Differential gene expression in white and brown preadipocytes

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1German Institute of Human Nutrition in Potsdam, 14558 Bergholz-Rehbrücke, Germany; 2Department of Biology, Philipps University, 35043 Marburg, Germany; and 3State Institute for Quality Control of Agricultural Products (RIKILT), Department of Safety and Health of Food, 6700 AE Wageningen, The Netherlands

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Boeuf, Stéphane, Martin Klingenspor, Nicole L. W. van Hal, Tatjana Schneider, Jaap Keijer, and Susanne Klaus. Differential gene expression in white and brown preadipocytes. Physiol Genomics 7: 15–25, 2001. First published August 8, 2001; 10.1152/physiolgenomics.00048.2001.—White (WAT) and brown (BAT) adipose tissue are tissues of energy storage and energy dissipation, respectively. Experimental evidence suggests that brown and white preadipocytes are differentially determined, but so far not much is known about the genetic control of this determination process. The aim of this study was to identify differentially expressed genes involved in brown and white preadipocyte development. Using representational difference analysis (cDNA RDA) and DNA microarray screening, we identified four genes with higher expression in white preadipocytes (three different complement factors and 6-6 fatty acid desaturase) and seven genes with higher expression levels in brown preadipocytes, of which three are structural genes implicated in cell adhesion and cytoskeleton organization (fibronectin, α-actinin-4, metarginidin) and four that might function in gene transcription and protein synthesis (vigilin, neadin, snRNP polypeptide A, and a homolog to human hepatocellular carcinoma-associated protein). The expression profile of these genes was analyzed during preadipocyte differentiation, upon β-adrenergic stimulation, and in WAT and BAT tissue in vivo compared with references such as peroxisome proliferator-activated receptor-γ (PPARγ), uncoupling protein 1 (UCP1), cytochrome c oxidase.

Differential gene expression in white and brown preadipocytes which involves sequential activation of numerous transcription factors from several families like different members of the CCAAT/enhancer binding proteins (C/EBP) and peroxisome proliferator-activated receptors (PPAR) (1, 15, 30, 44, 52). However, most of these studies focused on differentiation of white preadipocytes using established white preadipocyte cell lines such as 3T3-L1 and 3T3-F442A cells (37). One of the remaining questions is how and at which stage of development the differentiation of BAT vs. WAT is regulated, of which very little is currently known. Brown and white adipocytes show distinct morphological and biochemical phenotypes in vivo (9). When differentiated in vitro, brown adipocytes show a higher respiratory capacity than white adipocytes and express the BAT specific uncoupling protein 1 (UCP1), which is considered to be a marker for brown adipocytes (21). It is still not clear whether BAT and WAT derive from the same adipose precursor cells or arise independently from distinct mesenchymal stem cells (44), although recently PGC-1, a coactivator of PPARγ, has been identified, which induces genes important in the development of brown adipocyte phenotype (41).

We have performed parallel cultures of stromal vascular fraction isolated from WAT and BAT where we observed distinct phenotypes in WAT and BAT cultures even under identical culture conditions (21, 22, 25). We therefore hypothesized that at some time point after commitment of multipotent stem cells toward the adipose lineage a further commitment occurs to either white or brown adipocyte lineage. However, at what point this occurs and what the underlying molecular mechanisms are is still completely unknown (21, 22, 23). Also, so far brown and white preadipocytes in culture cannot be discriminated either morphologically, biochemically, or through marker genes. The aim of this study was therefore the identification of genes that are differentially expressed in white and brown preadipocytes. To identify these genes, representational difference analysis (RDA) (19, 26) was applied to semiconfluent brown and white preadipocytes raised in primary cultures. RDA is a sensitive and efficient polymerase chain reaction (PCR)-based subtraction method that has recently been employed to identify...

