BBA Research Letter

Identification and Expression of Bovine Ucp1 Variants

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Short title: Bovine Ucp1 variants

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Abstract

- UCP1 is a transporter responsible for nonshivering thermogenesis in mammalian brown/beige fat.
- We identified four alternative splice variants of bovine Ucp1 (variant 1-4).
- Bovine *Ucp1* comprises a substantial proportion of variant 1/3.
- The gene products from *Ucp1* variant 2-4 are efficiently degraded by the proteasome system.

Brown/beige adipocytes dissipate chemical energy as heat when exposed to cold [1]. Uncoupling protein (UCP) 1, predominantly expressed in brown/beige adipocytes, is primarily responsible for energy expenditure [1]. Because functional brown/beige adipocytes occur in adult humans [1], these cells may be a target for preventing and treating human obesity. We previously observed the presence of brown/beige adipocytes in the white fat of mature cattle [2]. We also demonstrated unexpected expression of UCP1 in cattle skeletal muscle tissues and myogenic cells [3]. Although small numbers of adipocytes are generally resident in bovine skeletal muscles, these adipocytes were negative for UCP1 and myogenic cells expressed UCP1 [3]. In this study, we characterized the expression of the bovine *Ucp1* gene.

Animal care and experiments were approved by the Animal Care Committee of Azabu University (140902-4, 160516-9, 160516-10, and 190306-3), and were conducted in accordance with the approved guidelines. Thirteen fat depots from 12 cattle hospitalized in the Azabu University Veterinary Teaching Hospital were obtained (Table S1). One sample (sample #1) was used in our previous study [3].

Total RNA from the perirenal fat of a newborn Jersey calf (sample #1, Table S1) treated with RNase-free DNase I was used to isolate *Ucp1* mRNA. The 5′- and 3′-ends of the

bovine *Ucp1* mRNA were determined by the 5′- and 3′-rapid amplification of cDNA end (RACE) method, respectively, as described previously [4].

Based on the predicted information of bovine *Ucp1* (NM_001166528), PCR primers were set at exon 4 (primer A: 5′-AATGTCATCATCAACTGTACAGAGC-3′) and exons 4 and 5 (primer B: 5′-AGGGCACATCGTCTGCTAAT-3′) of the bovine *Ucp1* gene to amplify the *Ucp1* fragment. The 5′-RACE method was performed using 1×Universal Primer Mix and the primer 5′-gattacgccaagcttGCAGGGCACATCGTCTGCTAATAGTTTG -3′ set at exons 4 and 5, where the capitalized characters represent the bovine *Ucp1*-specific sequence and small characters are part of the pRACE vector for cloning. The 3′-RACE method was performed using 1×Universal Primer Mix and the primer 5′-gattacgccaagcttGGAATGTCATCATCAACTGTACAGAGCTAG-3′ set at exon 4. Fragments obtained by the 5′-RACE and 3′-RACE methods were cloned into the pRACE vector, and the nucleotide sequence was determined.

The full-length and variants of bovine *Ucp1* mRNA were isolated by PCR using bovine brown fat cDNA as the template and the primer set at 5′-UTR (primer C: 5′-CGGAGTCCGCGTTGAGTCAGG-3′) and 3′-UTR located downstream of exon 6 (primer D: 5′-TCCGTCTTCTTCCCGAGGAGGA-3′). PCR was also performed using primer C and the primer set at the 3′-UTR located downstream of exon 5 (primer E: 5′-CATTGTAGATACCGAAGAACACATC-3′), and the nucleotide sequence of the PCR product was determined.

