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# An intronic single base exchange leads to a brown adipose tissue-specific loss of Ucp3 expression and an altered body mass trajectory

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<sup>1</sup>Molecular Nutritional Medicine, ZIEL Research Center for Nutrition and Food Sciences, and <sup>2</sup>Molecular Nutritional Medicine, Else Kröner-Fresenius-Zentrum für Ernährungsmedizin, Technische Universität München, Freising; <sup>3</sup>Department of Animal Physiology, Faculty of Biology, Philipps University, Marburg; and <sup>4</sup>Genome Analysis, Leibniz Institute for Age Research, Fritz Lipmann Institute, Jena, Germany

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Fromme T, Hoffmann C, Nau K, Rozman J, Reichwald K, Utting M, Platzer M, Klingenspor M. An intronic single base exchange leads to a brown adipose tissue-specific loss of Ucp3 expression and an altered body mass trajectory. Physiol Genomics 38: 54-62, 2009. First published April 21, 2009; doi:10.1152/physiolgenomics.00249.2007.-Uncoupling protein 3 (Ucp3) is a transport protein of the inner mitochondrial membrane and presumably is implicated in the maintenance or tolerance of high lipid oxidation rates. Ucp3 is predominantly expressed in skeletal muscle and brown adipose tissue and is regulated by a transcription factor complex involving peroxisome proliferator-activated receptor-a, MyoD, and COUP transcription factor II. By analysis of a mutant Djungarian hamster model lacking Ucp3 transcription specifically in brown adipose tissue, we identified a putative transcription factor-binding site that confers tissue specificity. A naturally occurring intronic point mutation disrupting this site leads to brown adipose tissue-specific loss of Ucp3 expression and an altered body weight trajectory. Our findings provide insight into tissue-specific Ucp3 regulation and, for the first time, unambiguously demonstrate that changes in Ucp3 expression can interfere with body weight regulation.

uncoupling protein 3; intronic binding site

UNCOUPLING PROTEIN (Ucp)3 belongs to the group of anion carrier proteins and is located within the inner mitochondrial membrane. It is predominantly expressed in skeletal muscle and brown adipose tissue (BAT) (1, 31). Initially described as a homolog of classical brown fat thermogenic Ucp1, it was soon established that Ucp3 has a function other than thermogenesis. One major argument against a thermogenic role was the positive transcriptional regulation of the Ucp3 gene in response to starvation in which activation of an energy-wasting mechanism seems unlikely (13). Many studies have described Ucp3 upregulation in response to further physiological situations associated with increased plasma fatty acid levels and lipid utilization including cold exposure (32), acute exercise (8), and streptozocin-induced diabetes (25) as well as direct lipid infusion (35). The inferred relevance of this anion carrier protein in lipid metabolism is the cornerstone of the hypothesis of Ucp3 being a fatty acid anion exporter, a biochemical function necessary to eliminate free fatty acids from the mitochondrial matrix and not assigned to a specific protein previously (20). This molecular function of Ucp3, however, is subject to an ongoing debate (16). Despite its obviously different role in vivo, certain biochemical properties of the protein as measured in mitochondrial proton leak assays resemble those of Ucp1, i.e., causing a GDP-sensitive inducible proton leak (27). Therefore, other major hypotheses consider Ucp3 to primarily be a proton translocator, pyruvate transporter, superoxide transporter, constituent of a radical defense system, and constituent of the mitochondrial  $Ca^{2+}$  uniporter (for reviews, see Refs. 16 and 28).

Apart from the controversy about the actual molecular function, there is general consent that Ucp3 is implicated in the maintenance or tolerance of high lipid oxidation rates by either being an integral part of fatty acid metabolism or an accompanying phenomenon. In addition to its localization in tissues with high  $\beta$ -oxidative capacities and its regulation by serum free fatty acid levels, there is complementary evidence for a role in lipid metabolism from a different perspective. In a number of association and linkage studies, polymorphisms in the human UCP3 locus have been reported to influence markers of obesity like the body mass index and waist-to-hip ratio (2, 17). Since Ucp3 and Ucp2 share the same genomic locus and are well within linkage disequilibrium, it has been problematic to assign association of the respective phenotype to a polymorphism in either of the paralogs (2, 33).

In our breeding colony of Djungarian hamsters (*Phodopus sungorus*), we identified animals lacking *Ucp3* mRNA expression exclusively in BAT without phenotypic consequences for the resting metabolic rate (11). When challenged by cold exposure, these animals display defective BAT function and decreased cold tolerance limit accompanied by a global down-regulation of metabolic BAT gene expression (15). We further analyzed this mutation on a broad genetic background to elucidate the affected regulatory mechanism and the phenotypic consequences.

