BFIT, a unique acyl-CoA thioesterase induced in thermogenic brown adipose tissue: cloning, organization of the human gene and assessment of a potential link to obesity

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We hypothesized that certain proteins encoded by temperature-responsive genes in brown adipose tissue (BAT) contribute to the remarkable metabolic shifts observed in this tissue, thus prompting a differential mRNA expression analysis to identify candidates involved in this process in mouse BAT. An mRNA species corresponding to a novel partial-length gene was found to be induced 2–3-fold above the control following cold exposure (4 °C), and repressed $\approx 70\%$ by warm acclimation (33 °C, 3 weeks) compared with controls (22 °C). The gene displayed robust BAT expression (i.e. $\approx$ 7–100-fold higher than other tissues in controls). The full-length murine gene encodes a 594 amino acid ($\approx$ 67 kDa) open reading frame with significant homology to the human hypothetical acyl-CoA thioesterase KIAA0707. Based on cold-inducibility of the gene and the presence of two acyl-CoA thioesterase domains, we termed the protein brown-fat-inducible thioesterase (BFIT). Subsequent analyses and cloning efforts revealed the presence of a novel splice variant in humans (termed hBFIT2), encoding the orthologue to the murine BAT gene. BFIT was mapped to syntenic regions of chromosomes 1 (human) and 4 (mouse) associated with body fatness and diet-induced obesity, potentially linking a deficit of BFIT activity with exacerbation of these traits. Consistent with this notion, BFIT mRNA was significantly higher ($\approx$ 1.6–2-fold) in the BAT of obesity-resistant compared with obesity-prone mice fed a high-fat diet, and was 2.5-fold higher in controls compared with *ob*/*ob* mice. Its strong, cold-inducible BAT expression in mice suggests that BFIT supports the transition of this tissue towards increased metabolic activity, probably through alteration of intracellular fatty acyl-CoA concentration.

Key words: fatty acids, hydrolase, metabolic rate.

INTRODUCTION

Remarkable shifts in the energetic profile of brown adipose tissue (BAT) occur upon exposure of rodents to certain environmental challenges (reviewed in [1,2]). Notably, non-shivering thermogenesis and a concomitant increase in fuel uptake and combustion are triggered in BAT upon exposure to a cold environment, thus providing a source of metabolic heat in defence of body temperature. A major catalyst of these events is uncoupling protein 1 (UCP1), which increases mitochondrial membrane proton permeability and thus accelerates flux of fuel-derived reducing equivalents in the electron transport chain. Sympathetic outflow to BAT increases in the cold, eliciting rapid biochemical changes supporting thermogenesis (i.e. stimulation of lipolysis). Such rapid changes are augmented by genetic regulation critical for the full manifestation of non-shivering thermogenesis in BAT. For example, expression of BAT UCP1 is rapidly and dramatically up-regulated following cold exposure in rodents [3], and its loss in UCP1 knockouts renders mice cold-intolerant [4]. Cold-induced proliferation and differentiation of BAT are more chronic events which, by definition, require changes at the level of gene regulation. Generally similar patterns of rodent BAT thermogenic activation may also occur in response to palatable high-fat diets [5,6], probably as a means to combat excessive weight gain under these conditions.

A more complete understanding of the molecular pathways underlying metabolic regulation will ultimately lead to optimal therapeutic regimens to treat obesity, diabetes and other disorders. Due to its metabolic malleability in response to thermoregulatory and nutritional cues, BAT is an attractive venue for studying the molecular basis of energy expenditure and fuel partitioning. BAT genes induced by cold exposure, but repressed in response to a warm environment, are likely to encode proteins involved in the transition of this tissue towards a more thermogenic state. Indeed, cold-induction of the recently described peroxisome proliferator-activated receptor-γ (PPARγ) co-activator-1 (PGC-1) fits this pattern, and this BAT-abundant transcription regulator probably plays a central role in sparking changes important to the regulation of metabolism in a variety of tissues [7,8].

The experiment described herein was based on the premise that metabolically relevant proteins will be discovered through analysis of genes expressed differentially in BAT derived from mice exposed to a variety of environmental temperatures. In particular, proteins encoded by genes upregulated at least 2-fold after cold exposure (4 °C, 48 h) were evaluated as potential candidates for their involvement in thermogenesis or intermediary metabolic regulation, based on their domain structures, homologies to known metabolic proteins and tissue-expression patterns. A gene encoding a BAT-abundant, novel murine acyl-CoA thioesterase termed brown-fat-inducible thioesterase (BFIT) was discovered, and found to be induced or repressed by cold and warm exposure, respectively. Murine BFIT (mBFIT) is homologous to a marginally characterized putative human

Abbreviations used: BAT, brown adipose tissue; UCP1, uncoupling protein 1; BFIT, brown-fat-inducible thioesterase; mBFIT, murine BFIT; hBFIT, human BFIT; WAT, white adipose tissue; RT-PCR, reverse transcriptase PCR; QEA, Quantitative Expression Analysis; ORF, open reading frame; NEFA, non-esterified fatty acid.

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protein KIAA0707 [9] (herein termed human BFIT1, or hBFIT1), whose novel splice variant (termed hBFIT2) is orthologous to mBFIT. [During the preparation of this article, protein and DNA sequences were submitted for an 'unnamed protein product' (GenBank accession no. AK023937) by the Helix Research Institute, Chiba, Japan. These sequences correspond to hBFIT2]. Evidence for an important role for BFIT in supporting enhanced metabolism includes its cold-induction in BAT, thioesterase and lipid-binding domains, co-localization with chromosomal sites associated with body fat in humans and mice, and significantly depressed BAT expression level in obesity-prone mice compared with their lean counterparts.

EXPERIMENTAL PROCEDURES

Animals and tissue collection

All experiments were done in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee at Genentech (South San Francisco, CA, U.S.A.). Differential gene-expression experiments were carried out using newly weaned male FVB-N mice (Taconic, Germantown, NY, U.S.A.), received at 3 weeks of age and housed at 2 mice/cage until tissue harvest at 6 weeks of age. Mice were fed standard rodent chow *ad libitum* (Chow 5010, Ralston Purina, St. Louis, MO, U.S.A.), and housed under a 12 h:12 h light/dark cycle (lights on at 06:00 h). Control and cold-challenged mice were housed at an ambient temperature of 22 °C during this period, whereas warm-acclimated mice were housed at 33 °C, within their thermoneutral zone. Cold-challenged mice were transferred to a 4 °C room for 48 h prior to tissue harvest. Warmth acclimation led to a $\approx 50\,\%$ reduction in food intake (results not shown). This prompted the addition of a food-restricted control group, to test whether warmth-induced changes in gene expression were specific to temperature or diminished food intake. The food-restricted group was fed a meal each morning (09:00 h) for 3 weeks prior to tissue harvest, given the amount of chow equal to the mean intake of a cohort of warm-housed mice for the previous 24 h. Tissues were harvested from this group at $\approx$ 4–6 h after their meal on the final day. For all groups, following CO_2-induced euthanasia in the afternoon, interscapular BAT was excised, cleaned of visible blood vessels, white adipose tissue (WAT) and connective tissue, and snap-frozen for subsequent RNA preparation. For differential expression studies, three independent BAT samples/treatment were generated for analysis; each sample was composed of BAT pooled from 10 mice. Follow-up studies examining the time course of cold-inducibility of BAT genes were carried out on cold-challenged mice exposed to 4 °C (1, 6 and 24 h), whereas studies examining the tissue distribution and cold-inducibility across tissues employed pooled samples (at least 3/tissue) from additional mice.