The coding region of the bovine *Ucp1* variants, structurally related *Ucp2* or *Ucp3*, was inserted into HA-tagged pcDNA3 to produce C-terminal HA-tagged proteins. The 3′-UTR (437 bp) of the bovine *Ucp1* variant 1/3 spanning from downstream of the stop codon to the poly-A signal (5′-aataaa-3′) was inserted downstream of the *Renilla*

luciferase gene regulated under the control of the thymidine kinase promoter (pGL4.74[hRluc/TK]) to prepare Luc-Ucp1(v1,3:437). We also prepared Luc-Ucp1(v2,4:200), which inserted 3′-UTR (200 bp) of the bovine *Ucp1* variant 2/4, spanning from downstream of the stop codon to the poly-A signal-related sequence (5′-agtaaa-3′ [5]) into pGL4.74[hRluc/TK]. The cytomegalovirus promoter (nt 232-819) was inserted into pGL4 to produce firefly luciferase under the control of the cytomegalovirus promoter (CMV-pGL4).

RT-qPCR was performed as described previously [6]. The following PCR primers were used: Primers A and B for total *Ucp1*, Primer F at exons 3 and 4 (5′-GCTTGACGGGGCTTTGGAAAGGGAC-3′) and Primer G at exons 5 and 6 (5′-GTCGCAAGAAGGAAGGTACAAATCCTT-3′) for variant 1, Primers F and H at exon 5 and downstream region of exon 5 (5′-AGATACCGAAGAACACATCCTACCCTT-3′) for variant 2, Primer I at exons 2 and 4 (5′-TCTTCACCACAGGGAAAGAAGGGACTA-3′), Primer G for variant 3, and Primers I and H for variant 4. Gene expression levels were determined by the standard curve method and were normalized by the expression of *Hprt1*, which was determined using primers 5′-CCAGTCAACAGGCGACATAAAAG-3′ and 5′-GCATTGTCTTCCCAGTGTCAATTA-3′.

Luciferase-based reporter assays were performed as described previously [7]. HB2 brown preadipocytes were transfected with a reporter with 3′-UTR of *Ucp1* together with CMV-pGL4, followed by treatment with forskolin. *Renilla* luciferase activity was normalized to firefly luciferase activity.

When cDNAs from bovine, mouse, and rat brown fat were used as the template, agarose electrophoresis of RT-PCR to amplify full-length *Ucp1* revealed two bands in bovine

brown fat but one band in mouse and rat brown fat (Fig. 1A). Two main bands were also detected in cDNA from bovine white fat and skeletal muscle, suggesting *Ucp1* variants in these bovine tissues (Fig. 1B). The 5′-RACE, 3′-RACE, and subsequent RT-PCR to isolate the *Ucp1* gene indicated four alternative splice variants, as shown in Figs. 1C and S1. Full-length *Ucp1* corresponding to mouse and rat *Ucp1*, which was registered in the NCBI nucleotide database as LC271253 (variant 1), presented a variant with extension of exon 5 and skipping exon 6 (variant 2, LC271254), a variant skipping exon 3 (variant 3, LC271255), and a variant with extension of exon 5 and skipping exons 3 and 6 (variant 4, LC271256). The nucleotide sequence of the *Ucp1* variant determined in this study was almost the same as the predicted nucleotide in NM_001166528, except for exon 1; 12 bp located at the 3′-terminus of exon 1 in NM_001166528 was deleted in *Ucp1* variant 1, which is compatible with exon 1 in other animal species, such as humans (NM_021833), mice (NM_009463), rats (NM_012682), and dogs (NM_001003046).

We explored the relative expression of *Ucp1* variants using PCR primers to detect individual *Ucp1* variants and total *Ucp1* (Fig. S2). We prepared a plasmid encoding each *Ucp1* variant and used it as the template for PCR. The PCR primers designed to detect each variant specifically amplified the expected *Ucp1* variant, and PCR primers to detect all *Ucp1* variants equally amplified *Ucp1* variants. Using these PCR primers, we evaluated the relative expression of *Ucp1* in adipose tissues from fetal or neonatal calves. The relative expression of total *Ucp1* was not statistically different among the breeds (Fig. 1D). Next, we quantified the expression levels of each *Ucp1* variant. The expression levels of *Ucp1* variant 2/4 were below the detection limits (data not shown). The breed did not affect the expression levels of the *Ucp1* variant 1/3 (Fig. 1E, F). Expression of variant 1 was 5–14 times greater than that of variant 3, and breed did not affect the ratio of variant 1 to variant 3 (Fig. 1G).