The expression of Ucp3 is controlled by an interacting network of multiple factors including peroxisome proliferatoractivated receptor (PPAR)- $\alpha$ /retinoid X receptor (RXR)- $\alpha$  heterodimers, MyoD, COUP-transcription factor II (COUP-TFII), and several more (7, 23). The function of all these known components of the machinery controlling Ucp3 transcription is mediated by the genomic region upstream of Ucp3 exon 1. Here, we report on a novel regulatory element in intron 1 that is essential for brown fat Ucp3 transcription and thereby confers tissue specificity of Ucp3 expression. We show that disruption of this element leads to BAT-specific loss of Ucp3expression and an altered body mass trajectory.

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#### EXPERIMENTAL PROCEDURES

DNA sequence analysis. We chose primers for comparative sequencing of the hamster Ucp3 locus with Primer3 software (see Ref. 19) based on GenBank entry AY523564 to yield overlapping PCR fragments of ~500 bp in length. Amplicons were Sanger sequenced from both ends. Sequences were aligned and analyzed with the GAP module of the Staden package (24). Sequence positions and variations are named following nomenclature recommendations and were based on GenBank entry AY523564 (4). Accordingly, positions in intron 1 are called intervening sequence 1 (IVS1)+x, counting from the first base (+1) of this intron. Positions outside introns are given relative to the A (+1) of start codon ATG in AY523564. Analyses of transcription factor-binding sites were conducted with MATCH and PATCH (both Biobase) and MatInspector (Genomatix). Information about transcription factor-binding sites was extracted from MatBase Matrix Library 8.0 (Genomatix).

For two sequence variations located in *Ucp3* intron 1, we developed restriction fragment length polymorphism (RFLP) assays. The region surrounding IVS1+1505A constitutes a recognition site for *Aar*I that is absent when the IVS1+1505G allele is present. At IVS1+2668A, there is an *Eco57I* site that is lacking if the IVS1+2668G allele is present. We amplified a genomic fragment including both polymorphic sites with primers 5'-TGTCCTGATGGCTCCTCTCA-3'/5'-CCCCAGGAACT-TCACAACTG-3' and digested fragments with either *Aar*I (Fermentas) or *Eco57I* (Fermentas).

To genotype a larger number of animals, we used the Pyrosequencing technique with the PSQ 96MA system (Biotage AB). We first amplified a long intronic DNA fragment with primers 5'-CAAG-TCACCCAGCAGCCTA-3'/5'-GGCGGCATCTATCTGGAATA-3'. In a second nested PCR, we used primers 5-'CAGTACCTCCTGCT-GGGAAG-3' and 5'-biotinylated Biotin-AGTCAGACCTTGGCTC-TCCA-3' to amplify a 248-bp-long region encompassing IVS1+1505. The total yield of biotin-labeled PCR product was immobilized on 3-µl steptavidin-labeled Sepharose beads (GE Healthcare) at room temperature. Single-stranded DNA was prepared at a Vacuum Work Station (Biotage) and transferred into a PSO reaction plate (Biotage), where the sequencing primer 5'-GCCTGAATAAGTGTTTTCT-3' was annealed. Pyrosequencing reactions were performed using the PyroGold SNP reaction kit (Biotage) following the manufacturer's instructions. The sequence to analyze was defined as 5'-TAACACRCCTG-3' and the dispensation order for the single nucleotide injection was calculated to be 5'-GTACACGAGC-3' (where bold letters indicate the polymorphic site) by PSQ 98MA software (version 2.1, Biotage). Results were calculated and analyzed by PSQ 98MA software.

Animal growth, body mass, and composition. We genotyped position IVS1+1505 of animals randomly chosen from our colony. To study the phenotypic consequences of the two alleles of Ucp3 intron 1 in vivo, on a broad genetic background and in all genotypes, we crossed heterozygous hamsters. The progeny of 14 heterozygous breeding pairs displayed an allele frequency of 0.5 for IVS1+1505G and 0.5 for IVS1+1505A in 171 animals, as expected. The genotypes were in Hardy-Weinberg equilibrium. Body weights of all animals were continuously monitored on a weekly basis. On day  $81 \pm 3$  after birth, 66 randomly selected hamsters were transferred to an ambient temperature of 4°C to pronounce differences that may arise from altered BAT physiology. They were killed after 2 days of cold exposure.

All determinations of body fat mass and femur length were performed under isoflurane anesthesia with dual-energy X-ray absorptiometry (DEXA; PIXImus2 scanner, software version 1.46.007, GE Medical Systems). The head of the animal was excluded from measurements. Bone mass is not included in DEXA-provided values for total body mass (head excluded) and lean mass.

Animal experiments were conducted in accordance with German animal welfare law.

Quantitative PCR. We extracted total RNA from BAT and skeletal muscle with TRIzol (Invitrogen), and RNA was photometrically quantified. RNA was reverse transcribed with the Superscript III First Strand Synthesis Kit (Invitrogen). To quantify transcript levels by quantitative PCR, we used ImmoMix (Bioline) supplemented with SYBR green (Bioline) and 20 nM fluorescein (Bio-Rad) on an iCycler (Bio-Rad). The efficiency of amplification was calculated based on dilution series standard curves by Bio-Rad iCycler IQ 3.0 software and used to determine starting quantity levels (PCR baseline subtracted) normalized to  $\beta$ -actin levels. Primers were as follows:  $\beta$ -actin, 5'-AGAGGGAAATCGTGCGTGAC-3' and 5'-CAATAGTGATGACCT-GGCCGT-3'; and Ucp3, 5'-AGGAAGGAATCAGGGGCTTA-3' and 5'-TCCAGCAGCTTCTCCTTGAT-3'. Results were statistically analyzed by *t*-tests (SigmaStat 3.1, Systat Software).