Some experiments employed a high-fat diet regimen to compare BAT gene-expression differences in model systems of obesity-prone versus obesity-resistant mice. The diet, containing 58 % of calories as fat, was based on that formulated by R. D. Surwit et al. [10] (Research Diets, New Brunswick, NJ, U.S.A.). Additional mice were fed a lower-fat diet containing 10.5 % of energy as fat [10]. Male mice received at 4 weeks of age were immediately placed on the diets, with interscapular BAT harvest occurring at 7 weeks of age. The first study compared expression in AKR/J and SWR/J mice, whereas the second study compared expression in C57BL/6J and A/J mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.). Additional studies compared expression in 9 week-old male *ob*/*ob* mice with lean *Ob*/? controls, fed standard rodent chow *ad libitum*. Tissues were harvested in the afternoon immediately after CO_2 euthanasia.

Differential gene-expression analysis and quantitative real-time reverse transcriptase PCR (RT-PCR)

Interscapular BAT mRNA samples were prepared, reverse-transcribed and subjected to CuraGen Quantitative Expression Analysis (QEA). The details of QEA analysis are described elsewhere [11]. Analysis focused on identification of genes regulated at least 2-fold by changes in ambient temperature. Follow-up analyses, using quantitative real-time RT-PCR (TaqMan) under conditions described previously [12], employed specific primers and 6-carboxyfluorescein/6-carboxytetramethylrhodamine-labelled probes recognizing mBFIT or hBFIT as follows (all are $5' \rightarrow 3'$): mBFIT, forward, TGAAGGATACCGGAACCCC; probe, CGGAGGTGCAGATGAGCCAGCTG; reverse, TACTGCCCTGCCACACCAA. hBFIT1, forward, TGCTGGGTTAGGGTCTCCCT; probe, ACTGAGCTGGTCTCGGCAAGTGGC; reverse, TCTATTCCTGGGGGCTCGA. hBFIT2, forward, TCTCTTGGACAACCGGAATGA; probe, TGGCCCCCAGCCTCCAGACC; reverse, TCTAGATGCCCTCAGTGGCC.

All quantitative RT-PCR data were normalized using 18 S RNA abundance to account for loading differences, using commercially available primer/probe sets (PE Applied Biosystems, Foster City, CA, U.S.A.). Human tissue mRNAs analysed by real-time quantitative RT-PCR were derived from the following sources: a human tissue mRNA panel (Clontech, Palo Alto, CA, U.S.A.), WAT (Zenbio, Research Triangle Park, NC, U.S.A.) and a human hibernoma sample derived from a 52 year-old female and procured from the University of Michigan. Hibernomas share some characteristics with BAT, a notion supported by the finding of substantial expression of the BAT-specific UCP1 in our sample (results not shown).

Radiation hybrid analysis

The hBFIT gene localization was carried out using the BFIT-specific oligonucleotide sets described above. Real-time RT-PCR was performed on the Stanford G3 Radiation Hybrid Panel of 83 human/hamster somatic clones. Positive clones were identified as having a signal above background and with a cycle threshold below 33 on the PCR amplification plot. The data were submitted to the Stanford server (http://www-shgc.stanford.edu/RH/G3index.html), and results were returned with linkage on chromosome 1 with a LOD score of 15.7 (see the Results section). Cytogenetic localization was obtained through the Genome Database (http://www.gdb.org). The mBFIT chromosomal locus was identified through radiation hybrid analysis of 93 hybrids of the Mouse/Hamster T31 Radiation Hybrid Panel (Research Genetics, Huntsville, AL, U.S.A.), employing PCR primers designed from the g1i0-182 fragment sequence (see Results) as follows ($5' \rightarrow 3'$): forward, GTAAGAAGGGAGCCTGGGAG, and reverse, TCTAGACCACCCTTTCTCCG. A collective set of scores (0 = no amplification; 1 = amplification; 2 = uncertain) were used to assess the radiation hybrid vectors, and analyses were performed in duplicate. Chromosomal placement of the mBFIT gene was accomplished using the Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research website (http://www.genome.wi.mit.edu/cgi-bin/mouse-rh/rhmap-auto/rhmapper.cgi). The LOD score for linkage was 15.

Statistics

Comparisons between any two treatments were made using a Student's *t* test, whereas statistical differences across temperature treatments were assessed using a Newman–Keuls treatment with $P < 0.05$ considered statistically significant. All values presented

are means ± S.E.M. from between three and five samples per treatment unless otherwise noted.

RESULTS

Identification of a novel, cold-induced gene in BAT

A 182 bp cDNA fragment (g1i0-182) corresponding to an mRNA up-regulated in the BAT of cold-challenged mice was identified through QEA (Figure 1). Sequencing of this short fragment suggested that it represented a portion of the 3′ untranslated region of a novel gene, as it lacked homology with genes or proteins in the public database, and did not display an obvious open reading frame (ORF; results not shown). The fragment sequence was utilized in strategies to obtain extended sequences from clones derived from an FVB/N murine BAT cDNA library. A number of clones contained a 594-amino-acid ORF with high homology (81 % identity, 83 % similarity) to a human acyl-CoA clone KIAA0707 (GenBank accession no. AB014607), predicted to encode a protein with a molecular mass of 68 kDa (Figure 2). None of the isolated clones contained an in-frame stop codon upstream of the first Met, but contig analysis identified matching overlapping murine expressed sequence tags (W64130 and AA051663) which extended the sequence in the 5′ direction to an in-frame stop codon. Furthermore, the human genomic region containing the entire ORF of KIAA0707 (see below) also contains an in-frame stop prior to the Met (results not shown).