All *Ucp1* variants had a common transcriptional initiation site; however, the terminal site differed between variants 1/3 and 2/4 (Fig. 1C). We hypothesized that the difference in the 3′-UTR was partly related to the lower expression levels of the variant 2/4 mRNAs than variant 1/3 mRNAs. Thus, we evaluated the role of the 3′-UTR in *Ucp1* expression using luciferase-based reporter assays. The expression of the reporter with 3′-UTR of the *Ucp1* variant 1/3 was higher than that with 3′-UTR of *Ucp1* variant 2/4 (Fig. 1H). Forskolin, an activator of the protein kinase A pathway, transcriptionally stimulates *Ucp1* expression [1]. Forskolin did not affect luciferase expression irrespective of the 3′-UTR of *Ucp1*. These studies suggest that mRNAs of *Ucp1* variant 2/4 are less stable than those of *Ucp1* variant 1/3. Further studies are needed to verify using bovine brown adipogenic cells.

We further evaluated the protein expression of UCP1 in cells transfected with an equal amount of plasmid encoding each *Ucp1* variant (Fig. 1I). Expression of UCP1 variant 1 was detected and was comparable to that of UCP2 and UCP3 (Fig. 1I, lanes 2, 6, and 7). Expression levels of UCP1 variant 2-4 were lower than that of variant 1, and expression level of variant 4 was lower than that of variant 2/3 (Fig. 1I, lanes 2–5). MG-132 and chloroquine are inhibitors of proteasomes and autophagy, respectively. Treatment with MG-132, but not chloroquine, increased UCP1 variant 2-4, whereas expression of UCP1 variant 1, UCP2, and UCP3 was not affected. These results suggest that UCP1 variant 2-4 are efficiently degraded by the proteasome system.

Previously, several SNPs of *Ucp1* gene have been reported in dairy cows, which related to milk traits [8]. Additionally, two SNPs with an amino acid substitution in exons were detected in the human *UCP1* gene [9], and variants differing in the 3′-UTR were shown in murine *Ucp1* [10]. Furthermore, alternative splice variants of *Ucp1* have been shown in genetically engineered mice [11-13] (Fig. S1B). The present study revealed alternative

splice variants of *Ucp1* in genetically intact cattle. In addition, the patterns of alternative splice of *Ucp1* are novel in mammals; the spliced regions in bovine *Ucp1* gene are distinct from those in murine *Ucp1* gene [11-13] (Fig. S1B).

The effect of autophagy on the degradation of UCP1 protein remains controversial [14]. Alternatively, rat UCP1, which is expected to correspond to bovine UCP1 variant 1, was degraded via the ubiquitin-proteasome pathway with a half-life of 30–72 h [15]. The present study showed protein degradation of bovine UCP1 variant 2-4 via the proteasomal pathway. UCP1 variant 2-4 are likely to degrade more rapidly than UCP1 variant 1.

Here, we identified four variants of *Ucp1*; *Ucp1* variant 2/4 mRNAs were relatively unstable, leading to lower expression. Additionally, UCP1 variant 3 was rapidly degraded. Thus, we consider that variant 1 protein is the main UCP1 protein in cattle. Nevertheless, other variants, possibly variant 3 of UCP1, may exhibit some functions in bovine tissues. Future studies should be directed to clarify the relevance of expression of *Ucp1* variants to efficiency of weight gain as well as metabolic and clinical status. Furthermore, the presence of *Ucp1* variants should be also evaluated in neonatal humans.