Vector construction. Using the genomic DNA of animals either homozygous for (IVS1+1505G, IVS1+2668A) or (IVS1+1505A, IVS1+2668G), we PCR amplified fragments of 4,338 bp spanning position -4234 to +103 of the Ucp3 genomic locus with primers 5'-CCTTGGGAAGTCAATGTCCT-3'/5'-CCAGGGGGAAGGTGAG-TAGGTCT-3' and *Pfu* polymerase (Fermentas). The amplicon included a part of the promoter, exon 1, intron 1, and most of exon 2, including the start codon. Amplicons were used in a nested *Pfu*-PCR to amplify the region between positions -4027 to +14 with primers 5-'GGGG-GAAGGGAAACGGGGAGAC-3'/5'-GAAGTCCAACCATGGTGC-CCAGCAG-3', the latter of which is heterologous in one base position (underlined), creating a NcoI recognition site around the endogenous start codon of the Ucp3 gene. Respective amplicons were digested with BglII and *NcoI*, and the resulting fragment spanning the region between -3555to +3 was gel purified and cloned into the respective restriction sites of pGL3-basic (Promega). By sequencing, we identified clones without PCR-introduced errors representing the two alleles (IVS1+1505G, IVS1+2668A) and (IVS1+1505A, IVS1+2668G) (corresponding to vectors 1 and 2 in Fig. 2A).

We next generated constructs representing the two alleles not present in our colony, (IVS1+1505A, IVS1+2668A) and (IVS1+1505G, IVS1+2668G) (corresponding to *vectors 3* and 4 in Fig. 2A). To do so, we cut *vectors 1* and 2 into two fragments each with *Kpn*I (Fermentas), with both fragments either containing IVS1+1505 or IVS1+2668. These fragments were gel purified and ligated crosswise, yielding *vectors 3* and 4.

Reporter gene assays. We conducted reporter gene assays in the hibernoma-derived mouse brown adipocyte cell line HIB1B and the mouse myoblast cell line C2C12 established from normal leg muscle. HIB1B cells were grown in DMEM-F-12 (Gibco) containing 10% FCS (Biochrom) until confluence. To differentiate the cells, we decreased the FCS content to 7% and added 17 nM insulin (Sigma) for  $\sim$ 10 days. C2C12 cells were grown in DMEM containing 10% FCS until confluence and then switched to DMEM with 2% horse serum to induce differentiation.

Both cell lines were transfected by the nucleofection method (Amaxa) according to the manufacturer's recommendations for the respective cell line. After nucleofection, cells were incubated for an additional 24 h with fresh medium. Rosiglitazone (20  $\mu$ M, PPAR- $\gamma$  agonist) dissolved in ethanol and Wy14,643 (10  $\mu$ M, PPAR- $\alpha$  agonist) in DMSO were added during this medium change where stated. These compounds have previously been shown to increase Ucp3 expression (12, 14). In all measurements without stimulant, medium was supplied with a comparable volume of DMSO and ethanol only. Photinus luciferase activity was measured with components of the Dual Luciferase Reporter Assay System (Promega). We deliberately refrained from usage of a Renilla luciferase reference construct as explained previously (7). All measurements were replicated at least three times independently including nucleofection.

*EMSA*. Besides HIB1B and C2C12 cells, we also used an immortalized BAT cell line in EMSA that was kindly provided by B. Spiegelman (29). All cell lines were grown on a 10-cm culture plate and harvested after full differentiation and either 24 h of stimulation with 20  $\mu$ M

rosiglitazone and 10 µM Wy14,643 or left untreated. Nuclear protein was isolated, and complementary oligonucleotides were annealed as previously described (7). To prepare nuclear extracts from C2C12 cells, we used a different published protocol (21). Sequences of the double-stranded oligonucleotide probes (sense strand) were as follows: IVS1+1505G, 5'-GTGTTTTCTTAACACGCCTGCACTGTTGGTA-3'; IVS1+1505A, 5'-GTGTTTTCTTAACACACCTGCACTGTTGGTA-3'; NF-кB binding, 5'-TTGGCTGTGGTACTTTCCAACGGAA-3', and MyoD binding, 5'-CCCCCAACAGCTGTTGCCTGA-3'. The sense strand of each probe carried a 5'-Cy5 modification for nonradioactive detection. For gel retardation assays, we incubated 3–5  $\mu$ g of nuclear extract with  $\sim$ 50 fmol of probe for 20 min on ice in a final volume of 10 µl containing 4% (vol/vol) glycerol, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris·HCl (pH 7.5), and 50 µg/ml poly(dIdC)·poly(dIdC). Samples were analyzed by electrophoresis with a 5.2% nondenaturing polyacrylamide gel at 4°C and 200 V for 3 h in 0.5× TBE. The gel was documented using a Typhoon TRIO+ imaging station (GE Healthcare).

