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ACKNOWLEDGEMENTS. We thank P. Karieva and D. Mech for commenting on earlier drafts. This work was supported in part by grants from the United States National Science Foundation, Division of Mathematical Sciences.

Development of obesity in transgenic mice after genetic ablation of brown adipose tissue

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BROWN adipose tissue, because of its capacity for uncoupled mitochondrial respiration^{1,2}, has been implicated as an important site of facultative energy expenditure^{3–5}. This has led to speculation that this tissue normally functions to prevent obesity^{3–5}. Attempts to ablate or denervate brown adipose tissue surgically have been uninformative because it exists in diffuse depots and has substantial capacity for regeneration and hypertrophy⁶. Here we have used a transgenic toxigenic approach^{7,8} to create two lines of transgenic mice with primary deficiency of brown adipose tissue. At 16 days, both lines have decreased brown fat and obesity. In one line, brown fat subsequently regenerates and obesity resolves. In the other line, the deficiency persists and obesity, with its morbid complications, advances. Obesity develops in the absence of hyperphagia, indicating that brown fat deficient mice have increased metabolic efficiency. As obesity progresses, transgenic animals develop hyperphagia. This study supports a critical role for brown adipose tissue in the nutritional homeostasis of mice.

Regulatory elements of the gene for the uncoupling protein (UCP), a specific protein marker for brown adipose tissue (BAT)^{2,9}, were used to drive expression of diphtheria toxin A-chain (UCP-DTA) or an attenuated mutant (UCP-176) (Fig. 1a). Two lines of mice were created, UCP-DTA and UCP-176, both of which have ablated BAT and obesity (Fig. 1). To quantify the degree of BAT ablation, immunoblotting with UCP antisera was used to assess the interscapular BAT depot content of UCP. The interscapular depot is well circumscribed and represents the largest single collection of BAT in mice⁵. Interscapular UCP content was decreased modestly in UCP-DTA mice and markedly in UCP-176 mice (Fig. 1b,c). In 16-day-old UCP-DTA transgenic mice, UCP content was decreased by 68% whereas in

UCP-176 transgenic mice it was reduced by 96%. One difference between the UCP-DTA and UCP-176 lines is that the former has microphthalmia and cataracts but the latter does not; this suggests that the eye abnormality is related to the site of transgene integration and is unrelated to the ablation of BAT.

Total body lipid in 16-day-old mice was increased by 31% in UCP-DTA mice and by 68% in UCP-176 mice (Fig. 1d), thus

