Emergence during development of the white-adipocyte cell phenotype is independent of the brown-adipocyte cell phenotype

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In mammals, two types of adipose tissue are present, brown and white. They develop sequentially, as brown fat occurs during late gestation whereas white fat grows mainly after birth. However, both tissues have been shown to have great plasticity. Thus an apparent transformation of brown fat into white fat takes place during post-natal development. This observation raises questions about a possible conversion of brown into white adipocytes during development, although indirect data argue against this hypothesis. To investigate such questions *in vivo*, we generated two types of transgenic line. The first carried a transgene expressing Cre recombinase specifically in brown adipocytes under the control of the rat UCP1 promoter. The second corresponded to an inactive *lacZ* gene under the control of the human cytomegalovirus promoter. This dormant gene is inducible by Cre because it contains a Stop sequence between two *loxP* sequences, separating the promoter from the coding sequence. Adipose tissues of progeny derived by crossing independent lines established from both constructs were investigated. LacZ mRNA corresponding to the activated reporter gene was easily detected in brown fat and not typically in white fat, even by reverse transcriptase PCR experiments. These data represent the first direct experimental proof that, during normal development, most white adipocytes do not derive from brown adipocytes.

Key words: adipose tissue, Cre/loxP, lineage, plasticity, transgenic.

INTRODUCTION

Two functionally different types of fat are known in mammals: brown adipose tissue (BAT), containing specialized thermogenic brown adipocytes, and white adipose tissue (WAT), containing white adipocytes specialized in triglyceride metabolism [1]. Both tissues are strongly involved in energy balance but their respective functions are quite different. Brown fat participates in cold- as well as diet-induced thermogenesis whereas white fat is mainly involved in energy storage. The thermogenic capability of brown fat is due to the presence of a mitochondria-uncoupling protein, UCP1, which is considered unique and specific to brown adipocytes [2]. Growth and development of these tissues have been studied extensively *in vitro* and some fundamental molecular mechanisms leading to adipose cell differentiation have been described [3–5]. Nevertheless, few data except old histological investigations are available regarding *in vivo* adipose-tissue development during embryogenesis and after birth [6]. The developmental patterns of both fat tissues are quite different. In most mammals, brown fat develops during fetal life and possesses all the features of mature tissue at birth when the requirements for non-shivering thermogenesis are greater than at any other time of life [7]. On the contrary, WAT development takes place after birth and its mass considerably increases during post-natal life, particularly after the animal has suckled, to reach a great percentage of body mass in animals or humans. The exact relationships between brown and white fat during development remain an open question. At the present time, the view of brown and white fat as distinct tissues is only supported by different indirect arguments: (i) the anatomical location of macroscopically identified brown fat is different from that of white fat [8], and (ii) *in vitro* precursor cells isolated from brown or white fat primarily differentiate into brown and white adipocytes respectively [9–11]. In fact, no previous arguments are conclusive. The presence of brown adipocytes can be detected among all white fat pads and their proportions differ according to the localization of the deposit, the ambient temperature and the species [12–14]. During post-natal development, the transformation of typical brown fat into white fat can occur rapidly in numerous species and deposits, suggesting a possible transformation of brown into white adipocytes [15,16]. In adult mice as well as in adult dogs, all white deposits can be rapidly and massively converted into brown fat [13,17]. We proposed the term plasticity to describe and summarize all of these properties [18]. However, an exhaustive and complete study investigating transient expression of UCP1 during white-fat differentiation has never been conducted.

In order to get direct proof of the exact relationship between brown and white fat during development, we developed a cell-lineage approach. This approach took advantage of the *Cre/loxP* DNA-recombination system, which permitted us to achieve spatially and temporally regulated somatic mutations [19]. The expression of the Cre recombinase catalyses an intramolecular recombination reaction from two *loxP* sequences located in tandem in the same orientation, resulting in the deletion of the *loxP*-flanked sequence. If the presence of this *loxP*-flanked sequence prevents expression of a reporter gene, even under the control of a constitutive promoter, the deletion will definitely induce the expression of the dormant gene [20,21]. Its expression will tag all the cells that express the Cre enzyme, even if they do so in a transient manner. We used a promoter region derived from the rat *UCP1* gene that is expressed specifically in brown

Abbreviations used: BAT, brown adipose tissue; CMV, cytomegalovirus; RT-PCR, reverse transcriptase PCR; WAT, white adipose tissue.