Acknowledgements

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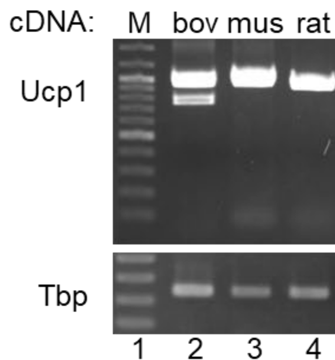
Figure legends

Fig. 1. Characterization of bovine *Ucp1* variants

(A, B) Identification of *Ucp1* variants in bovine brown fat, white fat, and skeletal muscle. Total RNA was isolated from the brown fat of indicated species (A) or the indicated bovine tissues (B), and RT-PCR was performed to detect full-length *Ucp1*. Primers used were primer C and primer D for bovine *Ucp1*, 5′-CACTCACGCCTCTCTGCCCTCCAAGCCAGG-3′ and 5′-CCCAATGATGTTCAGTATCTCTTCCTCCAAGTTGC-3′ for mouse *Ucp1*, and 5′-ATGGTGAGTTCGACAACTTCCGAAGTGCAA-3′ and 5′-CTATGTGGTGCAGTCCACTGTCTGCCGGGAC-3′ for rat *Ucp1*. The PCR products were electrophoresed in agarose gels and stained with ethidium bromide. A representative result is shown. M: marker, bov: bovine, mus: mouse, rat: rat, BAT: brown adipose tissue, WAT: white adipose tissue, SM: skeletal muscle, neg: negative control. (C) Gene structure of four variants of bovine *Ucp1*. *Ucp1* variants were isolated by 5′-RACE and 3′-RACE, and a total of four variants were determined by nucleotide sequence after RT-PCR using Primers C and D and Primers C and E. Gene structures of four variants of *Ucp1* are shown. (D-G) Expression levels of total *Ucp1* (D), *Ucp1* variant 1 (E), and *Ucp1* variant 3 (F) in fat from neonatal calves of the indicated breed were examined by RT-qPCR analysis. The relative expression in Jersey calves was set to 100. The data are presented as the mean ± SE (n = 3–4). (G) The expression level ratio of *Ucp1* variant 1 to *Ucp1* variant 3 was calculated for each breed. Data are presented as the mean ± SE (n = 3–4). Relative gene expression was log-transformed to approximate a normal distribution before analysis, and differences among breeds were evaluated by the Tukey–Kramer method. Differences were considered significant at $P < 0.05$, and no differences were detected. (H) Comparison of 3′-UTR of *Ucp1* variant 1/3 to that of *Ucp1* variant 2/4 on *Ucp1* expression was explored. HB2 brown adipogenic cells were transfected with the indicated reporter and treated with or without forskolin (10 μM) for 6 h. *Renilla* luciferase activity

