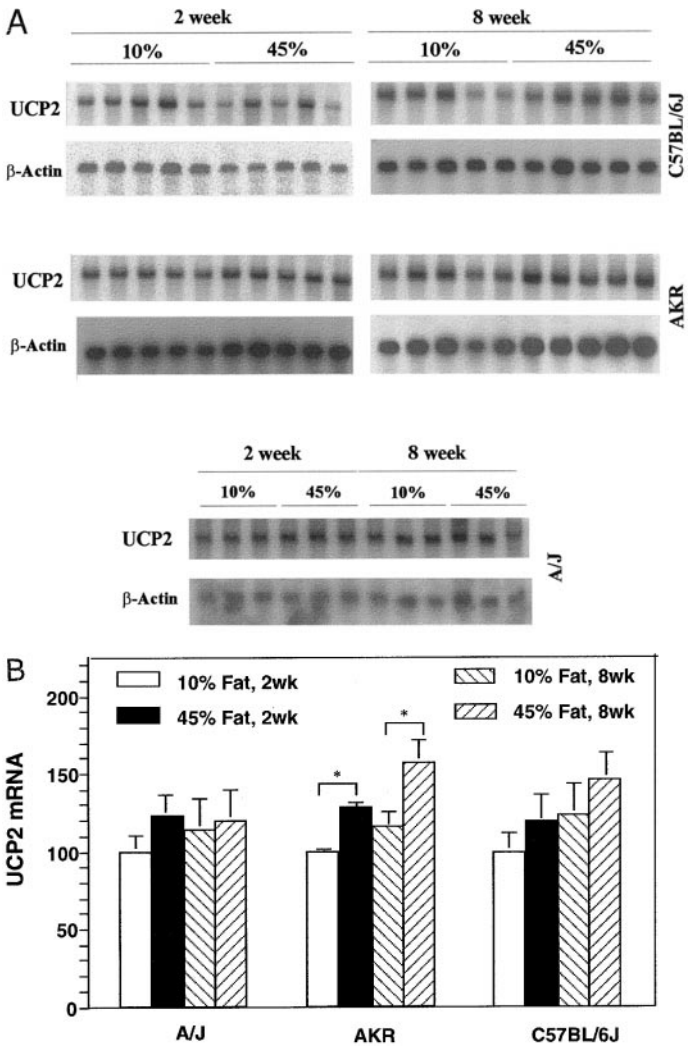

FIG. 3. A. UCP2 mRNA levels in white adipose tissue. Each lane contains RNA from an individual mouse fed the 10 or 45% fat diet for 2 or 8 weeks, as indicated. Blots were probed successively for UCP2 and β-actin. B. Quantitation of UCP2 mRNA levels. See Fig. 2B for details.

[1]. Long term (18 week) feeding of the high fat diet increased UCP2 in both strains. In contrast, our experiments did not show a significant increase in epididymal UCP2 mRNA in A/J mice at either 2 or 8 weeks. Our findings that muscle UCP2 mRNA levels did not change on a high fat diet agrees with those reported [32].

Surwit et al. observed that UCP3 mRNA levels were not changed by a high fat diet [32]. In contrast, we observed that UCP3 mRNA levels were 2-fold higher in C57BL/6J mice. A less dramatic increase was seen in AKR mice, and no change was observed in the A/J mice. A high fat diet also increased muscle UCP3 2-fold increase in Sprague-Dawley rats [33]. Notwithstanding the differences between laboratories and strains, muscle UCP3 mRNA levels tend to increase with a high fat diet. This may be due to the elevation of free fatty acid levels that occurs with obesity, since free fatty acids are thought to increase UCP3 mRNA levels [15] and UCP2 and UCP3 may facilitate fatty acid metabolism.

There are plausible explanations for the somewhat divergent data on UCP2 and UCP3 mRNA levels. Most importantly, the magnitude of the diet-induced increases in UCP3 and UCP2 expression are modest compared to changes caused by other manipulations, such as starvation, exercise, and thyroid hormone. Thus experimental variation may contribute to the divergent observations.

There are three conclusions to be drawn from our data. First, a high fat diet indeed causes changes in UCP2 and UCP3 mRNA levels, but the changes are subtle. Second, the animal's underlying genotype influences the changes in mRNA levels. Third, it is not clear whether these mRNA changes are a primary determinant of body fat content, or alternatively, a reflection of metabolic responses to changes in fat intake and body composition. Notably, reduced UCP2 and UCP3 expression was not observed in the strains susceptible to diet induced obesity, suggesting that it is not an initial reduction in expression that causes the obesity.

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