Western blot analysis. Total protein was prepared from skeletal muscle and BAT of control hamsters, and protein concentrations were determined using the Bradford method. Protein  $(30 \ \mu g)$  was loaded by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-C extra, Amersham Biosciences) in a semidry electroblotting chamber. Successful protein transfer was controlled by Ponceau staining of the membrane. Ucp3 protein was detected using a rabbit Ucp3 antibody (kindly provided by R. Porter), which was, in turn, bound by a goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (Dako) and developed by a chemiluminescence reagent (Supersignal, Pierce).

*Statistical analysis.* We statistically analyzed the body weight development of hamsters with different genotypes. Raw body mass data were included into two-way repeated-measures ANOVA with "sex" (male or female) and "genotype" (IVS1+1505: AA, GA, or GG) as factors for between-group comparisons and "age" as withingroup comparison factors (33 measurements, Statistika '99 Edt., StatSoft). All complete individual data sets were included and were collected between *weeks 4* and *36* of age.

mRNA expression levels of the *Ucp3* gene as measured by quantitative PCR were tested for group differences with the Mann-Whitney rank sum test (SigmaStat 3.1, Systat Software).

# RESULTS

*The hamster Ucp3 gene exhibits two alleles.* To detect sequence variations associated with the lack of Ucp3 expression in BAT, we sequenced 12,721 bp of the *P. sungorus Ucp3* gene including all seven exons, all introns, and 3,632 bp of the promoter region. We compared sequences derived from two wild-type hamsters with two Ucp3-deficient ("mutant") hamsters. Sequences were identical except for two positions within the first intron (Fig. 1A). At position IVS1+1505, both wild-type hamsters were heterozygous G/A, whereas both mutant animals were homozygous for allele A. At position IVS1+2668, wild-type hamsters were heterozygous G/A, whereas mutant animals were homozygous for allele G (Fig. 1A).

We developed RFLP assays for both sequence variations IVS1+1505G>A and IVS1+2668A>G and genotyped 43 hamsters of our breeding colony. The genotype distribution indicated the presence of two haplotypes: (IVS1+1505G, IVS1+2668A) and (IVS1+1505A, IVS1+2668G).

A single base exchange is responsible for the expression phenotype. To elucidate whether these two polymorphic sites are responsible for the expression phenotype, we constructed four different reporter gene vectors harboring a minimal *Ucp3* promoter, the first exon, the complete first intron, and the untranslated region of the second exon. The endogenous ATG



Fig. 1. The uncoupling protein  $(Ucp)^3$  gene of *Phodopus sungorus* exhibits two sequence variations. *A*: genomic organisation of the seven  $Ucp^3$  exons. There are two polymorphic sites at IVS1+1505 and IVS1+2668 in the first intron. TSS, transcriptional start site; ATG, start codon; Stop, stop codon. *B*: gel images of the restriction fragment length polymorphism genotyping method.

was used as the start codon for the luciferase open reading frame. The four vectors differed in haplotypes of the two intron 1 polymorphic sites, as shown in Fig. 2A, i.e., *vector 1* (IVS1+1505G, IVS1+2668A; GA), *vector 2* (IVS1+1505A, IVS1+2668G; AG), *vector 3* (IVS1+1505A, IVS1+2668A; AA), and *vector 4* (IVS1+1505G, IVS1+2668G; GG).

Nucleofection of these vectors into differentiated HIB1B brown adipocytes revealed allele-specific reporter gene expression (Fig. 2*B*). The basal activity of vector 1 (GA) and vector 4 (GG) was 10-fold higher than of vector 2 (AG) and vector 3 (AA). Induction by PPAR agonists led to a strong increase of expression in vector 1 (GA) and vector 4 (GG) but not in vector 2 (AG) and vector 3 (AA). In this condition, the two vectors exhibiting IVS1+1505G displayed an ~100-fold higher reporter gene activity than vectors with IVS1+1505A. Thus, both basal activity and induction by PPAR agonists were dependent on the presence of allele G at position IVS1+1505.

In differentiated C2C12 myotubes, we did not observe differences in basal expression levels of the four vectors. Upon stimulation with PPAR agonists, however, the expression of *vectors 1* and 4 with IVS1+1505G was slightly upregulated, whereas *vectors 2* and 3 with IVS1+1505A retained their basal levels (Fig. 2*B*).