was normalized to firefly luciferase activity, and the relative activity in cells transfected with Luc-Ucp1(v1,3:437) and treated without forskolin set at 1 (n = 3). (I) COS7 cells were transfected with the indicated plasmid encoding HA-tagged *Ucp1* variant and treated with MG-132 (20 μM) or chloroquine (100 μM) for 4 h. Cell lysates were immunoprecipitated with anti-HA (12CA5) antibody, followed by western blot analysis using anti-HA(3F10) antibody. An equal portion of cell lysates was also subjected to western blot analysis to detect β-actin expression.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Tbp
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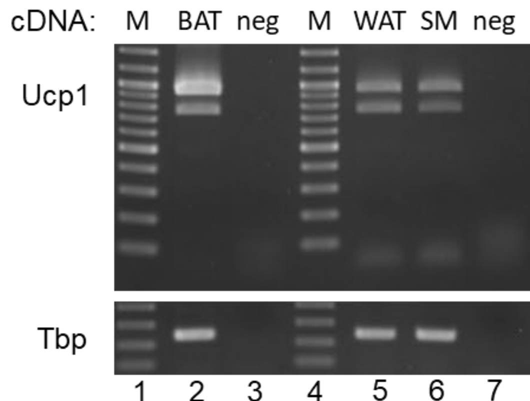
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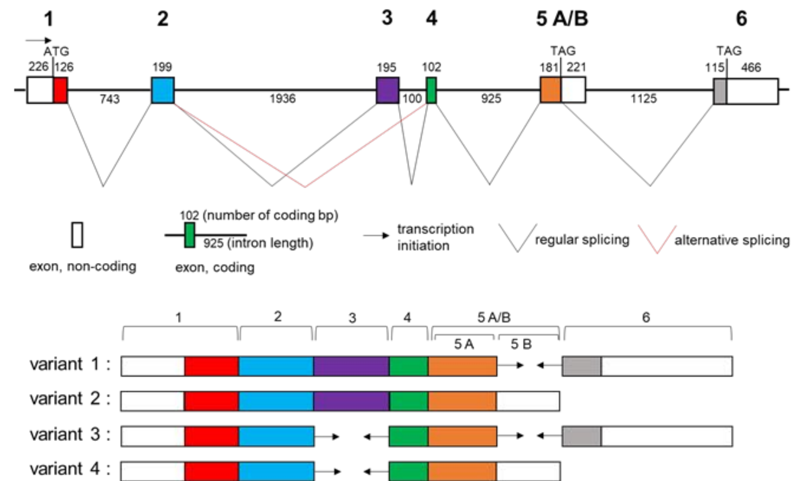
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TAG
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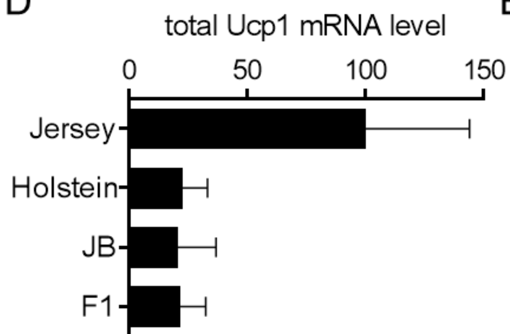
regular splicing
alternative splicing
variant 1 :
variant 2 :
variant 3 :
variant 4 :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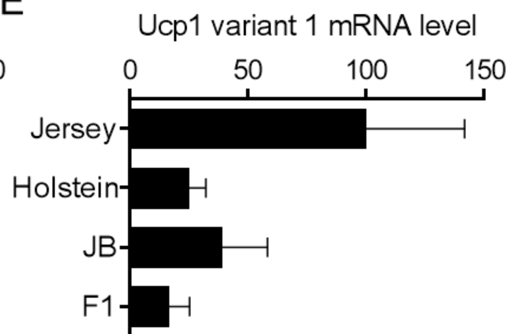
Holstein
JB
F1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Jersey
Holstein
JB
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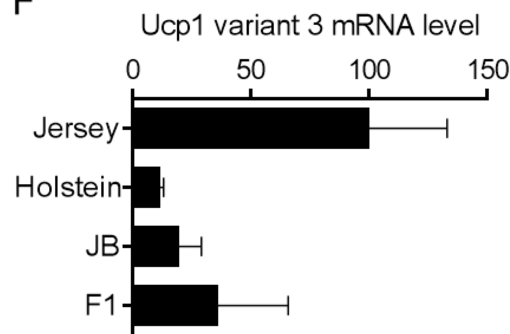
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JB
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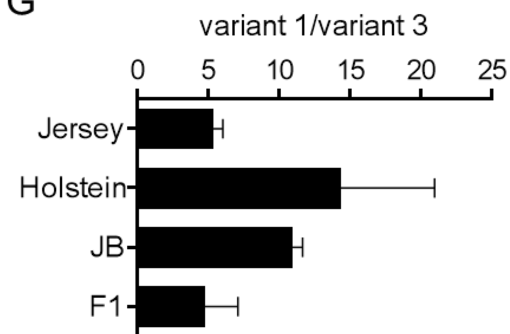
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JB
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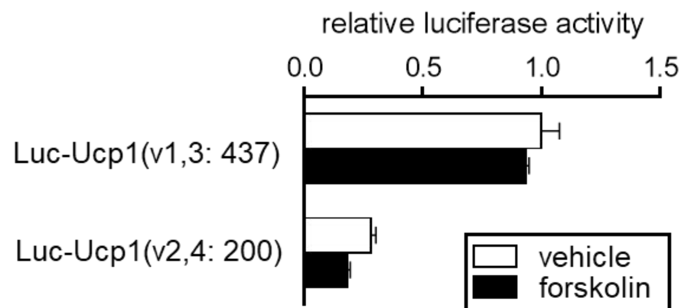
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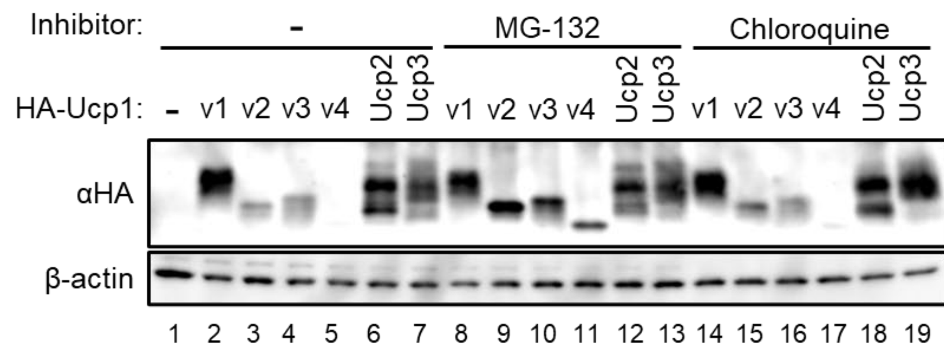
vehicle
forskolin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αHA
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Supplementary information