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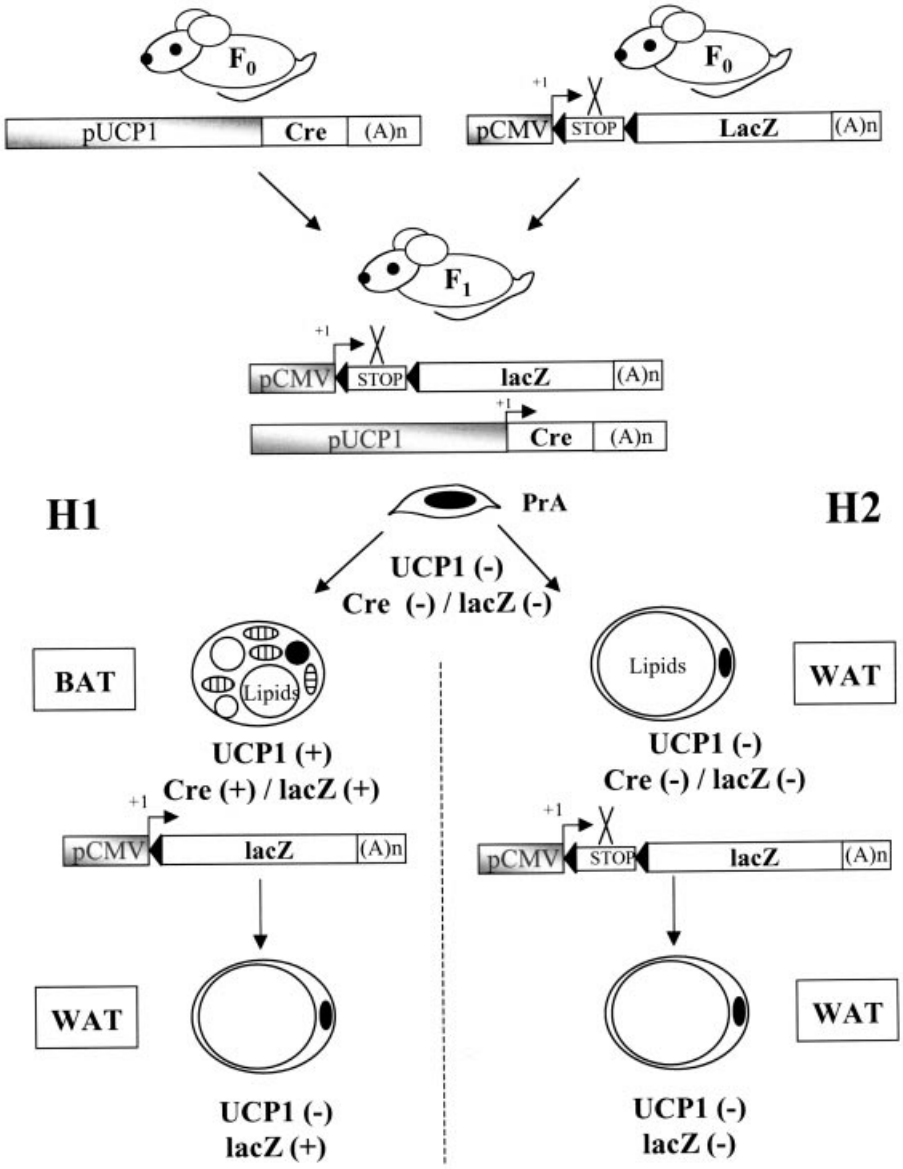