Ucp3 expression is silenced by allele IVS1+1505A in vivo. We analyzed Ucp3 expression of 2-day cold-exposed hamsters at the age of  $83 \pm 3$  days with respect to genotypes at positions IVS1+1505 and IVS1+2668. As the polymorphisms are coupled (see above), we give results for functional IVS1+1505 only. In BAT, Ucp3 mRNA expression showed marked differences based on the genotype (Fig. 3A). Heterozygous animals had a 30% lower mRNA abundance than homozygous IVS1+1505G animals, whereas homozygous IVS1+1505A hamsters were virtually devoid of Ucp3 mRNA. In skeletal muscle tissue, Ucp3 mRNA expression was lowest in homozygous IVS1+1505A animals and significantly higher in homozygous IVS1+1505G animals with an intermediary Ucp3 mRNA level in heterozygous hamsters.



Fig. 2. Reporter gene assays revealed the point mutation responsible for the lack of Ucp3 expression to be at position IVS1+1505. A: schematic representation of the four reporter gene vector constructs used (vectors 1-4). All four possible haplotypic combinations were tested. B: luciferase activity is shown relative to the activity of the unstimulated construct (vector 1) representing the wild-type allele. Errors are standard deviations. In HIB1B cells, both constructs containing IVS1+1505G showed 10-fold higher basal activity than constructs harboring IVS1+1505A, whereas position IVS1+2668 did not seem to play a role. When cells were stimulated with peroxisome proliferator-activated receptor (PPAR) agonists, this difference increased to 100-fold. In C2C12 cells, the basal activity of all constructs was comparable. When stimulated, IVS1+1505G constructs increased activity by  $\sim$ 50%, whereas IVS1+1505A constructs did not change expression.

The Ucp3 protein level was determined later and, thus, in hamsters of considerably greater age that we did not expose to cold. Nevertheless, the differences in protein levels very well reflected the mRNA data. There were clear genotype- and tissue-specific differences in expression (Fig. 3*B*).

The mutant allele is naturally occurring. To determine allele frequencies in our hamster colony, we cumulated RFLP results from the 43 animals initially genotyped and of 90 animals genotyped to establish heterozygous breeding pairs. By pyro-



Fig. 3. Ucp3 expression in vivo was silenced by IVS1+1505A. A: Ucp3 mRNA abundance in tissues from cold-exposed animals as measured by quantitative PCR relative to homozygous IVS1+1505G. Errors are standard deviations. In brown adipose tissue (BAT), Ucp3 mRNA abundance was significantly different from each other in all three genotypes with homozygous IVS1+1505A hamsters being virtually devoid of expression. In skeletal muscle (SKM), only homozygous animals differed significantly from each other with an intermediate Ucp3 mRNA level in heterozygotes. n = 53 (17 GG/26 AG/20 AA) for BAT and 46 (15 GG/15 AG/16 AA) for SKM. \*\*\*P < 0.001; \*P < 0.05. B: Western blots of protein isolated from tissues of animals under control conditions. The above difference in mRNA amounts was reflected in the protein level. Homozygous IVS1+1505A hamsters did not show any detectable Ucp3 protein in BAT. In SKM, the Ucp3 protein level of these animals was slightly decreased.

sequencing, we genotyped an additional 97 animals randomly chosen from our breeding colony totalling 230 hamsters. Of these, 105 hamsters were homozygous IVS1+1505G, 104 hamsters were heterozygous, and 21 hamsters were homozygous IVS1+1505A, resulting in an allele frequency of 0.68 for IVS1+1505G and 0.32 for IVS1+1505A (Fig. 4*A*). The observed genotypes were in Hardy-Weinberg equilibrium.

To further evaluate the distribution of IVS1+1505A, we collected samples from five different breeding colonies of P. sungorus in the United States, Canada, and Europe (Fig. 4A). Two of those had been established independently from the Marburg colony. Most Djungarian hamsters in research laboratories nowadays can be traced back to the K. Hoffmann and J. Figala colony at the Max Planck institute in Andechs, Germany (26). This is true for our animals (allele frequency for IVS1+1505A = 0.37) as well as for the commercial supplier, Wrights (Essex, UK), where J. Mercer (0.97) and A. Loudon (1.00) obtained their founder animals (personal communications and Ref. 5). S. Steinlechner (0.03) and C. Wynne-Edwards (0.23) trapped wild animals in Siberia and established independent colonies in their laboratories that have never been interbred with animals of a different origin (personal communications and Ref. 36). In samples of hamsters from both colonies, we identified the mutant allele. Since in S. Steinlechner's samples we only found a single heterozygous animal, we confirmed our results by genotyping the offspring of this animal and could again identify IVS1+1505A.

T. Bartness' hamsters (IVS1+1505A = 0.07) originate from the Hoffmann-Figala line and were interbred with animals from S. Steinlechner and C. Wynne-Edwards (personal communications).

We confirmed a strongly decreased Ucp3 transcript abdundance in BAT of mutant hamsters from J. Mercer and A. Loudon compared with heterozygous and wild-type hamsters from C. Wynne-Edwards (Fig. 4B).