Identification and Expression of Bovine Ucp1 Variants

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Figure S1-2

Table S1

References

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Figure S1. Nucleotide sequence of bovine *Ucp1* variants and comparison with amino acid sequence of murine UCP1

(A) Nucleotide sequence of cDNA encoding the bovine *Ucp1* variant. The translational initiation site ATG is underlined. The common sequence among all variants are shown in black, and those only between variants 1 and 2 are shown in blue. The common sequence only between variants 1 and 3 are shown in red, and those only between variants 2 and 4 are shown in green. The stop codon is wavy-underlined in red or green, and the putative poly-A signal is boxed. A dot "." indicates sequence identity to variant 1, and a dash "-" indicates a gap in the sequence. (B) Comparison of amino acid sequence between bovine UCP1 and murine UCP1. Amino acid sequence of bovine UCP1 variant 1 was compared with that of murine UCP1. Amino acid sequence spanning exon 1-6 is shown in red, blue, violet, green, yellow, and gray, respectively. An amino acid at exon-exon junction is underlined, and color of the amino acid matches to that of exon including two nucleotides of the triplet. Amino acids surrounded by solid line lack in bovine UCP1 variant 3/4, and those surrounded by dashed line lack bovine UCP1 variant 2/4. Dashed lines under murine UCP1 indicate deleted amino acids resulting from alternative splicing, which was shown in previous studies: #1 and 3: reference [1], #2: references [2, 3]. A dot "." indicates sequence identity to bovine UCP1 variant 1, and a dash "-" indicates a gap in the amino acid sequence.