WHITE AND BROWN ADIPOSE TISSUES represent counter actors in energy partitioning, channeling lipid energy either to accumulation in white fat (WAT) or to oxidation, i.e., dissipation in brown fat (BAT) a highly thermogenic tissue (23). Throughout the last years considerable progress has been made in elucidating the molecular mechanisms of adipocyte differentiation...
genes specifically expressed in visceral adipose tissue of obese, diabetic rats in contrast to diabetes-resistant rats (18). Using RDA in two directions, i.e., with cDNA derived from white preadipocytes subtracted from brown preadipocytes and the other way around, over 250 cDNA fragments were cloned. For screening of differentially expressed genes these clones were spotted on DNA microarrays and hybridized with cDNA from either white or brown preadipocytes. Microarray screening revealed 11 genes of which 4 showed higher expression in white preadipocytes and 7 showed higher expression in brown preadipocytes. Their expression was furthermore analyzed during preadipocyte differentiation, upon β-adrenergic stimulation, and in WAT and BAT in vivo.

MATERIALS AND METHODS

Animals. Djungarian (also termed Siberian) dwarf hamsters (Phodopus sungorus) were obtained from our own breeding colony. Animals were kept at 23°C (thermoregulat-

ity) in long photoperiod (18:6, light:dark). Hamsters aged 4–6 wk were used for primary cell cultures. For tissue preparation animals were anesthetized with CO₂ and killed by cardiac puncture. P. sungorus is a widely used animal model in the study of BAT thermogenesis because it shows very high cold-induced, adaptive increase in BAT thermogenesis and abundant BAT. Four-week-old dwarf hamsters have about 1 g of BAT, a mouse less than 0.1 g. To our knowledge it is also the only model system for which parallel cultures of white and brown preadipocytes are established and well characterized (21, 22, 25). As possible disadvantage of this model is the fact that much more DNA sequence data are available for mouse and rat. However, cDNA sequences obtained so far show very high homology to other rodent sequences (51).

Primary cell culture. Inguinal WAT, which represents the largest subcutaneous fat depot in the young hamster, and several subcutaneous depots of BAT (axillar, suprasternal, interscapular, dorsocervical) from Djungarian hamsters were used for parallel primary cell cultures of white and brown preadipocytes. Fat depots of four to six animals were pooled for every culture to minimize possible artifacts resulting from differences between different fat depots and individual differences. In our experience, in vivo, differences between individuals tend to be higher than differences between different BAT depots.

The stromal-vascular fraction, containing the preadipo-
cytes, was obtained after collagenase treatment as described before (24). Cells were inoculated in petri dishes at 1,500–2,000 cells/cm². Cells were grown at 37°C in air with 5% CO₂ content and 100% relative humidity in cell culture medium (50% modified Eagle’s medium [GIBCO-BRL] and 50% Ham’s F12 medium [GIBCO-BRL]) supplemented with NaHCO₃ (1.2 g/l), biotin (4 mg/l), calcium panthotenate (2 mg/l), glutamine (5 mM), glucose (4.5 g/l), and HEPES (15 mM, pH 7.4), and penicillin G (6.25 mg/l) and streptomycin (5 mg/l). The medium was supplemented with 10% FCS (BioWhit-taker). Medium was changed at days 1 and 3. Semiconfluent preadipocytes were harvested at day 4, and confluent preadipocytes were harvested at day 6. To induce differentiation, insulin (17 nM, Sigma) and triiodothyronine (1 nM, Sigma) were added, and FCS supplement was reduced to 7%. For chronic β-adrenergic stimulation, isoproterenol (10 μM, Sigma) was added at the start of differentiation. This concentration has been shown to be maximally effective for long-term stimulation of UCP1 expression, whereas acute effects could be seen at much lower concentrations (24, 25). Differentiating adipocytes (i.e., cells treated with insulin) were harvested at day 6, mature adipocytes, with or without isoproterenol treatment, at day 10.