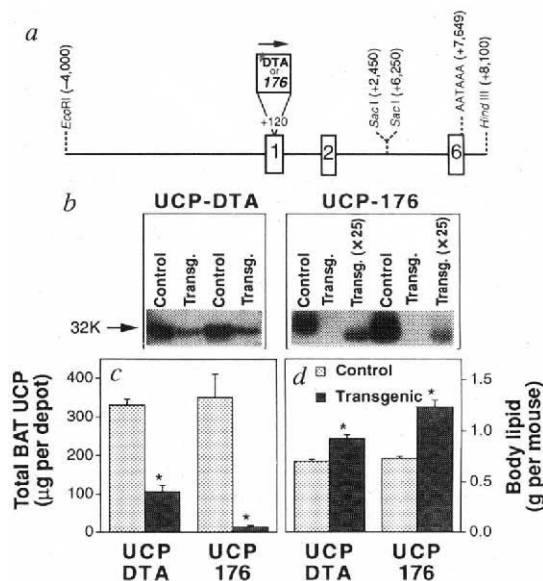


FIG. 1 a, UCP-DTA and UCP-176 transgenes. UCP-1 is a UCP minigene which has previously been shown in transgenic mice to be expressed exclusively in BAT²³. The genetic ablation vectors were constructed to include all of UCP-1 plus an additional 850 base pairs of 5'-distal UCP sequence. Full-strength DTA²⁴ or a 50-fold less toxic, attenuated mutant termed 176 (refs 25, 26), were inserted into the first exon of the UCP minigene at position +120 to create UCP-DTA and UCP-176, respectively. The entire vector was excised from the plasmid, gel purified and injected into the pronuclei of fertilized FVB/N mouse embryos (Taconic Labs, Gormantown, New York). Care of mice was in accordance with institutional guidelines. Numbers shown are relative to the UCP transcription start site and the arrow represents the orientation of DTA and 176 coding sequence. b–d, Sixteen-day-old UCP-DTA and UCP-176 mice. Heterozygous transgenic mice (Transg.) and their nontransgenic littermates (Control) were analysed at age 16 days, before weaning (UCP-DTA: control = 12 (7 female, 5 male), transgenic = 13 (7 female, 6 male); UCP-176: control = 8 (5 female, 3 male), transgenic = 7 (4 female, 3 male). Body weight of transgenic mice at age 16 days was not significantly different from controls (mean \pm s.e. = 7.3 ± 0.2 g). b, Immunoblotting of BAT homogenates for UCP. Each lane contains 1/2,000th of the total depot protein content except for lanes labelled ($\times 25$) where 25 times that amount was loaded. c, Quantification of UCP. Immunoblots of BAT interscapular homogenates were prepared with 5 µg of homogenate protein per lane and purified mouse UCP standards (25–1,000 ng) and analysed with a phosphor-imager. Total interscapular BAT depot UCP was determined by adjusting for total interscapular depot protein content. d, Total body lipid content. Lipid content was assessed using alcoholic potassium hydroxide digestion with saponification of all fats, neutralization and then enzymatic determination of glycerol^{22,27}. Body lipid was determined by multiplying mols of glycerol per mouse and the average relative molecular mass of a triglyceride molecule (860 g mol^{-1}). All values are shown as mean \pm s.e. (* $P < 0.01$).

METHODS. BAT was homogenized as previously described²⁸. Homogenate protein and purified mouse UCP standards were added to a 2% SDS loading buffer and then separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide). The proteins were transferred to nitrocellulose, blocked in 5% milk and probed with rabbit anti-hamster UCP antisera diluted 1:200. The bound antibody was detected with ¹²⁵I-labelled goat anti-rabbit IgG (NEN-Dupont). The blots were analysed using a phosphor-imager equipped with Image Quant software (Molecular Dynamics, Benton, New Jersey).

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TABLE 1 Summary of data obtained on older UCP-DTA mice

| | Total BAT UCP (μg per depot) | Body weight (g) | Total body lipid (g) | Food intake (g per week) | Serum analyses | | | |
|-------------------|---|--------------------|-------------------------|-----------------------------|-----------------|------------------|--|---------------------------------------|
| | | | | | Glucose (mM) | Insulin (pM) | Triglyceride (mg dl ⁻¹) | Cholesterol (mg dl ⁻¹) |
| Male (8 weeks) | | | | | | | | |
| Control (n=5) | 246 \pm 21 | 25 \pm 1 | 2.3 \pm 0.2 | 33 \pm 1 | 15 \pm 1 | 380 \pm 90 | 42 \pm 3 | 101 \pm 12 |
| Transgenic (n=5) | 32 \pm 2* | 39 \pm 1* | 8.8 \pm 0.9* | 53 \pm 2* | 24 \pm 2* | 1,910 \pm 800 | 60 \pm 43† | 143 \pm 9† |
| Female (8 weeks) | | | | | | | | |
| Control (n=13) | 330 \pm 34 | 20 \pm 1 | 1.4 \pm 0.1 | 31 \pm 1 | 13 \pm 1 | 270 \pm 50 | 78 \pm 8 | 75 \pm 3 |
| Transgenic (n=4) | 130 \pm 23* | 25 \pm 1* | 3.5 \pm 0.7* | 37 \pm 3† | 17 \pm 1* | 920 \pm 310* | 155 \pm 47* | 90 \pm 1† |
| Female (19 weeks) | | | | | | | | |
| Control (n=3) | 255 \pm 51 | 27 \pm 2 | 4.5 \pm 1.2 | — | 14 \pm 1 | 510 \pm 350 | 56 \pm 8 | 59 \pm 9 |
| Transgenic (n=3) | 74 \pm 12† | 52 \pm 2* | 28.1 \pm 2.1* | — | 29 \pm 4† | 5,690 \pm 260* | 155 \pm 19* | 138 \pm 23† |