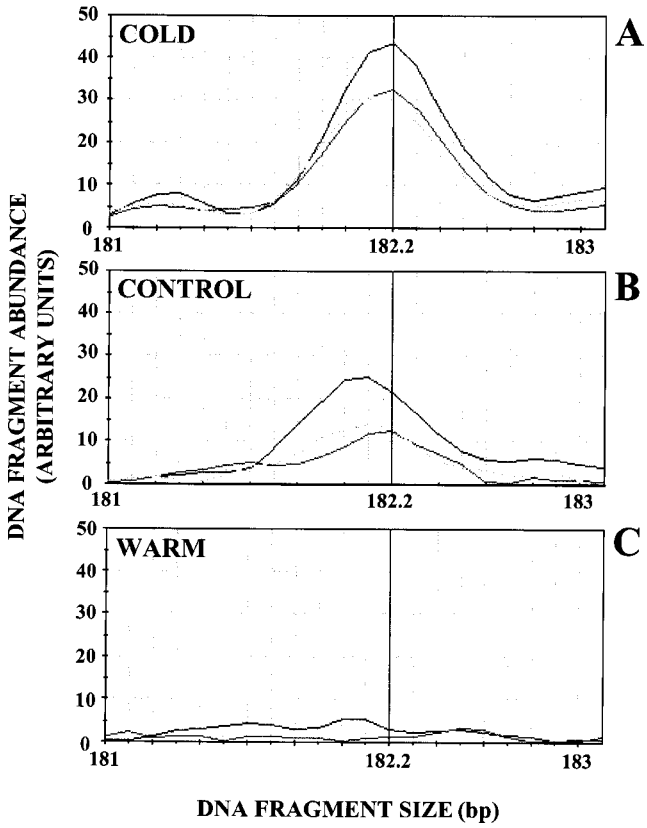

Figure 1 Identification of a cold-inducible gene in BAT by QEA

BAT mRNA samples derived from 6 week-old male cold-challenged mice (4 °C, 48 h), controls (22 °C) or warmth-acclimated mice (33 °C, 3 weeks) were subjected to a QEA analysis designed to uncover temperature-sensitive genes. Peak height corresponds to the abundance of a ≈ 182 bp cDNA fragment, which in turn represents the abundance of a specific mRNA species in the original sample. The abundance of the fragment, later determined to represent mBFIT (see Results), is highest in the BAT from cold mice (**A**), lower in controls (**B**) and negligible in warm mice (**C**). The bp values on the *x* axis are derived from gel location.

Interestingly, there appeared to be significant sequence divergence in the C-terminus of the murine protein compared with KIAA0707, beginning at residue 544 of the human protein (Figure 2). This result raised the possibilities that a separate gene may encode a protein orthologous to the version found in mouse BAT, or that alternative splice variants exist for the human gene. The latter possibility was confirmed as follows. The divergent mouse C-terminal sequence was used to search the public database for orthologous human ESTs, two of which were identified and found to include a stop codon towards their 3′ ends (ESTs AA587744 and AI732328). Employing a reverse primer specific to this region (5′-CCAGACCCTCTAGATGCC CTCA-3′) and a forward primer designed from the AB014607 sequence (5′-ATGATCCAGAATGTCGGAAATCAC-3′), a 1794 bp cDNA fragment containing the entire ORF of this variant was generated by PCR using human hibernoma cDNA as a template, and subcloned into the pcDNA3.1/V5-His-TOPO TA vector (Invitrogen, Carlsbad, CA, U.S.A.). A 1878 bp cDNA encoding the entire ORF of KIAA0707 was also PCR-generated and cloned from hibernoma cDNA, using the AB014607 forward primer and the reverse primer (5′-AGACACCTGAAACCTTA TCATGAGCC-3′). The predicted N-terminal sequences of the proteins encoded by these cDNAs matched that of KIAA0707, but the sequences diverged at the C-terminus, analogous with the mouse sequence compared with KIAA0707 (Figure 2). Both forms of the protein possess two acyl-CoA thioesterase domains and a START lipid-binding domain (where START stands for steroidogenic acute regulatory protein-related lipid transfer; Figure 2), indicative of involvement with lipid metabolism. Thus, based on domain structure and the expression pattern in the mouse, we have re-named the KIAA0707 protein hBFIT1, with the novel variants termed hBFIT2 and mBFIT2 in humans and mice, respectively. A mBFIT1 has not yet been identified. hBFIT1 was found to share the highest homology (30–35 % identity, 50–55 % similarity) with a variety of acyl-CoA thioesterases identified in *Chlamydophila* and *Bacillus* (i.e. AP002547_137 and AP001509_236, respectively), but also appeared related to the human or rat thioesterase CTE_2 (CTE2_HUMAN and CTE2_RAT, respectively) at 22 % identity and 41 % similarity.

Follow-up analysis indicated that mBFIT2 expression was induced by 2.5-fold in the BAT of cold-challenged animals (Figure 3A). Furthermore, acclimation to a warm environment reduced its expression by at least ≈ 70 % (Figure 3), thus confirming the results from the initial QEA (Figure 1). Interestingly, BFIT mRNA was also higher in the BAT of food-restricted/meal-fed mice (Figure 3A). The gene was found to display particularly robust expression in BAT compared with other mouse tissues examined, and the cold-induction of the gene was most apparent in BAT (Figure 3B).

BFIT association with chromosomal markers associated with body fat

Radiation hybrid analyses were carried out to identify the chromosomal localizations of hBFIT and mBFIT. Interestingly, mBFIT was co-localized with the markers D4Mit168 and D4Mit43 on chromosome 4, which rest in the region defined by the quantitative trait locus (QTL) Dietary-obese 1 (*Do1*) described by West et al. [13]. This QTL was discovered in a genetic analysis of obesity-prone AKR/J mice versus obesity-resistant SWR/J mice. *Do1* was hypothesized to contain a gene which plays a role in driving the large differences in body fat content between the strains, observed after feeding a high-fat diet [13].