Figure 1 Principle of brown adipocyte lineage

Two types of transgenic animal were obtained. pUCP1-*Cre* animals (top left) were produced by injection of a construct corresponding to 4.55 kb of the rat UCP1 promoter region (pUCP1) fused to Cre recombinase cDNA (Cre). This promoter specifically monitors Cre expression in brown adipocytes of transgenic mice. pCMV-Stop-*lacZ* mice (on the right) carried an inactive *lacZ* reporter gene under control of the human CMV promoter, containing a 1.3 kb Stop sequence, which is sensitive to deletion by Cre excision and which blocks transcription of the pCMV-Stop-*lacZ* transgene. Adipose tissues were analysed for LacZ expression in double-transgenic lines obtained by crossing both types of line. We tested two hypotheses of adipocyte development from a pre-adipocyte (PrA) with a fibroblastic phenotype. Such a precursor, which will not express UCP1 or the Cre enzyme, is LacZ-negative. Hypothesis 1 (H1): during development, white unilocular adipocytes exist as brown multilocular adipocytes before acquisition of their definitive phenotype. The expression of UCP1 due to the brown phenotype activates Cre expression driven by the UCP1 promoter and this expression leads to the deletion of the Stop sequence and the expression of LacZ, which can be detected. Afterwards, if conversion of brown into white adipocyte occurs during development, UCP1 disappears whereas LacZ expression persists. Hypothesis 2 (H2): brown and white adipocyte lineages are distinct during normal development. White adipocytes never express UCP1, which is an indicator of the brown phenotype. No recombination event can occur and the dormant reporter gene is never expressed. A(n), metallothionein poly(A)$^+$ sequence.

adipocytes to monitor Cre recombinase expression in transgenic animals. Then we developed other transgenic lines corresponding to an inactive reporter *lacZ* (bacterial β-galactosidase) gene under control of the human cytomegalovirus (CMV) promoter containing a 1.3 kb Stop sequence, sensitive to deletion by Cre excision, and crossed both types of line. The detection or not of *lacZ* gene expression in white fat would demonstrate the presence (hypothesis 1, Figure 1) or the lack (hypothesis 2, Figure 1) of a transient brown-fat phenotype during white-adipocyte differentiation during development.

MATERIALS AND METHODS

Construction of transgenes and generation of transgenic mice

The pSP73.45 plasmid containing the UCP1 promoter was kindly

provided by Ricquier et al. [22]. The *Cre* coding sequence followed by the metallothionein poly(A)$^+$ sequence was isolated from plasmid pBS185 (Gibco-BRL) and cloned into the *Xho*I and *Hin*dIII sites of the pBSKS+ plasmid (Stratagene; the plasmid was named pBS-Cre). A *Sac*II-*Pvu*II-*Pst*I-*Kpn*I-*Not*I linker was inserted into the *Sac*II/*Not*I restriction sites of pBS-Cre and the 2900 bp *Kpn*I fragment of pBS-Cre was cloned into the *Kpn*I restriction site of pSP73.45. This plasmid was designated pUCP1-Cre.

For the dormant reporter-transgene CMV construct, the *loxP*-Stop-*loxP* sequence was isolated from plasmid pBS302 (Gibco-BRL) and cloned into the *Eco*RI and *Not*I sites of the pBSKS+ plasmid (to create pBS-Stop). A *Sac*II-*Bam*HI-*Sma*I-*Sal*I-*Not*I linker was inserted into the *Sac*II/*Not*I restriction sites of pBS-Stop and the 1500 bp *Sal*I fragment of pBS-Stop was cloned into the *Xho*I restriction site of pCMV (Clontech). Before injection, DNA was linearized and plasmid sequences were removed. The injection into the pronuclei of fertilized mouse eggs and subsequent steps to obtain the parental transgenic mice were performed by the Service d'expérimentation animale et de transgénèse (CNRS, Villejuif, France).

The genotyping of mice was performed by PCR with *Taq* polymerase purchased from Promega. For this purpose, DNA (50 ng) was subjected to 35 amplification cycles (30 s at 94 °C, 30 s at 53 °C for pUCP1/*Cre* primers and 55 °C for pCMV/Stop primers) on a 9600 thermal cycler (Perkin-Elmer). The 5′ and 3′ primers used for detection were: for the pUCP1-Cre fusion, pUCP1 (5′-CAGGGCTTTGGGAGTGACG-3′) and Cre (5′-AGCCCGGACCGACGATGAAG-3′), for the pCMV-Stop-*lacZ* fusion, pCMV (5′-AGTGAACCGTCAGATCGCCT-3′) and Stop (5′-TTATGGCTGAACTGAGCGAA-3′), and for the detection of the actin gene, actin-1 (5′-ATCGTGGGCCGCCCTAGGCACCA-3′) and actin-2 (5′-TTGGCCTTAGGGTTCAGAGGGG-3′). The sizes of predicted reverse transcriptase PCR (RT-PCR) products were 668, 499 and 540 pb for pUCP1-Cre, actin and pCMV-Stop-*lacZ* transgenes respectively.