Offspring of the UCP-DTA founder were weaned at age 2.7 weeks and housed separately in plastic cages at 24 °C with free access to food (Purina Formulab Chow 5008, 6.5% fat by weight). Transgenic mice and their control littermates were killed at the ages indicated. Food intake during the week before death is shown. Food was weighed at the beginning and end of each week and the differences were assumed to represent grams of food eaten per week. The cages were inspected carefully for spillage and none was noted. Total BAT UCP and total body lipid were determined as described in Fig. 2 legend. Serum glucose was assessed using an NADH enzyme-linked assay²² and serum insulin was determined using a rat insulin radioimmunoassay kit obtained from Linco Research, Inc. Serum cholesterol (procedure 339) and triglyceride (procedure 339) were quantified using kits obtained from Sigma. Because the triglyceride assay detects all serum glycerol (free and triglyceride derived), true serum triglyceride values were obtained by enzymatically assaying for free glycerol and adjusting the triglyceride value accordingly²². All values are shown as mean \pm s.e.

* $P < 0.01$; † $P < 0.05$.

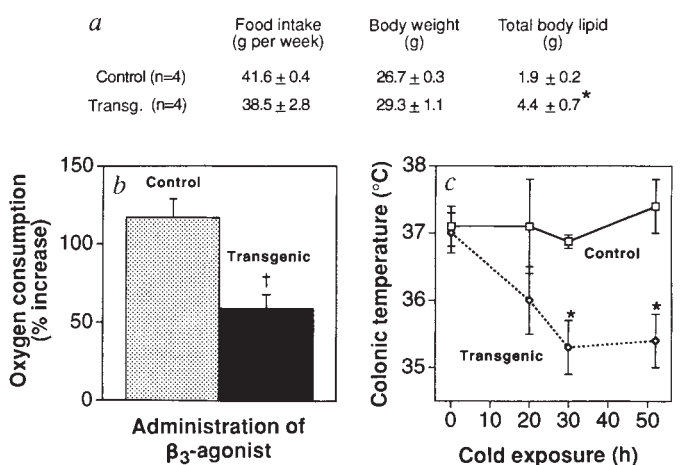
correlating with their relative deficiency of BAT. This degree of obesity at 16 days is comparable to that found in other murine models of genetic (*ob/ob*, *db/db*) or hypothalamic lesion (monosodium glutamate) induced obesity^{10,12}. As UCP-DTA mice age, they maintain their relative deficiency of BAT and obesity progresses (Table 1). By 19 weeks, UCP-DTA mice are extremely obese (>50% body fat). The two transgenic lines differ with respect to the status of BAT ablation over time. Whereas the UCP-DTA line has persistent BAT deficiency, the UCP-176 line has near complete regeneration of BAT by age 8 weeks (data not shown). Tissue regeneration following toxigen ablation has previously been observed and possible mechanisms have been discussed¹³. Regardless of its cause, whereas UCP-176 mice are extremely obese at 16 days, total body lipid stores have returned to normal by 8 weeks (data not shown). Regeneration of BAT followed by reversal of obesity provides compelling evidence that obesity in these mice is a consequence of BAT deficiency.

With advancing obesity, UCP-DTA mice develop several phenotypic abnormalities (Table 1); these include insulin resist-

ance as evidenced by hyperglycaemia and hyperinsulinaemia, hypertriglyceridaemia and hypercholesterolaemia. Corticosterone, which is elevated in nearly all genetic models of rodent obesity¹⁴, is normal in UCP-DTA mice (data not shown). Unexpectedly, UCP-DTA mice also develop hyperphagia, which begins at age 5–7 weeks and is uniformly observed in UCP-DTA mice over the age of 7 weeks. In the UCP-176 line, in which BAT regenerated, food intake was normal. As hyperphagia has so far been observed in only one line, it is possible that this phenotype is unrelated to ablation of BAT. One anatomical site where cellular ablation might cause hyperphagia is the hypothalamus¹⁴. To address this possibility, we have examined the brains of UCP-DTA mice histologically and found the appearance and volume of all hypothalamic nuclei to be normal. Additional evidence against a hypothalamic lesion is the fact the UCP-DTA mice are fertile (males and females) and grow normally (nasal anal length), processes that are sensitive to hypothalamic disturbance and impaired in other genetic models of rodent obesity¹⁴. Although provocative, the possibility of a