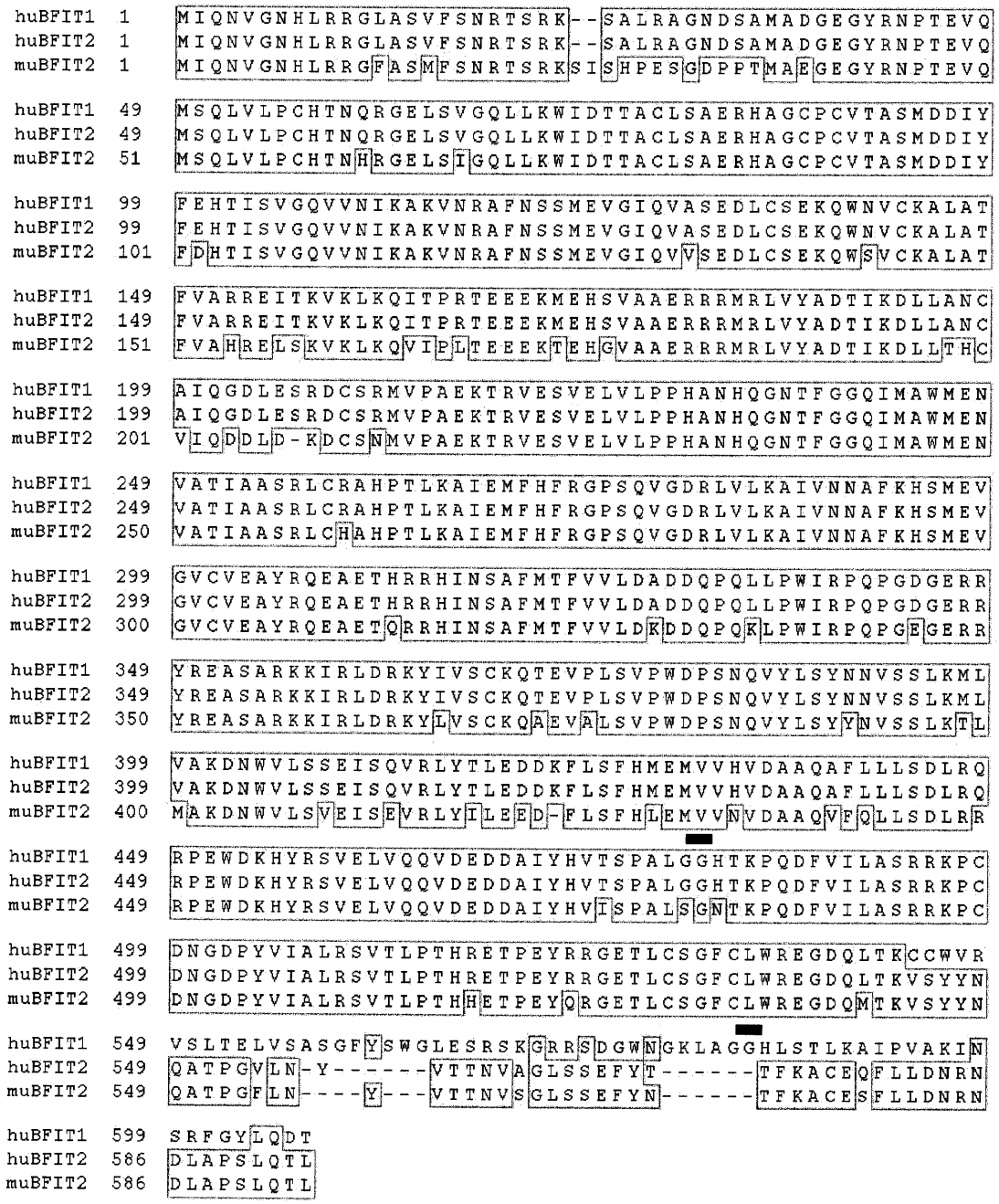

Figure 2 Amino acid sequences of human and murine BFIT variants

Alignment of BFIT sequences from human (huBFIT) and mouse (muBFIT) indicates the strong conservation of BFIT across species, and the presence of alternative splicing events. The divergence of hBFIT1 and hBFIT2 sequences at the C-terminus is due to alternative splicing of the hBFIT gene (see Results). Bars denote GXH motifs conserved in some thioesterase proteins. The following Pfam domains were identified: START lipid-binding domain (residues 384–590 and 384–571 in hBFIT1 and hBFIT2, respectively); two acyl-CoA thioesterase domains (residues 29–166 and 199–341 in both hBFIT variants). The GenBank cDNA accession numbers for hBFIT1, hBFIT2 and mBFIT2 are AF416921, AF416922 and AF416923, respectively.

hBFIT was localized to a syntenic region of chromosome 1 (1p32.3), near the Stanford Human Genome Center marker SHGC-58180 (between D1S509 and D1S3255). This marker is in proximity to a potential human *Do*1 region linked to body fatness in the Québec Family Study cohort (which includes the markers D1S193, D1S200 and D1S476) [14].

The chromosomal position of BFIT, its likely involvement with lipid metabolism and its high abundance and induction in thermogenic BAT pointed to BFIT as a candidate gene defining the *Do1* locus. To test this postulate, a comparison was made of the full-length ORF of mBFIT2 in AKR/J and SWR/J mice, using BAT cDNAs from each strain as templates in multiple, independent PCR reactions. The fragments obtained were subcloned into pcDNA3.1/V5-His-TOPO TA and sequenced. The cDNA and protein sequences corresponding to the mBFIT2 ORF did not differ between strains (results not shown), and matched that obtained previously from FVB/N mice (Figure 2). Because a putative strain-related difference in the BFIT gene sequence could occur outside the coding region and hence lead to expression differences among strains, we explored whether

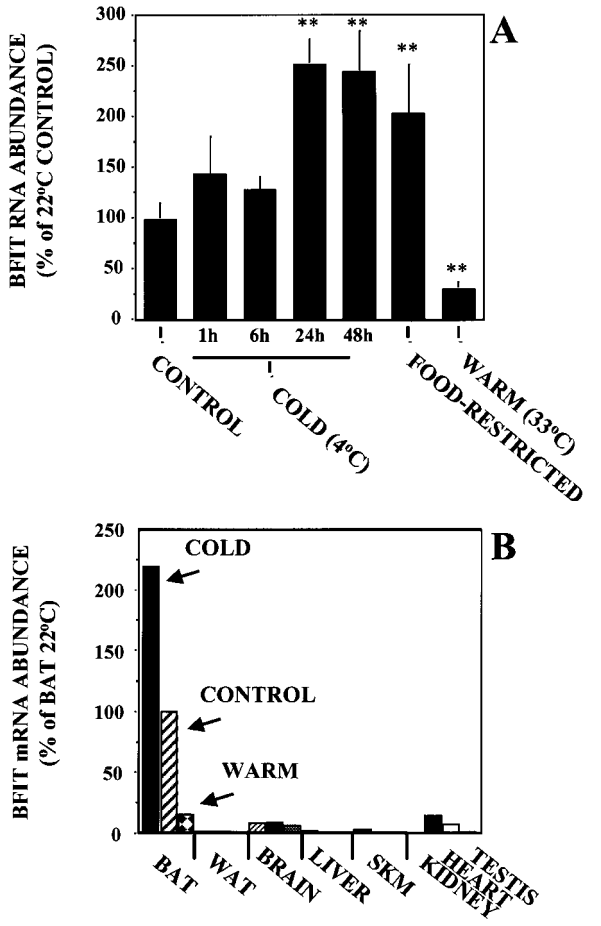

Figure 3 BFIT is induced in the BAT of mice exposed to cold, repressed by warm temperature and influenced by meal-feeding

(**A**) BFIT mRNA level in BAT was induced by 24 h of cold exposure (4 °C) in adult male mice, and remained elevated for at least 48 h in the cold. Samples derived from chronically (3 weeks) food-restricted/meal-fed mice at 4–6 h post-feeding also displayed elevated BFIT mRNA. In contrast, acclimation to a thermoneutral environment (33 °C, 3 weeks) depressed expression by 70% (**$P < 0.01$ versus control). (**B**) BFIT mRNA is most abundant in mouse BAT, and its cold-inducibility is strikingly apparent in this tissue. The Figure depicts expression in pooled mRNAs from each tissue noted, and represent different samples from those in (**A**). Expression levels after cold exposure, in control conditions or upon warmth acclimation are indicated for WAT, whole brain, liver and hindlimb skeletal muscle (SKM); kidney, heart, and testis values were derived from control mice.

BAT expression of BFIT was altered in AKR/J versus SWR/J mice fed a high-fat or lower-fat diet for 3 weeks. As expected, a high-fat diet led to a substantial difference in body fat accretion between strains, as judged by the 4-fold higher epididymal fat-pad weight (as a percentage of body weight) in AKR/J mice ($4.0 \pm 0.29\%$) compared with SWR/J mice ($1.0 \pm 0.11\%$, $P < 0.01$; also see [13]). The abundance of BFIT was significantly elevated $\approx$ 1.6-fold in the BAT of SWR/J mice compared with obesity-prone AKR/J mice fed the high-fat diet (Figure 4A). Similar patterns emerged from additional lean/obese comparisons of BFIT expression in different established models. For instance, a comparison between obesity-resistant A/J versus obesity-prone C57BL/6J mice [10] fed a high-fat diet for 3 weeks revealed a 2-fold higher expression of BFIT in the A/J mice (Figure 4B). It is notable that BFIT expression in the BAT of both obesity-prone strains was attenuated even under low-fat conditions. Notably, BFIT expression in the BAT of lean *Ob*/? mice was significantly higher compared with obese *ob*/*ob* mice ($257 \pm 20\%$ of mean *ob*/*ob* mRNA abundance; $P < 0.001$).