Analysis of the transgenic animals

The animals were housed in a controlled environment (12 h light/dark cycle, 21 °C) with free access to water and standard chow diet. After CO_2 anaesthesia, the mice (12–14 weeks old) were killed by cervical dislocation. Tissues (liver, muscle, inguinal white fat, interscapular brown fat) were removed immediately and dissected carefully. Samples were rapidly frozen in liquid nitrogen and stored at −80 °C until RNA analysis. Total RNA was extracted from various tissues by the guanidinium thiocyanate procedure as described previously [23]. Total RNA (1 μg) was reverse transcribed using SuperscriptII reverse transcriptase (Gibco-BRL) and then subjected to 35 amplification cycles of PCR (30 s at 94 °C, 30 s at 55 °C). The primers used were Cre-F (5′-TCACTGGTTATGCGGCGGAT-3′), Cre-E (5′-TGCCCCTGTTTCACTATCCA-3′), lacZ-1 (5′-GTCGTTTTACAACGTCGTGACT-3′), lacZ-2 (5′-GATGGGCGCATCGTAACCGTGC-3′), actin-1 (5′-ATCGTGGGCCGCCCTAGGCACCA-3′) and actin-2 (5′-TTGGCCTTAGGGTTCAGAGGGG-3′) for the detection of LacZ, Cre and actin mRNAs respectively. The sizes of predicted RT-PCR products were 633, 273 and 244 bp for Cre, UCP1 and actin mRNAs respectively. RT-PCR products were loaded and electrophoresed on a 2% agarose gel together with a 1 kb ladder (Eurogentec). After migration, the gel was stained with ethidium bromide and observed on a UV transilluminator. The specificity of *lacZ* PCR product was confirmed by cloning and sequencing.

RESULTS

The pCMV-Stop-*lacZ* dormant reporter construct was designed to contain the 1.3 kb Stop fragment with *loxP* sequences, inserted between the CMV promoter and *lacZ* gene. The pUCP1-Cre construct contained the 4.5 kb rat UCP1 promoter fused to the cDNA coding for Cre recombinase. Transgenic mice were produced by injecting separately both transgenes as a linear fragment into the pronuclei of fertilized mouse eggs that were then transferred to oviducts of pseudopregnant foster mothers. Three independent transgenic mouse founder lines harbouring each construct were obtained. No morphological abnormalities among founders with both transgenes were observed. Of the three founder lines carrying the dormant pCMV-Stop-*lacZ* construct, one of them failed to efficiently reproduce and was not used. Reproduction parameters were not statistically different between the other different trangenic lines although a tendency of lower fertility in pUCP1-Cre animals compared with pCMV-Stop-*lacZ* mice was observed.

RT-PCR experiments and an *in vitro* test ensured the validity of the pCMV-Stop-*lacZ* transgenic lines. RT-PCR experiments on RNA from BAT as well as white fat of pCMV-Stop-*lacZ* mice failed to detect any signal corresponding to *lacZ* or to the Stop-*lacZ* fragment (results not shown). This demonstrated the efficiency of the Stop sequence to block transcription and no detectable transcription was associated with the transgene. To test the efficiency of our construct, we performed primary culture of cells from animals of each line and transfected them with an expression plasmid corresponding to the Cre coding sequence fused to the promoter of CMV. Blue cells can be distinguished only after transfection by the expression vector compared with empty vector.

The specific pattern of Cre expression driven by the UCP1 promoter was tested by RT-PCR experiments in different tissues. As shown in Figure 2, a signal corresponding to Cre RNA was obtained in RNA from interscapular BAT. Under these conditions, no signal was observed in RNA from other tissues, such as muscle, liver or inguinal WAT, although all of them were positive for actin RNA amplification. The single band at 244 bp detected in these last experiments corresponded to coding sequence and demonstrated no DNA contamination.