RNA isolation. Total RNA from cultured cells and tissue was extracted using the single-step acid phenol-guanidine protocol (7). All RNAs from cultured cells used for RDA or array hybridization were pooled from at least two different cell cultures. RNA from the WAT of 16 adult animals and from the BAT of 18 adult animals was pooled to obtain tissue RNA.

cDNA RDA. All of the following steps in the RDA protocol were carried out in parallel with semiconfluent preadipocytes from WAT and BAT. RNA, 200 μg, was used for each type of preadipocytes, originating from two separate cell cultures. RNA from preadipocytes was digested with RNase-free DNase (Promega) to remove eventual traces of genomic DNA. mRNA was isolated by poly(A)⁺ selection using magnetic beads (PolyATract System IV, Promega) and double- stranded cDNA was synthesized (cDNA-synthesis-system, GIBCO-BRL). The cDNAs were digested by DpnII, and RNA was applied to them as described (31, 19, 26), subtracting white from brown cDNAs and the other way around.

The first difference product (DP1) was generated by subtracting one cDNA pool from the other at a ratio of 1:100 (tester:driver) followed by selective PCR amplification of DP1. The DP1 was then subjected to two more rounds of subtractive hybridization applying tester:driver ratios of 1:400 and 1:16,000 for DP2 and DP3, respectively. Prior to ligation of new adapters DPs were gel purified (QiAquick Gel Extraction kit, Qiagen).

DP2 and DP3 (size range of 200 to 900 kb) were separated on a 3% agarose gel (Metaphor agarose, FMC), cut into 11–12 size fractions, and extracted from the gel slices (QiAquick Gel Extraction kit, Qiagen) to reduce redundancy of highly abundant cDNAs and avoid bias toward small fragments in the subsequent cloning step. The fractions were ligated into a TA cloning vector (pGEM-T, Promega), transformed into Escherichia coli DH5-α and plated on LB-ampicillin agar. Thereby, we obtained 12 cDNA libraries for brown preadipocytes and 11 for white preadipocytes.

Northern blots. For Northern blots, total RNA was separated by electrophoresis in a 1.2% agarose gel containing formaldehyde and blotted by capillary transfer to a nylon membrane (Hybond N, Amersham). The blots were probed with 32P-labeled cDNA inserts found represented in the DPs in a hybridization solution containing sodium phosphate (0.5 M), EDTA (1 mM), SDS (7%), and BSA (1%) at 42°C over night, and washed twice with 2× SSC/0.1% SDS for 10 min at room temperature, twice with 0.1× SSC/0.1% SDS for 20 min at 42°C, and twice with 0.1× SSC/0.1% SDS for 20 min at 68°C. A phosphor-imager (BA2000, Fuji) was used for analysis and quantitation of radiolabeled signals.

Microarray manufacturing. We chose 256 cloned DPs from the cDNA libraries and 47 reference clones with established functional relevance to adipocyte development (Research Genetics, http://www.resgen.com, vector: pTT7T3-D-P with modified polylinker) for arraying. In addition, as positive and negative controls, luciferase and Salomonella cDNAs were arrayed. A set of luciferase clones, the 5’ end, the middle part, the 3’ end, as well as the complete luciferase sequence, were printed on the arrays as positive controls. Luciferase mRNA was spiked into all mRNA samples to check the quality of the labeling reaction. Additionally, Salomonella cDNAs were arrayed as negative controls. To increase the reliability of the results, all clones were printed in duplicate
onto the arrays. In the data analysis, mean values of duplicate spots were used.