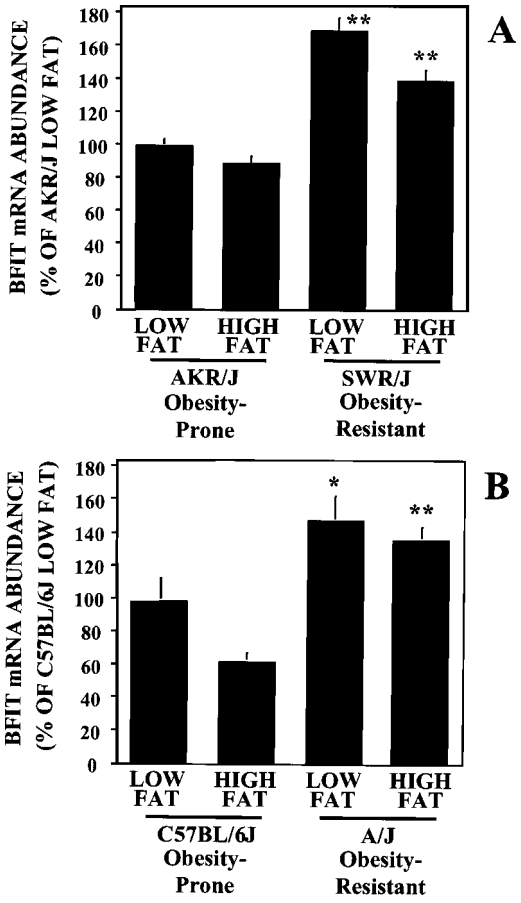

Figure 4 Mouse strains prone to obesity display diminished BFIT mRNA in BAT compared with their lean counterparts

The BAT abundance of BFIT mRNA was significantly depressed (**$P \leqslant 0.001$ and *$P < 0.05$ compared with lean mice within diet) in at least two models of diet-induced obesity, compared with their lean counterparts, (**A**) AKR/J compared with SWR/J mice and (**B**) C57BL/6J versus A/J mice.

Organization of the hBFIT gene, and evidence for tissue-specific regulation of hBFIT splicing events

The presence of two hBFIT cDNA variants and a single chromosomal location for BFIT as shown by our radiation hybrid analysis (see above) suggested the presence of alternative splicing events in the hBFIT gene. A $\approx$ 155 kb assembly encompassing the hBFIT genomic region was made *in silico*, using information contained in two human unordered, non-directional phase I genomic clones from the public database (AC025990 and AC011736), which contained the hBFIT1 and hBFIT2 ORFs. It became immediately clear that hBFIT variants arise from

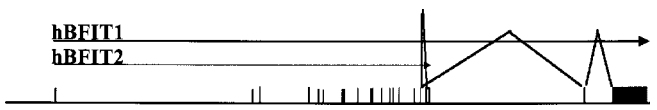

Figure 5 Schematic representation of the hBFIT genomic organization and presence of alternative splice sites

The model was generated through assembly analysis of human genomic clones covering a $\approx$ 155 kb span (see Results), and illustrates exons (upward ticks) encoding hBFIT1 and hBFIT2 splice variants, plus intervening introns represented by the horizontal line. Both variants share the initial 15 exons of the gene, but hBFIT2 is generated from an alternate exon (see bottom arrow) not utilized in hBFIT1 transcription. In contrast, hBFIT1 is generated from the use of two downstream exons (see top arrow) not used to generate the hBFIT2 sequence.

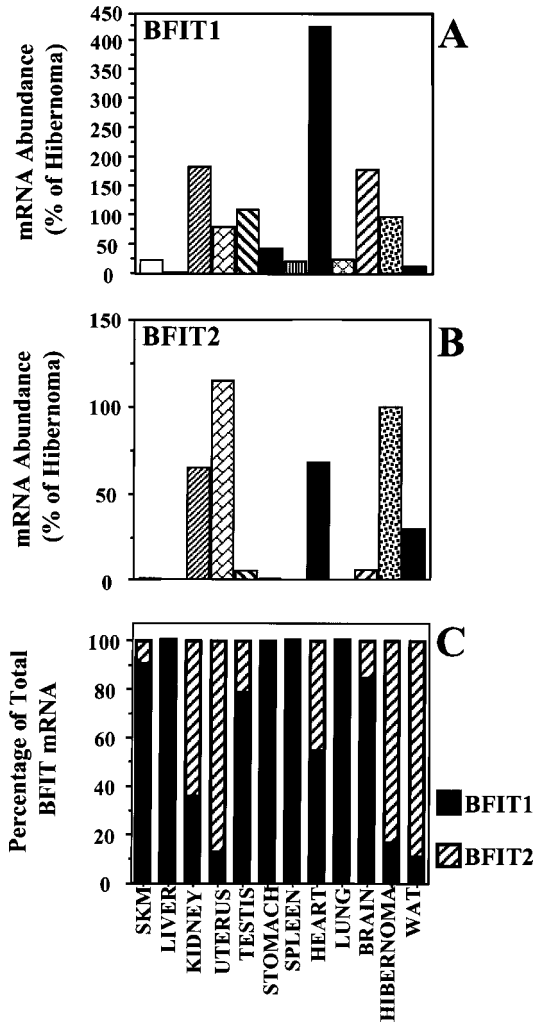

Figure 6 Evidence for broad expression of BFIT in humans, and tissue-specific regulation of gene-splicing events

(**A**) BFIT1 mRNA, expressed as a percentage of that determined in hibernoma (a benign tumour with characteristics of BAT), was especially abundant in heart, but was also apparent in numerous other tissues (bars are identified in **C**). (**B**) BFIT2 mRNA distribution was more confined than BFIT1, but nevertheless expressed in numerous tissues. (**C**) The ratio of BFIT splice variants was quite variable, with tissue-specific patterns indicative of differential regulation of transcription. Values represent the percentage of total BFIT mRNA (BFIT1 plus BFIT2 mRNAs) attributable to either variant in each tissue examined (e.g. hibernoma predominantly expressed BFIT2, represented by the cross-hatched bar). SKM, skeletal muscle.

alternative splicing events, as depicted schematically in Figure 5: hBFIT1 is predicted to arise from 17 exons, compared with the 16 exons predicted to give rise to BFIT2.

In humans, an interesting tissue-specificity of hBFIT splicing patterns emerged from quantitative real-time RT-PCR assays employing variant-specific primers and probes. Detectable expression of hBFIT mRNA in one form or another appeared in numerous tissues, although its signal was relatively low in human skeletal muscle, liver, stomach, spleen and lung compared with other sites (Figures 6A and 6B). Of the total hBFIT expressed, hBFIT1 predominated in skeletal muscle, liver, testis, stomach, spleen, lung and brain (Figure 6C). In contrast, hBFIT2 was the primary variant expressed in kidney, uterus, hibernoma and WAT; both variants were expressed equally in heart (Figure 6C).