Double-transgenic offspring harbouring both the CMV-Stop-*lacZ* and UCP1-*Cre* transgenes were easily identified on the basis

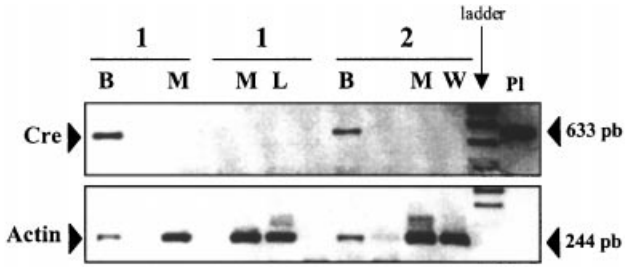

Figure 2 Representative Cre expression profiles in pUCP1/*lacZ* transgenic lines

The specificity of Cre expression driven by the rat UCP1 promoter was tested in interscapular BAT (B), muscle (M), liver (L) and inguinal WAT (W) of two offspring mice from two independent crossings (1 and 2). RT-PCR experiments were performed with specific primers for Cre and actin mRNAs (detection of the latter was used as a positive control). A plasmid containing Cre cDNA (PI) was used as a positive control for Cre cDNA amplification. Positive signals for Cre RNA were only detected in RNA from brown fat whereas signals for actin were obtained in all tested RNA samples.

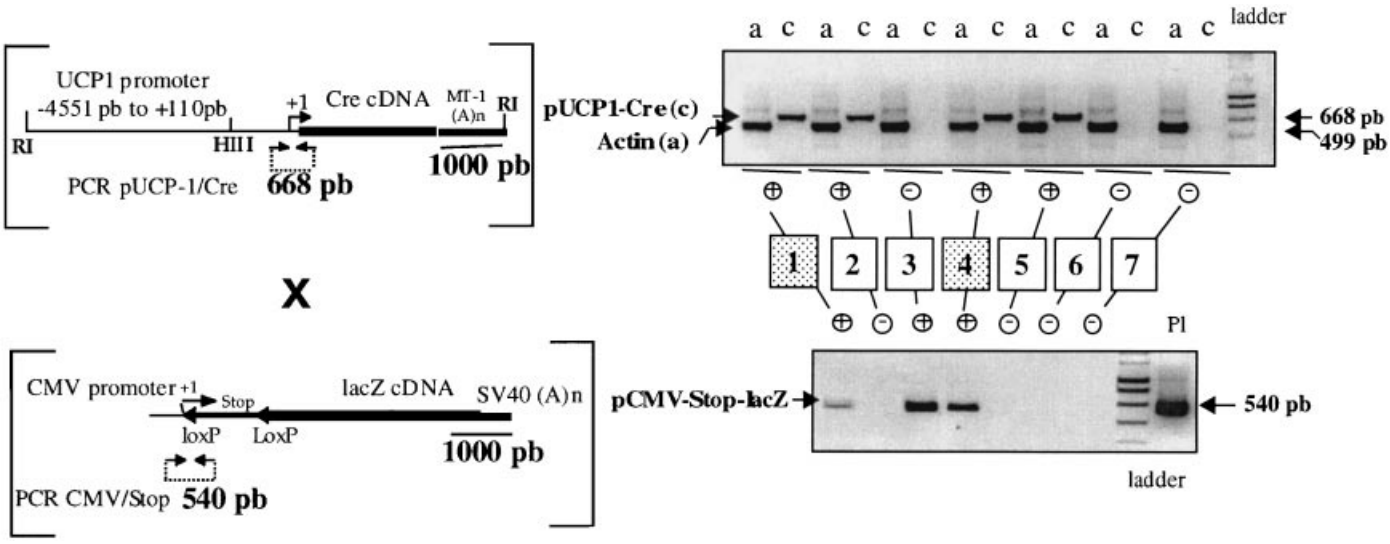

Figure 3 Selection of positive double-transgenic mice

Genomic DNA was purified from the tails of offspring and PCR experiments with specific primers were performed to detect both constructs. Actin gene amplification was used as a positive control (upper panel, lanes a, PCR fragment at 499 bp). PCR products were separated and stained on agarose gel with a size ladder to determine their respective sizes. The figure is representative of one litter. Of seven mice, four animals (mice 1, 2, 4 and 5) were positive for the pUCP1-Cre construct (upper panel, lines c, PCR fragment at 668 bp), three (mice 1, 3 and 4) were positive for the pCMV-Stop-*lacZ* construct (lower panel, PCR fragment at 540 bp), and two (mice 1 and 4) for both transgenes. MT-1 A(n), metallothionein poly(A)$^+$ sequence; RI, *Eco*RI.

of their genotypes, as confirmed by PCR (Figure 3). UCP1-*Cre* transgenic males mated inefficiently and had reduced fertility. For these reasons, in most cases, UCP1-*Cre* transgenic females were mated with a male harbouring the other construct.