# BROWN ADIPOSE TISSUE-SPECIFIC UCP3 EXPRESSION

Fig. 4. Both alleles are present in the wild population. *A*: allele frequencies of IVS1+1505G (shaded) and IVS1+1505A (solid) in six international colonies proved the occurrence of both alleles in the natural habitat. The colonies of Klingenspor, Steinlechner, and Wynne-Edwards are completely independent and have never been interbred. Hamsters from the Mercer and Loudon colonies can be traced back to the same source as our (Klingenspor) hamsters. The Bartness animals are a product of interbreeding hamsters from all these lines. *B*: hamsters of three different colonies were phenotyped (n = 3 each). Mutant hamsters from J. Mercer and A. Loudon displayed strongly decreased Ucp3 mRNA abundance compared with wild-type (n = 1) and heterozygous (n = 2) hamsters from C. Wynne-Edwards. a.u., Arbitrary units.



Taken together, the allele IVS1+1505A was found in every colony analyzed including all independently established ones.

Differential complex formation in EMSA. One potential link between a sequence variation and a defective transcriptional regulation is the loss or impairment of a transcription factorbinding site. To investigate this possibility, we performed EMSAs with probes representing the polymorphic intronic site IVS1+1505 and nuclear extracts isolated from several cell lines stimulated with Wy14,643 and rosiglitazone (Fig. 5). In HIB1B cells, this treatment has been proven to induce the allele-specific expression phenotype in our reporter gene assays.

Stimulated HIB1B nuclear extracts led to the formation of several protein-DNA complexes on the IVS1+1505G probe that were distinctly weaker on the IVS1+1505A probe (Fig. 5A). The same pattern was observed with extracts from a different immortalized BAT cell line (29). No complex formation on either probe was observed using extracts of stimulated C2C12 myotubes. All three nuclear extracts formed complexes on positive control probes representing published binding ele-



Fig. 5. Differential DNA-protein complex formation occurs on a sequence element surrounding IVS1+1505. Bands of interest are shown by arrowheads. A: we performed EMSA experiments with four different probes as shown in *D*, and nuclear extracts were prepared from three different cell lines that had been treated with Wy14,643 and rosiglitazone. In brown adipocyte cell line (HIB1B and BAT cells) extracts, five complexes formed on probe IVS1+1505G that were weaker on the A allele probe. These complexes did not form on nuclear extracts from C2C12 myotubes. All extracts used were able to shift the positive control probes for NF- $\kappa$ B and MyoD. *B*: in HIB1B cells, stimulation with Wy14,643 and rosiglitazone did not affect complex formation on either probe IVS1+1505G or probe IVS1+1505A. *C*: we competed labeled probe IVS1+1505G with unlabeled versions of this probe, IVS1+1505A, or NF- $\kappa$ B. IVS1+1505G displayed a markedly higher competition potential than the A allele probe. The negative control probe (NF- $\kappa$ B) did not compete. *D*: sequences of the four probes used in the EMSA experiments.

ments for transcription factors NF- $\kappa$ B and MyoD, respectively (10, 34).

Notably, the stimulation of HIB1B cells with Wy14,643 and rosiglitazone did not change the pattern of complexes formed by nuclear protein with either probe (Fig. 5*B*).

We investigated the specificity of this differential complex formation in EMSA with competing probes (Fig. 5*C*). Unlabeled IVS1+1505G probe itself was able to efficiently disrupt complex formation of HIB1B nuclear protein with its labeled counterpart. Unlabeled IVS1+1505A probe displayed a markedly lower competition potential. The probe for NF- $\kappa$ B was used as a negative control and did not compete for this complex at all.

Analysis of putative transcription factor-binding sites. The sequence element surrounding IVS1+1505 was identified in the first intron of the *Ucp3* gene in all analyzed vertebrates (Fig. 6A). Among these vertebrates, the G nucleotide at position IVS1+1505 is conserved. We derived a consensus sequence from this alignment and analyzed it for potential transcription factor-binding sites (see Supplemental Material). All three algorithms recognized a binding site for proteins of the forkhead factor family.

To verify binding of one of the more than 40 members of this family, we designed competitor oligonucleotides representing forkhead factor-binding sites. We extracted all 15 known binding matrixes from the MatBase database (Genomatix) and grouped them by similarity (Supplemental Material).<sup>1</sup> We were able to design 5 sequences (sequences A-E) to represent the 15 ambiguous matrixes. The predicted forkhead factor-binding site on our wild-type hamster probe was replaced by one of these five elements (A-E) and tried to compete with the protein-DNA interaction on the hamster wild-type probe in bandshift assays. Whereas unlabeled wildtype probe was clearly able to compete stronger than the unlabeled mutant probe in a control experiment, none of the five unlabeled forkehead factor-binding probes was able to compete stronger than the negative control (Supplemental Material).

<sup>1</sup> Supplemental Material for this article is available online at the *Physiological Genomics* website.