DISCUSSION

The presence of acyl-CoA thioesterase (hydrolase) activity has been recognized in a wide variety of tissues since at least the 1950s [15]. Despite extensive biochemical characterization of thioesterase proteins over the last few decades (i.e. [16–21]), and the molecular identification of numerous functional family members [22–36], a complete understanding of the physiological role of most thioesterases has remained elusive. Ultimately, efforts to clarify the biological functions of specific acyl-CoA thioesterases will benefit from studies placing the enzymes into a physiological context.

We have characterized a unique acyl-CoA thioesterase whose molecular and biochemical profile points to a role in modulating tissue metabolic status. The current study was based on the premise that genes differentially expressed in the BAT of cold-exposed rodents are good candidates for involvement in the transition of this tissue towards a thermogenic, metabolically amplified state. Thus the emergence of BFIT as a cold-induced, warmth-repressed gene is particularly exciting, due to its remarkably abundant BAT expression in rodents coupled with the pivotal participation of fatty acid metabolism in BAT thermogenesis [37,38].

The thioesterase domain structure of BFIT, coupled to its protein sequence homology with a broad range of acyl-CoA thioesterases (see Results), suggested that this enzyme catalyses hydrolytic thioester cleavage, thus yielding non-esterified fatty acids (NEFAs) and CoA. Consistent with this, initial assays using partially purified *Escherichia coli*-generated recombinant hBFIT proteins displayed acyl-CoA thioesterase activity towards a medium- (C_{12}-CoA) and a long-chain (C_{16}-CoA) fatty acyl-CoA substrate (S. H. Adams, R. Vandlen and D. Yansura, unpublished work). Some acyl-CoA thioesterases contain a GXH domain [24], postulated to be involved in modifying thioesterase activity, but which is nevertheless absent in numerous thioesterases [26,30,35,36]. In the case of BFIT, this domain was present in the C-terminal region of hBFIT variants (see Figure 2), but its absence in mBFIT2 and some other thioesterases suggests that it may not be essential for BFIT function. The START domain, observed in all BFIT variants and previously noted for the KIAA0707 (hBFIT1) protein [39], is thought to infer lipid-binding qualities [39]. We propose that the presence of a START domain, the acyl-CoA thioesterase character of BFIT, and the strong abundance and induction of the BFIT gene in thermogenic BAT points to an important role for BFIT in regulating lipid metabolism. It is reasonable to propose that modification of the cellular acyl-CoA profile is the mechanism by which BFIT activity impacts tissue function. Based on homology analysis, BFIT appears more similar to prokaryotic acyl-CoA thioesterases compared with known mammalian enzymes (see the Results section). Therefore, BFIT represents a member of a new subclass of the mammalian acyl-CoA thioesterase functional family.

Acyl-CoA hydrolysis may directly impact cellular metabolism in at least four ways. First, modulation of the finite pool of mitochondrial CoA, a cofactor required for fatty acid entry into the β-oxidative pathway, could impact fatty acid combustion. This is suggested by the strong diminution of mitochondrial β-oxidation upon introduction of poorly metabolizable acyl compounds which sequester matrix CoA (i.e. [40,41]). The uncoupled nature of BAT mitochondria from cold-exposed rodents, with concomitant acceleration of fatty acid flux, may place a strain on available CoA. Thus, cold-induction of thioesterases, with concomitant export of chain-shortened fatty acids and release of CoA, may be crucial in thwarting accumulation of mitochondrial

acyl-CoA [21]. Indeed, the presence of thioesterase enzymes with specificity towards short-, medium- and long-chain fatty acyl-CoA have been demonstrated in rat and hamster BAT mitochondria [16,19,21], the subcellular site of most thioesterase activity in brown adipocytes [19,21]. Interestingly, activities for each rise $\approx$ 2-fold in mitochondria isolated from cold-acclimated animals [19], reminiscent of what is observed with mBFIT gene expression (Figure 3). Second, thioesterase activity in peroxisomes may participate in lipid metabolism at that site, analogous with such activity in mitochondria [20]. Third, prior to entering various metabolic pathways in the cell, NEFAs must be 'activated' to form acyl-CoA moieties through the action of acyl-CoA synthetases (requiring ATP). Cytosolic acyl-CoA thioesterases in BAT [19,21] would counteract such activity and may therefore modulate the profile and abundance of acyl-CoA in cells. If coupled to acyl-CoA synthetase activity, enhancement of cytosolic thioesterase activity (i.e. after cold exposure) could drive a futile, ATP-consuming cycle and thus increase energy expenditure. Alternatively, cytosolic thioesterase modulation of NEFA availability could regulate the putative positive effects of NEFA on UCP1 activity [42] and hence cellular thermogenesis. Fourth, certain thioesterases elicit termination of chain elongation during *de novo* fatty acid synthesis [22–24], and the latter pathway is significantly induced in BAT under cold conditions in the current mouse model (X. X. Yu, W. Forrest, D. A. Lewin and S. H. Adams, unpublished work). However, the role of BFIT in modulating lipogenesis remains to be clarified.

Acyl-CoA thioesterases have also been implicated in the regulation of cell signalling, and trafficking of proteins. For instance, a novel human thioesterase (hTE or hACTE-III) was found to associate with the HIV-1 Nef protein [27,29], and blocking this interaction through mutational analysis of hTE abolished the ability of Nef to down-regulate CD4 [29]. An acyl-protein thioesterase 1 was found to deacylate α-subunits of G-proteins and Ha-Ras, events expected to result in changes in their membrane association and activities [34]. Indeed, the important role of fatty acids and/or acyl-CoAs as signals regulating gene expression, glucose-stimulated insulin secretion in pancreatic β cells, and other intracellular events cannot be overlooked [43]. Thus another role for BFIT may be to modulate certain acyl-CoA profiles affecting intracellular signalling pathways, thereby amplifying the metabolic state of BAT and other tissues.

We were intrigued by the close relationship between the human and mouse BFIT chromosomal locations and those of various markers previously linked to body fat in humans [14] or diet-induced obesity in rodents [13]. Despite this, there was no difference in the protein-coding region of mBFIT2 cDNAs in obesity-resistant SWR/J mice compared with obesity-prone AKR/J mice, indicating that a functional change in the activity of mBFIT2 protein itself does not underlie the phenotypic differences in these strains. Interestingly, BAT from the latter strain displayed significantly lower BFIT mRNA abundance under both dietary regimens (Figure 4A), raising the possibility that strain-specific differences in the BFIT gene may exist outside of the coding region, resulting in diminished expression in AKR/J mice. When assessing the importance of the changes in BFIT expression, one must take into account the multigenic nature of most obesity syndromes, including diet-induced obesity in AKR/J mice [13]. Thus the gene defining the *Do*1 locus in these mice is expected to contribute, but not wholly determine, their obesity phenotype. Whether BFIT represents the obesity-linked *Do1* gene in rodents [13], or the analogous site in humans [14], remains a possibility to be clarified further. Studies that compare the genomic span encompassing the BFIT gene across AKR/J and SWR/J strains, as well as experiments analysing expression and genomic differences between relevant obese and lean human populations, should answer this question. Regardless of the outcome of such genetic studies, it is important to note that obese mice from a variety of model comparisons express diminished levels of BFIT mRNA in BAT (Figure 4). This indicates that lowered BFIT mRNA abundance in this tissue correlates with the diminished BAT thermogenic activity observed in obese rodents [44].