cDNA fragments were prepared by PCR amplification of bacterial stocks. For amplification, the following primers were used: RDA adapters J-Bgl-24 for DP3 and N-Bgl-24 for DP2 (19); forward and reverse plasmid specific primers for the reference clones (forward, 5′-AGCGGATAAGTGGGTAAC-3′; reverse, 5′-AGCGGATAACATTTCACAC-3′). PCR reactions were performed in a total volume of 100 μl, containing 80 pmol primer (for reference clones: 40 pmol forward primer and 40 pmol reverse primer), 1.5 mM MgCl2 (Life Technologies), 1× PCR buffer (Life Technologies), 0.2 mM dNTPs, 5 U Taq polymerase (Life Technologies), and 1 μl of the bacterial glycerol stock. The PCR conditions for the reference clones consisted of a 2-min 94°C denaturation step followed by 35 cycles of 40 s at 94°C, 1 min at 55°C, 2.5 min at 72°C, and subsequently a final incubation of 10 min at 72°C. For the DPs, the annealing and the elongation step were replaced by 3.5 min at 72°C for DP3 and 3.5 min at 70°C for DP2. To check the quality of the PCR reactions, 1 μl of each PCR product was run on a 1% agarose/TBE gel (TBE is 89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA). Subsequently, amplification products were purified by QiAquick purification kit (Qiagen) using Millipore filtered water as an elution buffer; eluates were evaporated overnight on a hot plate (30°C) and dissolved in 10 μl spotting buffer (5× SSC). Microarrays were produced as described (Van Hal NLW, Vorst O, Kramer E, Hall RD, and Keijer J, unpublished observations). They were printed on silylated slides (CEL Associates, Houston, TX) using a PixSys 7500 arrayer (Cartesian Technologies, Durham, NC). Arrays were spotted by passive dispensing using quill pins (Chipmaker 3, Telechem), resulting in a spot diameter of 0.12 mm at a volume of about 0.5 nl. After printing, microarrays were allowed to dry at room temperature for at least 3 days. Free aldehyde groups were blocked with NaBH4 according to the method of Schena et al. (46).

Sample preparation and labeling. mRNA for microarray hybridization was isolated using an mRNA purification kit (Amersham Pharmacia). Sample mRNA was labeled by incorporation of either Cy3-dCTP or Cy5-dCTP during a reverse transcription (46). In short, 1 μg of sample poly(A)+ RNA was mixed with 2 μg oligo-dT primer (21-mer) in a final volume of 13.5 μl, heated for 3 min at 65°C (RNA denaturation) and 10 min at 25°C (primer annealing), and immediately put on ice. Then, a reverse transcription reaction was performed for 2 h at 37°C in a final volume of 25 μl. The reaction mixture contained the RNA template with the annealed oligo-dT primer, 1× first-strand buffer (Life Technologies), 10 mM dithiothreitol, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 0.04 mM dCTP, 0.04 mM Cy3-labeled dCTP (or Cy5-labeled CTP), 15 U RNase OUT (Life Technologies), and 150 U SuperScript II reverse transcriptase (Life Technologies). The obtained labeled cDNA was purified by an ethanol precipitation performed at room temperature. The pellet was dried in a SpeedVac and dissolved in 10 μl TE, pH 8.0 (TE is 10 mM Tris-HCl and 1 mM EDTA). After a 3-min boiling step, the cDNA was immediately put on ice, and 2.5 μl of 1 M NaOH was added. The cDNA was then incubated for 10 min at 37°C to break down the remaining RNA. To neutralize the pH, 2 μl of 1 M HCl and 2.5 μl of 1 M Tris-HCl (pH 6.8) were added. Finally, an additional ethanol precipitation at room temperature was performed, and the resulting cDNA pellet was dissolved in 20 μl hybridization buffer containing 5× SSC, 0.2% SDS, 5× Denhardt’s solution, 50% (vol/vol) formamide, and 0.2 mg/ml denatured herring sperm DNA. Prior to hybridization, the labeled cDNA was denatured for 3 min at 65°C and spun for 2 min at 12,000 g to remove nondissolved debris.

Microarray hybridization. The microarrays were hybridized with different mRNA probes as indicated in Table 1. Each mRNA sample was labeled twice (except the tissue samples) and hybridized to two different arrays. Prior to hybridization, microarrays were prehybridized in hybridization buffer [5× SSC, 0.2% SDS, 5× Denhardt’s solution, 50% (vol/vol) formamide, 0.2 mg/ml denatured herring sperm DNA] at 42°C for several hours. After prehybridization, slides were rinsed twice in Millipore filtered water, once in isopropanol, and dried by centrifugation (2 min, 470 g). The hybridization was performed in a Geneframe (1× 1 cm2, 25 μl hybridization volume; Westburg, Leusden, The Netherlands). A 1:1 (vol/vol) mixture of Cy3- and Cy5-labeled cDNAs was applied to each array. Arrays were hybridized overnight at 42°C in a humid hybridization chamber. After hybridization, slides were washed at room temperature first in 1× SSC/0.1% SDS (5 min) and subsequently in 0.1× SSC/0.1% SDS (5 min) and 0.1× SSC (1 min) and then dried by centrifugation (2 min, 470 g).