FIG. 2 Thermogenesis in heterozygous UCP-DTA mice. *a*, Total body lipid content of UCP-DTA mice before the development of hyperphagia. Male UCP-DTA mice and their control littermates were weaned at 2.7 weeks and group housed, two control and two transgenic mice per cage (four total per cage). At 5.5 weeks of age, they were separated and housed individually. Food intake was monitored over the following week after which the mice were killed and analysed for total body lipid content. *b*, Thermogenic response to CL 316,243 (CL), a β_3 -adrenoreceptor selective agonist. UCP-DTA (2 male and 8 female) and control (3 male and 4 female) littermates were weaned at age 2.7 weeks, then separated and housed individually at age 4.5 weeks. Mice were 62–69 days old at the time of CL administration. Male and female control mice responded similarly to CL. *c*, Colonic temperature during cold exposure. Female UCP-DTA ($n=6$) and control ($n=3$) littermates were weaned at age 2.7 weeks, individually housed at age 4.5 weeks (at 24 °C) and acutely exposed to cold to 4 °C at age 8 weeks. Colonic temperature was measured using a YSI model 43 telethermometer equipped with a series 500 probe (Yellow Springs Instrument Co., Inc., Ohio). All values are shown as mean \pm s.e. * $P < 0.01$; † $P < 0.05$.

METHODS. Oxygen consumption was measured with computerized equipment which included a 1-litre chamber maintained at 28 °C, an air flow of 500 ml per min (regulated with a mass flowmeter, Brooks Instrument Division, Emerson Electric) and an oxygen analyser (Beckman Industrial Oxygen Analyzer model 755)²⁹. Mice were awake and unrestrained for the study. The resting rate of oxygen consumption was assessed when the mice were curled up and still, usually 1–2 h



after they had been placed in the chamber. CL was dissolved in water (10 mg ml⁻¹) and diluted in saline immediately before injection (1 μg per g, s.c.). In preliminary experiments with other mice, this dose exerted maximum effects on oxygen consumption. After injection of CL, mice were awake but still stretched out and panting and a constant rate of oxygen uptake was achieved after about 1 h.

TABLE 2 Summary of data obtained from 26-week-old non-transmitting UCP-DTA founders

| | Sex | Status | Total BAT UCP (μg per depot) | Body weight (g) | Total body lipid (g) | Food intake (g per week) |
|--------------------|--------|-----------|---|--------------------|-------------------------|-----------------------------|
| Controls ($n=4$) | Male | — | 213 \pm 83 | 39 \pm 7 | 8 \pm 2 | 31 \pm 2 |
| UCP-DTA-2 | Male | Mosaic | 107 | 60 | 20 | 39 |
| UCP-DTA-3 | Male | Infertile | 83 | 65 | 26 | 39 |
| Controls ($n=6$) | Female | — | ND | 30 \pm 4 | 7 \pm 2 | ND |
| UCP-DTA-4 | Female | Mosaic | ND | 46 | 17 | ND |

All animals were weaned at age 3 weeks and the founder mice were mated with nontransgenic animals. Four male and six female nontransgenic mice were housed individually and used as controls. The male controls were littermates of founders UCP-DTA-2 and -3 and the female controls were age-matched animals studied at another time. UCP-DTA-2 and UCP-DTA-4 were presumed to be mosaic because each failed to produce transgenic offspring and UCP-DTA-3 was infertile. All animals were housed individually at age 17 weeks and killed at age 26 weeks. Food intake was assessed during the week before death. Total body lipid was determined as described in Fig. 2 legend. For the control groups, values are shown as mean \pm s.d. ND, not determined.

link between BAT deficiency and hyperphagia requires further study.