Hs	GAGAATCTGCTCTGTAAACAAGCA-GCTCAAGGGATCC
Pt	GAGAATCTGCTCTGTAAACAAGCA-GCTCAAGGGATCC
Cf	GAGAATCCACTCTGTAAACAAGCA-GCTCCAGGGATCC
Bt	AAGAATCCGGTCTGTAAACAAGTG-GCTCCAGGTACCC
Rn	GGAGGGTATTTCTCTAAACACGCCCACACCGCTATCCC
Mm	GGAGAGCATGTCTCTAAACATGCCTACCCTGCTCTTCC
Md	AATTGGGGAATGTCTGAACAAGTTATCGTATATGAATA
Ps	$GAATAA\underline{GTGTTTTCTTAACAC}\mathbf{G}\underline{CCTGCACTGTTGGTA}C$
	* * * **** *

#### TcTsTaAACAaGcnngC

Fig. 6. A: alignment of the responsible intronic element of several vertebrate species and the derived consensus sequence. \*Fully conserved position. Bold letters show IVS1+1505; underlined letters show the EMSA probe. Hs, *Homo sapiens*; Pt, *Pan troglodytes*; Cf, *Canis familiaris*; Bt, *Bos taurus*; Rn, *Rattus norvegicus*; Mm, *Mus musculus*; Md, *Monodelphis domestica*; Ps, *Phodopus sungorus*.

The G allele at position IVS1+1505 led to altered body weight development. We kept offspring of heterozygous breeding pairs at room temperature under long-day conditions (16: 8-h light-dark cycle) with ad libitum access to food and water and monitored body weights. At the age of  $81 \pm 3$  days, 66 hamsters were transferred to 4°C ambient temperature to pronounce any differences that may arise from altered BAT physiology. After 2 days of cold exposure, hamsters were killed and organ weights were determined. We weighed suprasternal BAT, interscapular BAT, inguinal white adipose tissue, and the spleen. None of these displayed significant differences with respect to genotype at IVS1+1505. The same was true for the difference in body mass before and after cold exposure (Supplemental Material).

We did, however, find a differential development of body weight (Fig. 7). Both male and female animals that were homozygous for IVS1+1505G displayed a lower body mass than carriers of IVS1+1505A. Heterozygous hamsters tended to increase body weight in a similar fashion as hamsters homozygous IVS1+1505A. This pattern of genotypespecific body weight trajectory was very similar in male and female hamsters. The development of body weight over time was significantly influenced by the genotype of the animals (Table 1).

To allocate this difference, we measured the body composition of 108 hamsters aged 259–289 days by DEXA, but we did not detect any significant genotype effects on body composition. As a measure for body size, we determined the lengths of the femur of all animals from these X-ray pictures and did not find a significant genotype effect on size (Supplemental Material). The significant difference in body mass trajectory was neither clearly attributable to fat or lean mass nor to an overall increase in body size.

## DISCUSSION

In our breeding colony of the Djungarian hamster (*P. sun*gorus), we identified animals exhibiting a heritable BATspecific lack of Ucp3 mRNA (11). We were able to pinpoint the underlying genetic variation by resequencing of the *Ucp3* gene in several animals. Surprisingly, it turned out to be a single-nucleotide variation located in the first intron (IVS1+1505), i.e., far away from all regulatory elements known so far, which are found in the proximal promoter region and further upstream (7, 22, 23). Differential complex formation around IVS1+1505 in EMSA and in silico analyses implied a function as a binding site for a transcriptional regulator.

In reporter gene assays as well as in vivo, allele IVS1+1505A led to complete loss of expression in BAT only, wheras in skeletal muscle cells only a mild phenotype was observed. Stimulation with PPAR- $\alpha/\gamma$  ligands led to a less-pronounced activation of the Ucp3 promotor in C2C12 cells, probably due to a different pattern of expressed PPAR iso-forms. The comparison of promoter construct activity in the absence of agonists, however, revealed that the difference between IVS1+1505G and IVS1+1505A was less pronounced in C2C12 cells. In EMSA experiments, nuclear extracts of two brown adipocyte cell lines led to allele-specific complex formation, whereas in myotubes the binding factor was obviously absent. We conclude that the affected element very probably is a regulator of tissue-specific *Ucp3* expression. So far, the



Fig. 7. Body weight development was dependent on the genotype of hamsters. Body weights of hamsters were monitored on a weekly basis beginning with weaning. Shown are the mean absolute body weights with standard errors. Wt, homozygous IVS1+1505G; het, heterozygous; mut, homozygous IVS1+1505A.

molecular basis of this tissue specificity is unknown, and differences in the activity of known transcription factors like PPAR- $\alpha$  and - $\delta$  have been implicated (18, 22). Interestingly, treatment of HIB1B cells with agonists for PPAR- $\alpha/\gamma$  led to increased wild-type promoter activity in reporter gene assays but not to increased complex formation in EMSA. Therefore, these PPARs and their direct targets do not seem to be constituents of the regulatory complex binding around IVS1+1505. Formation of a protein complex on this intronic element appears rather to be a switch allowing PPAR-dependent promoter transactivation, which itself is mediated by the proximal promoter (23).