Microarray scanning and DNA sequencing. Microarrays were scanned using a confocal laser scanner ScanArray 3000 (General Scanning) containing a GaHeNe 543 nm laser for Cy3 measurement and a RHeNe 633 nm laser for Cy5 measurement. Scans were made with a pixel resolution of 10 μm, a laser power of 90% and a photomultiplier tube voltage of 55%. The software package ArrayVision (Imaging Research, Ontario, Canada) was used for image analysis of the TIF files as generated by the scanner. Average spot intensities were collected for each individual spot and stored for further data processing in Microsoft Excel.

DPs were sequenced by SeqLab, Göttingen, Germany, using the universal primer 5′-TGTAACCGGCAGCCAGT-3′.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Insulin</th>
<th>Isoproterenol</th>
<th>Culture Day</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>pAd 6</td>
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<td></td>
<td>6</td>
</tr>
<tr>
<td>Ad 6</td>
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</tr>
<tr>
<td>Ad 10</td>
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<td></td>
<td>10</td>
</tr>
<tr>
<td>Ad iso</td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>“Tissue”</td>
<td></td>
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<td>10</td>
</tr>
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</table>

Table 1. cDNA samples used for hybridization to the arrays
Analysis and normalization of microarray data. There are several factors that should be taken into account when comparing the hybridization signals of multiple samples. First of all, there can be random fluctuations between arrays in the amount of DNA present in the spot, due to fluctuations in the spotted volume and in the fixation of the DNA on the slide (47). A variation in the amount of spotted DNA will result in variations in the hybridization signal (Van Hal NLW, Vorst O, Kramer E, Hall RD, and Keijer J, unpublished observations). Second, hybridization conditions might vary. There can be varying hybridization conditions within a slide, between slides, and between different experiments (i.e., when arrays are hybridized on different days). Third, differences in labeling efficiency will occur during mRNA sample labeling. Fourth, there will be inaccuracies in the amount of sample mRNA labeled. To be able to compare multiple mRNA samples and different hybridization experiments, we devised an experimental design that made it possible to correct for these factors.

To correlate all hybridization data with each other, a reference mRNA was used which was made by pooling the mRNAs from WAT of 16 animals and the BAT of 18 animals of different ages. The labeling of this reference cDNA was performed in a single reaction; thus exactly the same reference cDNA was used for all hybridizations. Each microarray was hybridized simultaneously with the reference cDNA labeled with the fluorescent cyanine dye Cy3 and the sample cDNA labeled with Cy5.

The data normalization procedure used consisted of three steps. First, a signal intensity threshold was set. All data points with a mean of Cy3 and Cy5 signals lower than twice the mean background of the Cy3 and Cy5 scans, respectively, were excluded from data analysis (representing 5% of spots). In a second step, the reference signals were used to correct for 1) differences in the amount of spotted PCR product, 2) local differences in the hybridization conditions within a slide, 3) differences in hybridization conditions between slides, and 4) differences in hybridization conditions between experiments performed on separate days. This correction was performed by calculating the Cy5/Cy3 ratio of each spot. After this normalization step, we eliminated also spots where the difference between the Cy5/Cy3 ratios of duplicate spots was higher than half their mean value (<5% of spots). For further analysis, Cy5/Cy3 ratios were calculated using the mean of the Cy3 signals and of the Cy5 signals from duplicate spots. In the third step of the normalization procedure, for each array median Cy5/Cy3 ratios of all spots on the arrays were calculated and used to correct for 1) differences in the amount of mRNA labeled and 2) differences in the labeling efficiency of the various sample mRNAs.