Specific components which regulate BFIT gene expression are not yet defined, and their discovery awaits more detailed studies. Its BAT induction and repression by cold or warm ambient temperature, respectively, suggests that sympathetic tone is the initial factor driving temperature-related changes in BFIT expression. However, we suspect that regulation of this gene is more complex, based on the results of a follow-up experiment designed to dissect whether repression of the BFIT gene in warmth-acclimated mice (Figure 3A) was attributable to specific temperature signals or due to their diminished food intake. BFIT expression in food-restricted mice was induced to $204 \pm 47\,\%$ of control values, similar in magnitude to observations in cold-challenged mice (Figure 3A). These results indicate that the warmth-related repression of the gene was due to physiological signals specific to ambient temperature. Furthermore, the data suggest that factors common to both long-term food restriction/meal-feeding and acute cold exposure trigger the gene. It is relevant to note that, in retrospect, the food-restriction regimen employed herein may, in some aspects, mimic fasting/re-feeding, in that tissues were collected a few hours post-feeding. Therefore, interpretations regarding BAT genes induced by this paradigm must be made with this in mind.

It is predicted that the BFIT promoter shares at least some functional elements with acetyl-CoA carboxylase 2 (ACC2), ATP citrate-lyase, stearoyl-CoA desaturase 2 (SCD2) and monoacylglycerol lipase, as there is remarkable co-ordinate expression of this cohort of metabolic genes in mouse BAT under the conditions used for this experiment (X. X. Yu, W. Forrest, D. A. Lewin and S. H. Adams, unpublished work). Such elements may include, for example, a sterol-response element, Sp1 and regions responsive to polyunsaturated fatty acids.

Assessments of BFIT's precise role in regulating the metabolic state of tissues will benefit greatly from future studies identifying the subcellular location(s) of the protein, its substrate specificity and its regulation by ADP/NADH [16,19–21] or other post-translational modifiers. Furthermore, the interplay between BFIT and other acyl-CoA thioesterases, such as CTE-1 (cytosolic thioesterase 1) and MTE-1 (mitochondrial thioesterase 1), known to be expressed in rodent BAT and having relatively broad specificity including medium- to long-chain fatty acyl-CoAs [32], warrants further consideration. Based on its abundant expression in thermogenic rodent BAT, BFIT expression in human BAT is an interesting avenue of research. Its relatively widespread expression in humans supports the idea that BFIT biological function extends to numerous tissues, and its body-wide metabolic role warrants further research. Removal or enhancement of BFIT activity through molecular strategies *in vitro* and *in vivo* should clarify further the full physiological impact of this protein.