In male and female double-transgenic mice, RT-PCR experiments were performed to detect RNA coding for LacZ. A positive signal at the expected band (273 bp) could be amplified in all double-transgenic brown-fat samples, whereas no equivalent PCR fragment appeared in inguinal fat (Figure 4A). Similar results were obtained with epididymal fat (results not shown). This signal revealed the presence of LacZ mRNA because no signal was observed when the reverse transcriptase was omitted (results not shown). Actin mRNA detection in all samples was performed as a positive control in the RT-PCR. When different adipose tissues were compared (Figure 4B), a gradient based on LacZ detection can be established from interscapular adipose tissue to inguinal fat with peri-renal, then retro-peritoneal adipose tissues as intermediates. In this last tissue, LacZ detection is very faint and cannot be detected in all animals. Whatever the tissue, no signal corresponding to a putative Stop-*lacZ* transcript was amplified (results not shown).

DISCUSSION

The *Cre*/*loxP* system is a powerful tool with which to target any gene recombination [21,24]. This technique is mostly used to specifically monitor a particular gene knockout in one tissue and investigate the physiological consequences at the whole-organism level [25]. The activation of a silent reporter transgene induced by Cre expression driven by a specific promoter can also be achieved [20]. This was previously described in adipose tissues [26,27]. As the Cre-mediated excision is cell-heritable, the marked cells and all their progeny should express the reporter gene at later stages, even after Cre is no longer expressed. In developmental studies, transient expressions of the gene in any cellular lineage or at the whole-organism level during physiological or physiopathological processes can be investigated using this technique. When the expression of these genes is closely linked to a particular cell type, the resulting data inform us about the lineage derived from this cell type [28,29].

In adipose tissues, this question is relevant concerning the relationship between brown and white fat tissue. Both phenotypes have numerous points in common, but their respective developments have opposite physiological roles in energy balance and associated disorders [1]. Up to now, the single and best-characterized means to clearly distinguish both cell types is the detection of *UCP1* gene expression [2,30]. This gene has been described as specific and unique to brown fat cells and its ectopic expression in non-brown fat cells induces a regulated mitochondrial uncoupling, unique to brown fat cells [2,31]. The promoter of the rat gene has been cloned and characterized using transgenic animals [22,32]. The 4.5 kb sequence contained all the elements necessary for brown-fat-cell-specific expression in transgenic mice. Two independent transgenic lines of the construct corresponding to Cre cDNA fused to this promoter have been used in this study. As expected, the Cre transcript can be easily detected in brown fat compared with white fat. Similar results were observed whatever the crossing between the different types of transgenic line. This excludes any artifact due to a positional effect of the transgene. To investigate a *UCP1*-positive cell lineage, the CMV promoter was used to drive a Cre-inducible reporter gene. The *in vitro* controls that we performed demonstrated that the construct was functional. Indeed, the inhibitory Stop sequence was removed and the silent *lacZ* gene was definitively activated.

In the double-transgenic mice, investigations at the mRNA level reveal that the induction of LacZ expression is due to Cre expression in brown fat. In all crossings, the same pattern was observed; LacZ mRNA can be easily detected in typical brown fat but not in inguinal or epididymal white fat. The absence of LacZ mRNA expression in white fat compared with brown fat demonstrates clearly that the majority of white adipocytes never