RESULTS

Generation of a cDNA library of candidate genes. To control the quality and the viability of the preadipocytes, for each cell culture some preadipocytes were kept in culture and differentiation was triggered. Adipogenic conversion (i.e., lipid accumulation) and specific UCP1 expression in brown adipocytes were verified for each culture (data not shown).

Three rounds of subtractive hybridization and selective amplification were performed for the white preadipocytes and for the brown preadipocytes, respectively, yielding three difference product pools (DP1–DP3). These DPs were separated by agarose gel electrophoresis. DP1 fragments from both cell types consisted of a continuous smear ranging from 200 to 1,000 bp and did not show much differences by size compared with the cDNA fragments of the amplicon. For DP2, and DP3 more markedly, the gel revealed discrete bands between 200 and 900 kb in size which showed different patterns between the two directions of subtraction (data not shown). Both DP2 and DP3 were then size separated into 11–12 pools and cloned to obtain cDNA libraries. From each of these libraries, consisting of cDNA fragments with similar sizes, 10–20 clones were randomly picked. Altogether, 256 individual clones (110 from DP2 and 146 from DP3) were isolated from these libraries for use on microarrays.

Microarray hybridization and their reproducibility. DNA microarrays are becoming widely used tools in gene expression analysis (50). To study differential gene expression in BAT and WAT, microarrays were produced containing the 110 clones from DP2 and 146 clones from DP3. In addition, 47 genes of potential interest in adipocyte function and differentiation were selected and added to the arrays as reference genes. In particular, a UCP1 clone was printed onto the array, since it is uniquely expressed in brown adipocytes (22). The microarrays were hybridized with mRNA isolated from 1) cultured white and brown preadipocytes at various stages of differentiation, 2) cultured adipocytes treated with isoproterenol, and 3) WAT and BAT from various animals (Table 1). To be able to compare multiple mRNA samples and different hybridization experiments, a reference mRNA was used to correlate all hybridization data with each other. After data normalization, duplicate spots showed very good correlation when plotted against each other (Fig. 1A). There was a high reproducibility throughout different hybridizations as shown in Fig. 1B, which shows the correlation of corrected ratios of two different arrays hybridized with two different labelings of the same mRNA. The slope of the correlation is 0.917 ($r^2 = 0.942$). Less than 2% of spots displayed a ratio difference of more than twofold.

White vs. brown preadipocytes. Figure 2A shows the comparison between an array hybridized with a white semiconfluent preadipocytes sample and an array hybridized with a brown semiconfluent preadipocytes sample. Repetition of labeling and hybridization using the same mRNA yielded a highly similar pattern (data not shown). Seventy-nine clones, representing 27.7% of the DP cDNA fragments, showed a white-to-brown preadipocytes ratio (W/B) or brown-to-white preadipocytes ratio (B/W) higher than 2 in at least one of the hybridizations. DP2 contained 44.5% positive clones, i.e., 49 clones representing 13 genes; DP3 contained only 15% positive clones, i.e., 22 clones representing 14 genes (6 genes were found both in DP2 and DP3). As these data suggest, we observed that DP2 generally contained less different genes with a higher number of copies, whereas DP3 showed a higher diversity of genes. As two hybridizations were performed, we considered as positive only clones with a mean W/B or B/W ratio of higher than 2. These cDNA fragments were se-
quenced, and sequences were matched to databases. One of them, homologous to ribosomal sequences as well as to untranslated regions of different mRNAs, could not be identified with certainty as a particular gene. The others represent 11 different genes, which are listed in Table 2. From five of these genes, only one copy was present among the difference products, whereas six genes were represented several times (e.g., complement factor B had 11 clones).

Northern blot analysis was used to confirm the differences in gene expression observed on the microarrays. Two examples are shown in Fig. 3. Quantitation of Northern blot signals showed complement factor C2 mRNA to be 3.3-fold more abundant in white preadipocytes, and hepatocellular carcinoma-associated protein (HCAP) 2.1-fold higher in brown preadipocytes. The other differentially expressed genes could also be confirmed by Northern blot (data not shown).