In addition to the above two lines, we have generated three non-transmitting UCP-DTA founders which were analysed at age 26 weeks (Table 2). These founders had diminished BAT as assessed by interscapular UCP content (not determined in UCP-DTA-4), were markedly overweight and had a large increase in total body lipid. Also, both UCP-DTA-2 and -3 were hyperphagic (not determined in UCP-DTA-4). The presence of brown fat deficiency, obesity and hyperphagia in these additional founder animals essentially rules out the possibility that these phenotypic features of the UCP-DTA line could be due to positional integration effects.

Because BAT is a site of thermogenesis, BAT deficiency might be expected to cause reduced energy expenditure, and experiments were performed to address this point (Fig. 2). When UCP-DTA mice were analysed before the development of hyperphagia (age 6 weeks), they were found to have increased total body lipid (Fig. 2a). The finding of increased energy stores in the absence of hyperphagia indicates that UCP-DTA mice have increased metabolic efficiency. Administration of β_3 -adrenoceptor agonists to rodents increases oxygen consumption by at least 100% (ref. 15). As β_3 -adrenoceptors are found predominantly in brown and white fat¹⁶⁻¹⁸, it has been suggested that this response is due primarily to activation of BAT. However, when CL 316,243, an extremely selective β_3 -adrenoceptor agonist¹⁹, was given to UCP-DTA mice, their thermogenic response was reduced by 50%, suggesting that these mice have a functional deficiency of BAT thermogenesis (Fig. 2b). Finally, when UCP-DTA mice were exposed to environmental cold, their core temperature gradually dropped by 1.7 °C, providing additional evidence of BAT dysfunction (Fig. 2c). The relatively small drop in body temperature is consistent with the partial BAT deficiency observed in UCP-DTA mice and with the view that brown fat mediates less than 40% of the thermogenic response to acute cold exposure²⁰, shivering being responsible for the remaining 60% (ref. 21).

The present study provides strong evidence that BAT plays a critical role in the regulation of energy balance in mice, and that BAT dysfunction can cause obesity. Also, the UCP-DTA mouse, by virtue of its normal fertility, linear growth and serum glucocorticoid levels, represents a valuable new model that may aid studies of the pathogenesis and treatment of human obesity and insulin resistance. □

Received 12 May; accepted 1 November 1993.

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ACKNOWLEDGEMENTS. We thank A.-L. Kates for UCP western blotting reagents and methodology, I. Maxwell for pIB1 130-DT-A and pIB1-176, R. Lechan for hypothalamic histology, J. Heck-enlively for ophthalmological expertise, J.-P. Flatt for help with carcass analysis, T. Claus (American Cyanamid Co.) for CL 316,243, the National Research Council of Canada for the computerized equipment for measuring oxygen uptake, K. Herzberg of the Beth Israel Transgenic Facility for help with the generation of transgenic mice, and B. Spiegelman for critically reading our manuscript. This work was supported by grants from the NIH to B.B.L., J.S.F. and L.P.K., Miles, Inc. to B.B.L. and J.S.F. and the MRC of Canada to J.H.-H. This paper is dedicated to the memory of Patricia Usher.

A role for Fyn tyrosine kinase in the suckling behaviour of neonatal mice

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NON-RECEPTOR-TYPE tyrosine kinases of the Src family, such as Src, Yes and Fyn, are strongly expressed in the brain and have been suggested to have an important function in the central nervous system¹⁻⁵. We generated Fyn-deficient mice by inserting the β -galactosidase gene (*lacZ*) into the *fyn* gene. The homozygous Fyn-mutant neonates from homozygous Fyn-deficient parents died because of a suckling problem. Neonates were, however, able to suckle milk normally when the homozygous mother's mammary glands had been activated by suckling of a heterozygous or wild-type pup. In these homozygous pups, the modified glomerular com-

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