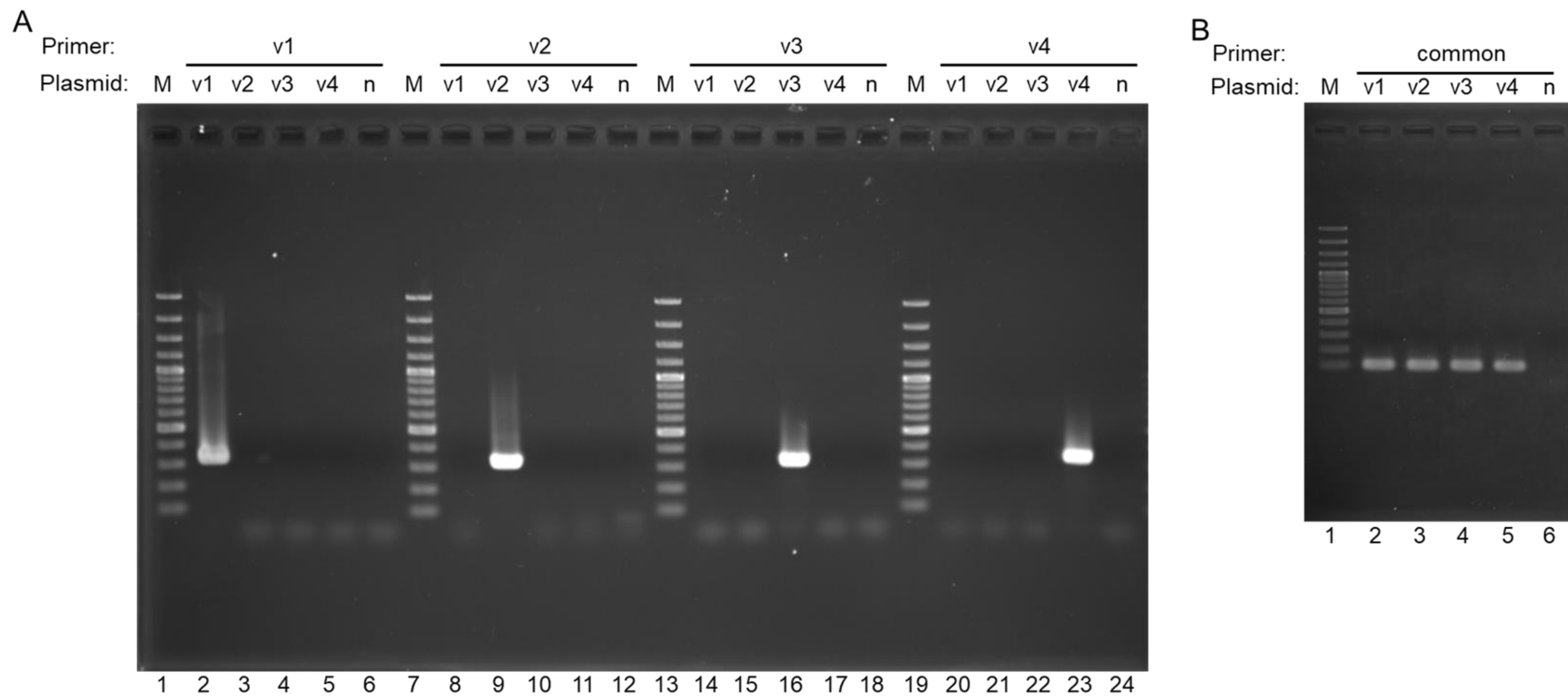

Figure S2. Establishment of detection of *Ucp1* mRNA

(A, B) Establishment of detection of each variant. Using the plasmid encoding the indicated *Ucp1* variant as the template, PCR was performed using variant-specific primers (A) or by primers amplifying all variants (B). The PCR products were electrophoresed in agarose gels and stained with ethidium bromide. A representative result is shown. M: GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific).

Table S1. Profile of samples analyzed fat depots

No	Breed	Sex	Age	Location	Reason and note
1	Jersey	male	5 dpn [1]	Perirenal fat	Practice of veterinary clinic for veterinary students
2	Jersey	male	5 dpn	Perirenal fat	Practice of veterinary clinic for veterinary students
3	Jersey	male	36 dpn	Perirenal fat	Practice of veterinary clinic for veterinary students
4	Jersey	male	10 dpn	Perirenal fat	Practice of veterinary clinic for veterinary students
5	Holstein	male	63 dpn	Perirenal fat	Arthritis of right hind limb tarsal joint
6	Holstein	unknown	296 dpc [2]	Perirenal fat	Prolonged gestation
7	Holstein	female	2 dpn	Perirenal fat	Imperforate anus malformation
8	Japanese Black	female	25 dpn	Perirenal fat	Unknown
9	Japanese Black	male	56 dpn	Perirenal fat	Otitis media
10	Japanese Black	unknown	170 dpc	Perirenal fat	Fetus of cattle suffered from fat necrosis
11	F1 [3]	female	13 dpn	Perirenal fat	Encephalitis: same cattle as sample #12
12	F1	female	13 dpn	Interscapular fat	Encephalitis: same cattle as sample #11
13	F1	female	43 dpn	Perirenal fat	Fracture of left forelimb radius

[1] days postnatal. [2] days post coitum. [3] crossbreds of Holstein cow sired by Japanese Black cattle.