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REFERENCES

1 Ricquier, D. and Mory, G. (1984) Factors affecting brown adipose tissue activity in animals and man. Clin. Endocrin. Metab. **13**, 501–520
2 Himms-Hagen, J. and Ricquier, D. (1998) Brown adipose tissue. In Handbook of Obesity (Bray, G. A., ed.), pp. 415–441, Marcel Dekker, New York
3 Ricquier, D., Mory, G., Bouillaud, F., Thibault, J. and Weissenbach, J. (1984) Rapid increase of mitochondrial uncoupling protein and its mRNA in stimulated brown adipose tissue. FEBS Lett. **178**, 240–244
4 Enerbäck, S., Jacobsson, A., Simpson, E. M., Guerra, C., Yamashita, H., Harper, M.-E. and Kozak, L. P. (1997) Mice lacking uncoupling protein are cold-sensitive but not obese. Nature (London) **387**, 90–94
5 Rothwell, N. J. and Stock, M. J. (1979) A role for brown adipose tissue in diet-induced thermogenesis. Nature (London) **281**, 31–35
6 Tulp, O. L., Frink, R. and Danforth, Jr, E. (1982) Effect of cafeteria feeding on brown and white adipose tissue cellularity, thermogenesis, and body composition in rats. J. Nutr. **112**, 2250–2260
7 Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M. and Spiegelman, B. M. (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell **92**, 829–839
8 Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C. and Spiegelman, B. M. (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell **98**, 115–124
9 Ishikawa, K., Nagase, T., Suyama, M., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N. and Ohara, O. (1998) Prediction of the coding sequences of unidentified human genes. X. The complete sequences of 100 new cDNA clones from brain which can code for large proteins *in vitro*. DNA Res. **5**, 169–176
10 Surwit, R. S., Feinglos, M. N., Rodin, J., Sutherland, A., Petro, A. E., Opara, E. C., Kuhn, C. M. and Rebuffé, M. (1995) Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. Metab. Clin. Exp. **44**, 645–651
11 Shimkets, R. A., Lowe, D. G., Tai, J. T., Sehl, P., Jin, H., Yang, R., Predki, P. F., Rothberg, B. E., Murtha, M. T., Roth, M. E. et al. (1999) Gene expression analysis by transcript profiling coupled to a gene database query. Nat. Biotechnol. **17**, 798–803
12 Yu, X. X., Barger, J. L., Boyer, B. B., Brand, M. D., Pan, G. and Adams, S. H. (2000) Impact of endotoxin on UCP homolog mRNA abundance, thermoregulation, and mitochondrial proton leak kinetics. Am. J. Physiol. Endocrin. Metab. **279**, E433–E446
13 West, D. B., Waguespack, J., York, B., Goudey-Lefevre, J. and Price, R. A. (1994) Genetics of dietary obesity in AKR/J X SWR/J: segregation of the trait and identification of a linked locus on Chromosome 4. Mammalian Genome **5**, 546–552
14 Chagnon, Y. C., Pérusse, L., Lamothe, M., Chagnon, M., Nadeau, A., Dionne, F. T., Gagnon, J., Chung, W. K., Leibel, R. L. and Bouchard, C. (1997) Suggestive linkages between markers on human 1p32-p22 and body fat and insulin levels in the Quebec Family Study. Obesity Res. **5**, 115–121
15 Srere, P. A., Seubert, W. and Lynen, F. (1959) Palmityl coenzyme A deacylase. Biochim. Biophys. Acta **33**, 313–319
16 Bernson, V. S. M. (1976) Acetyl-CoA hydrolase; activity, regulation and physiological significance of the enzyme in brown adipose tissue from hamster. Eur. J. Biochem. **67**, 403–410
17 Berge, R. K. and Døssland, B. (1979) Differences between microsomal and mitochondrial-matrix palmitoyl-coenzyme A hydrolase, and palmitoyl-L-carnitine hydrolase from rat liver. Biochem. J. **181**, 119–125
18 Lee, K. Y. and Schultz, H. (1979) Isolation, properties, and regulation of a mitochondrial acyl coenzyme A thioesterase from pig heart. J. Biol. Chem. **254**, 4516–4523
19 Alexson, S. E. H. and Nedergaard, J. (1988) A novel type of short- and medium-chain acyl-CoA hydrolase in brown adipose tissue mitochondria. J. Biol. Chem. **263**, 13564–13571
20 Alexson, S. E. H., Osmundsen, H. and Berge, R. K. (1989) The presence of acyl-CoA hydrolase in rat brown-adipose-tissue peroxisomes. Biochem. J. **262**, 41–46
21 Alexson, S. E. H., Svensson, L. T. and Nedergaard, J. (1989) NADH-sensitive propionyl-CoA hydrolase in brown-adipose-tissue mitochondria of the rat. Biochim. Biophys. Acta **1005**, 13–19
22 Safford, R., de Silva, J., Lucas, C., Windust, J. H. C., Sheddon, J., James, C. M., Sidebottom, C. M., Slabas, A. R., Tombs, M. P. and Hughes, S. G. (1987) Molecular cloning and sequence analysis of complementary DNA encoding rat mammary gland medium-chain S-acyl fatty acid synthetase thio ester hydrolase. Biochemistry. **26**, 1358–1364
23 Randhawa, Z. I. and Smith, S. (1987) Complete amino acid sequence of the medium-chain S-acyl fatty acid synthetase thio ester hydrolase from rat mammary gland. Biochemistry **26**, 1365–1373
24 Naggert, J., Witkowski, A., Mikkelsen, J. and Smith, S. (1988) Molecular cloning and sequencing of a cDNA encoding the thioesterase domain of the rat fatty acid synthetase. J. Biol. Chem. **263**, 1146–1150
25 Broustas, C. G., Larkins, L. K., Uhler, M. D. and Hajra, A. K. (1996) Molecular cloning and expression of cDNA encoding rat brain cytosolic acyl-Coenzyme A thioester hydrolase. J. Biol. Chem. **271**, 10470–10476
26 Yamada, J., Furihata, T., Iida, N., Watanabe, T., Hosokawa, M., Satoh, T., Someya, A., Nagaoka, I. and Suga, T. (1997) Molecular cloning and expression of cDNAs encoding rat brain and liver cytosolic long-chain acyl-CoA hydrolases. Biochem. Biophys. Res. Commun. **232**, 198–203
27 Watanabe, H., Shiratori, T., Shoji, H., Miyatake, S., Okazaki, Y., Ikuta, K., Sato, T. and Saito, T. (1997) A novel acyl-CoA thioesterase enhances its enzymatic activity by direct binding with HIV Nef. Biochem. Biophys. Res. Commun. **238**, 234–239
28 Engberg, S. T., Aoyama, T., Alexson, S. E. H., Hashimoto, T. and Svensson, L. T. (1997) Peroxisome proliferator-induced acyl-CoA thioesterase from rat liver cytosol: molecular cloning and functional expression in Chinese hamster ovary cells. Biochem. J. **323**, 525–531
29 Liu, L. X., Margottin, F., Le Gall, S., Schwartz, O., Selig, L., Benarous, R. and Benichou, S. (1997) Binding of HIV-1 Nef to a novel thioesterase enzyme correlates with Nef-mediated CD4 down-regulation. J. Biol. Chem. **272**, 13779–13785
30 Yamada, J., Suga, K., Furihata, T., Kitahara, M., Watanabe, T., Hosokawa, M., Satoh, T. and Suga, T. (1998) cDNA cloning and genomic organization of peroxisome proliferator-inducible long-chain acyl-CoA hydrolase from rat liver cytosol. Biochem. Biophys. Res. Commun. **248**, 608–612
31 Svensson, L. T., Engberg, S. T., Aoyama, T., Usuda, N., Alexson, S. E. H. and Hashimoto, T. (1998) Molecular cloning and characterization of a mitochondrial peroxisome proliferator-induced acyl-CoA thioesterase from rat liver. Biochem. J. **329**, 601–608
32 Lindquist, P. J. G., Svensson, L. T. and Alexson, S. E. H. (1998) Molecular cloning of the peroxisome proliferator-induced 46-kDa cytosolic acyl-CoA thioesterase from mouse and rat liver. Recombinant expression in *Escherichia coli*, tissue expression, and nutritional regulation. Eur. J. Biochem. **251**, 631–640
33 Finkielstein, C., Maloberti, P., Mendez, C. F., Paz, C., Cornejo Maciel, F., Cymeryng, C., Neuman, I., Dada, L., Mele, P. G., Solano, A. and Podestá, E. J. (1998) An adrenocorticotropin-regulated phosphoprotein intermediary in steroid synthesis is similar to an acyl-CoA thioesterase enzyme. Eur. J. Biochem. **256**, 60–66
34 Duncan, J. A. and Gilman, A. G. (1998) A cytoplasmic acyl-protein thioesterase that removes palmitate from G protein α subunits and $p21^{RAS}$. J. Biol. Chem. **273**, 15830–15837
35 Yamada, J., Kurata, A., Hirata, M., Taniguchi, T., Takama, H., Furihata, T., Shiratori, K., Iida, N., Takagi-Sakuma, M., Watanabe, T. et al. (1999) Purification, molecular cloning, and genomic organization of human brain long-chain acyl-CoA hydrolase. J. Biochem. (Tokyo) **126**, 1013–1019
36 Poupon, V., Bègue, B., Gagnon, J., Dautry-Varsat, A., Cerf-Bensussan, N. and Benmerah, A. (1999) Molecular cloning and characterization of MT-ACT48, a novel mitochondrial acyl-CoA thioesterase. J. Biol. Chem. **274**, 19188–19194
37 Bukowieki, L. J., Folléa, N., Lupien, J. and Paradis, A. (1981) Metabolic relationships between lipolysis and respiration in rat brown adipocytes. The role of long chain fatty acids as regulators of mitochondrial respiration and feedback inhibitors of lipolysis. J. Biol. Chem. **256**, 12840–12848
38 Guerra, C., Koza, R. A., Walsh, K., Kurtz, D. M., Wood, P. A. and Kozak, L. P. (1998) Abnormal nonshivering thermogenesis in mice with inherited defects of fatty acid oxidation. J. Clin. Invest. **102**, 1724–1731
39 Ponting, C. P. and Aravind, L. (1999) START: a lipid-binding domain in StAR, HD-ZIP and signalling proteins. Trends Biochem. Sci. **24**, 130–132
40 Alexson, S. E., Nedergaard, J. and Cannon, B. (1985) Inhibition of acetyl-carnitine oxidation in brown-adipose-tissue mitochondria by erucoyl-carnitine is due to sequestration of CoA. Biochim. Biophys. Acta **834**, 149–158
41 Lin, X., Adams, S. H. and Odle, J. (1996) Acetate represents a major product of heptanoate and octanoate β-oxidation in hepatocytes isolated from neonatal piglets. Biochem. J. **318**, 235–240
42 Klingenberg, M. (1999) Uncoupling protein – a useful energy dissipator. J. Bioenerg. Biomembr. **31**, 419–430
43 Symposium: The Role of Long Chain Fatty Acyl-CoAs as Signaling Molecules in Cellular Metabolism. (2000) J. Nutr. **130** (suppl.), 289S–314S
44 Himms-Hagen, J. (1979) Obesity may be due to a malfunctioning of brown fat. Can. Med. Assoc. J. **121**, 1361–1364

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