This distinct intronic regulatory element is responsible and essential for the BAT-specific expression of *Ucp3*. Analysis of the conserved consensus sequence based on the sequence comparison of several vertebrate species revealed a putative transcription factor-binding site that includes a conserved G nucleotide at hamster position IVS1+1505. Several programs consistently predicted a binding site for forkhead domain transcription factors among other possible candidates. However, competition experiments did not yield any supportive evidence for binding of a forkhead factor, although all known 15 binding matrixes were tested. Our group is currently establishing affinity chromatography and yeast one hybrid assays to identify the interacting protein.

The presence of Ucp3 in both skeletal muscle and brown adipose tissue has been reported for all species analyzed in this respect so far including mice (31) and rats (1). Since hamsters carrying the IVS1+1505G allele display this known distribution of Ucp3 and all analyzed sequences exhibit a G nucleotide at the orthologous intron position, we consider IVS1+1505G to be the ancestral allele and IVS1+1505A to be the derived allele. Our screening for IVS1+1505A and IVS1+1505G in several international breeding colonies of *P. sungorus* identified IVS1+1505A in all colonies including three independently founded ones. This clearly shows that the mutation generating IVS1+1505A did not occur in captivity but in the wild. In fact, it indicates a rather high allele frequency in the

wild population being incidentally trapped for three times at different times and places. Without any knowledge about the actual allele frequencies in the wild, however, we can merely conclude the lack of any dramatic negative consequences of IVS1+1505A regarding overall fitness. In the colonies of J. Mercer and A. Loudon, homozygous IVS1+1505A also led to the loss of BAT Ucp3 expression.

Beyond this study, the knowledge about the existence of IVS1+1505A and its primary expression phenotype may prove valuable to further study of the function of Ucp3. So far, there are  $Ucp3^{-/-}$  mouse models available and transgenic mice overexpressing the protein (1, 3, 6, 9, 30). With the Djungarian hamster, we now have access to an animal model with a tissue-specific lack of expression that can be applied to elucidate tissue-specific protein function. Furthermore, knowledge about IVS1+1505A will help researchers working with Djungarian hamsters to select animals of the appropriate genotype for their respective study.

The implication of Ucp3 in the regulation of body mass has long been disputed. There are several studies that have described an interrelation of polymorphisms within the human *UCP3* gene and markers of obesity, especially -55(T/C) located in the promoter region (2, 17, 33). The proximity of the *UCP2* gene has always raised the question of whether these effects can doubtlessly be ascribed to *UCP3* or possibly to additional polymorphisms within linkage disequilibrium (33). In this study, we demonstrate that a change in *Ucp3* expression can be responsible for altered body weight development. In this respect, heterozygous hamsters seemed to resemble homozygous IVS1+1505A hamsters rather than homozygous IVS1+1505G hamsters. This dominant body weight phenotype would imply that small changes of *Ucp3* expression levels could already lead to a full-scale impact on body weight development.

The involvement of Ucp3 in energy balance is substantiated by its transcription being correlated to serum fatty acid levels and its presence in tissues with exceptional  $\beta$ -oxidative capacities (35). The absence of a body weight phenotype in  $Ucp3^{-/-}$ mice (9, 30), however, questioned the relevance of Ucp3 in this context. Our demonstration of differential body weight development in response to altered Ucp3 expression reopens this discussion. In fact, it cannot be ruled out that comparable effects were simply overlooked in  $Ucp3^{-/-}$  mice. All published analyses of body weight development in these animal models used far fewer animals and shorter study periods than in this study and so would probably not have been sufficient to reveal an effect of this size. In contrast to  $Ucp3^{-/-}$  mice, however, our hamster model lacks Ucp3 specifically in BAT, whereas skeletal muscle expression is only mildly impaired.

 Table 1. Body weight data statistically analyzed by two-way

 repeated-measures ANOVA

Parameter	P Value
Sex	< 0.001
Genotype	0.010
Age	< 0.001
$Sex \times Genotype$	0.920
$Sex \times Age$	< 0.001
Genotype $\times$ Age	0.041

The genotype of an animal significantly influenced the development of body weight with age in both sexes.

Nevertheless, we cannot decide on the tissue that fundamentally accounts for the body weight divergence. Our group recently demonstrated that mutant hamsters display an impaired BAT function leading to reduced cold tolerance. This phenomenon is accompanied by a global decrease in BAT metabolic gene expression (15). Such consequences of a complete lack of Ucp3 in BAT seem tempting to be held responsible, but even a small change in skeletal muscle may significantly contribute to the observed phenotype given the huge fraction of body mass and metabolism it represents. In any case, it is surely worthwhile to comparatively resequence human *UCP3* intron 1 to identify possible single-nucleotide polymorphisms in this novel regulatory region.

Taken together, we identified an intronic element within the Ucp3 gene that is essential for BAT expression of this gene and constitutes a transcription factor-binding site. A naturally occurring mutation disrupting this element in the homozygous state leads to complete loss of BAT Ucp3 and altered body weight development.

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