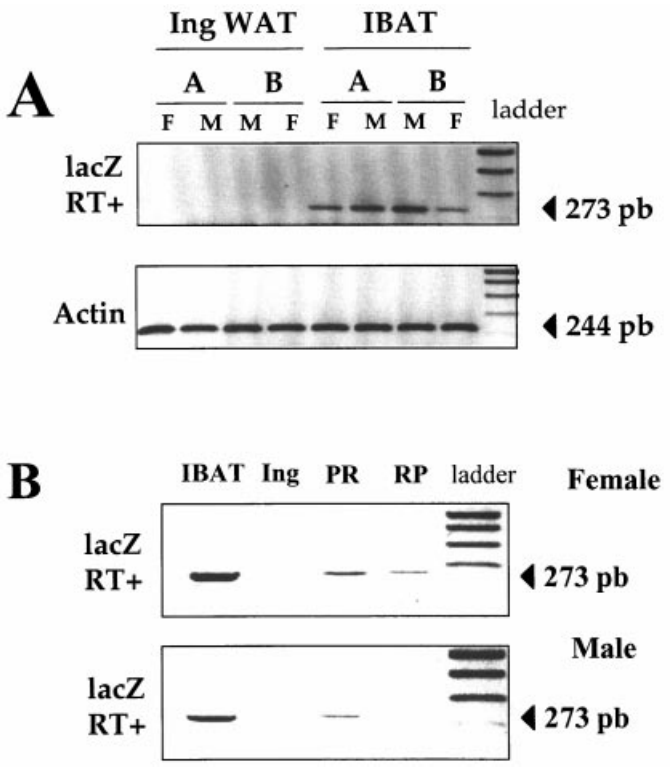

Figure 4 Representative pattern of *lacZ* gene expression in adipose tissues of double-transgenic animals

(**A**) In interscapular BAT (IBAT) of all double-transgenic mice, an RT-PCR fragment at 273 bp revealed *lacZ* gene expression (upper panel). Amplifications of actin mRNA (lower panel, RT-PCR fragment at 244 bp) were performed as positive controls. One male (M) and one female (F) of two independent crossings (A and B) are shown. Positive signals for LacZ mRNA were only detected in RNAs from brown fat whereas signals for actin were obtained in all tested RNA samples. Ing wat, inguinal WAT. (**B**) Representative pattern of LacZ mRNA detection in different adipose tissues of female and male double-transgenic mice. Whatever the animals, a gradient of LacZ expression can be observed. The strongest expression is detected in interscapular BAT. Lower signals were observed in peri-renal fat (PR), then in retro-peritoneal fat (RP) when a signal is detected. No LacZ mRNA was amplified in inguinal WAT (Ing).

express the *UCP1* gene. Because UCP1 expression is now considered as the unequivocal signature of the brown-adipocyte phenotype, the clear-cut difference between these adipose tissues reveals that no transient brown-adipocyte stage occurs during the emergence of white fat cells. Faint signals can be observed in peri-renal and sometimes in retro-peritoneal adipose tissues. The pattern of this expression can be explained easily when the heterogeneity of adipose tissue is considered. Indeed, scattered brown adipocytes are present in different fat that looks similar to white fat [13,33]. Their numbers vary according to localization and their presence is linked strictly with LacZ detection [13]. These results give further support to the independence of the white- and brown-adipocyte phenotypes during terminal differentiation.

Although indirect evidence agrees with the general conclusion [10,34,35], this work represents the first direct proof that, *in vivo*, most white adipocytes emerge independently from the brown adipocyte lineage during physiological development, and validate hypothesis 2 (Figure 1). This clearly demonstrates distinct lineages of brown and white adipocytes, at least during the acquisition of their respective phenotypes. When their precursors diverge, and whether trans-differentiation events between the mature adipocyte phenotypes exist, remain open questions which need further investigation to definitively establish the exact relationships between each lineage [18].

We thank S. Raimbault, F. Bouillaud, A. M. Cassard-Doulcier and D. Ricquier for kindly providing the promoter of the rat *UCP1* gene, D. Larrouy for discussion at the initiation of the project and B. Phillips for critical reading of the manuscript. We thank P. Guillou and J. Hillat for technical assistance, A. Belliure for secretarial work, and Ms Leuillet (Service d'expérimentation animale et de transgénèse, CNRS, Villejuif, France), and J. C. Couloumiers and Y. Barreira from the IFR31 husbandry service for the care of double-transgenic mice.

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Received 7 November 2000/5 February 2001; accepted 13 March 2001