Expression pattern of difference products in cell culture. To obtain information about the expression of the spotted genes during differentiation of preadipocytes, the microarrays were hybridized with mRNA probes from confluent preadipocytes, preadipocytes entering differentiation, mature adipocytes, and adipocytes treated with isoproterenol (Table 1). Figure 2B is the graphical representation of the comparison between arrays hybridized with white or brown mature adipocytes. The number of differentially expressed genes between white and brown adipose cells diminished during differentiation, and in mature cells there were almost no more differences between white and brown for the selected cDNAs. This indicates that clones were isolated with a difference in gene expression specific for preadipocytes.

Furthermore, the comparison of the different hybridizations gave the possibility to examine the expression pattern of single genes during the differentiation of the white and brown adipose cells. In Fig. 4, such expression patterns are shown for a selection of three standard (control) genes: uncoupling protein 1 (UCP1), cytochrome c oxidase chain I (COI), and PPARγ. UCP1 was found to be expressed only in mature cells, at a higher level in brown than in white adipocytes (Fig. 4).
PREADIPOCYTE GENE EXPRESSION

Table 2. Differentially expressed genes in white and brown preadipocytes

<table>
<thead>
<tr>
<th>No.</th>
<th>Spots</th>
<th>Expression</th>
<th>Accession No.</th>
<th>Length, bp</th>
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<th>Name</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>W/B</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>11</td>
<td>9.6</td>
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<td>mouse</td>
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<td>Δ6 fatty acid desaturase (FADSD6)</td>
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<td>12</td>
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<td>1,750*</td>
<td>rat</td>
<td>complement component C3</td>
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<tr>
<td>2</td>
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<td>310</td>
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<td>122 bp homology to complement component C2</td>
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<tr>
<td></td>
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<td>9</td>
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<td>rat</td>
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<td>2</td>
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<td>alpha actinin 4 (Actn4)</td>
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<td>4</td>
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<td>rat</td>
<td>metargdin (MDC15 gene)</td>
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<td>425</td>
<td>mouse</td>
<td>small nuclear ribonucleoprotein polypeptide A (Snrpa)</td>
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</table>

List of the difference products with a mean difference of expression level between white (W) and brown (B) preadipocytes higher than 2. For each difference product, the number of spots on the array with the same cDNA is indicated in the first column. The mean ratio of the expression levels from white preadipocytes compared with brown (expression W/B) or from brown preadipocytes compared with white (expression B/W) are shown for the white preadipocytes difference products and the brown preadipocytes difference products, respectively. The length referred to is the length of the cloned cDNA fragments. For the two genes marked with an asterisk, three different cDNA fragments were isolated by RDA; the length indicated corresponds to the whole length covered by these fragments. The homologies to BLAST records were all higher than 80%. If not specified otherwise, the homology covers the whole cDNA fragment.

Complement factor B is one of the genes with higher expression in white preadipocytes (Fig. 5A). In brown adipose cells, the expression level was higher in mature cells than in undifferentiated cells, whereas for white adipose cells there were higher expression levels in both preadipocytes and in adipocytes. For both kind of cells, the expression was repressed by isoproterenol; Δ6-fatty acid desaturase showed a similar pattern of expression (Fig. 5B).

Finally, Fig. 6 shows expression patterns for three genes with higher expression levels in brown preadipocytes, i.e., fibronectin (Fig. 6A), vigilin (Fig. 6B), and HCAP (Fig. 6C). They all showed similar expression patterns. The expression level in white adipose cells did not change significantly during differentiation. In brown preadipocytes, the expression level was high and diminished during differentiation. In mature cells, the expression level of brown adipocytes had reached the level of the white adipocytes and no effect of β-adrenergic stimulation was found either in white or brown adipocytes. In the tissue, there was no significant difference between WAT and BAT.

DISCUSSION

Starting from the hypothesis that brown and white preadipocytes represent different cell types, our aim was to identify genes differentially expressed in these cell types, since so far no molecular markers or distinguishing phenotypes had been established.

The cells used for comparison were obtained from parallel primary cell cultures of white and brown preadipocytes and cultivated until semiconfluence when RNA was extracted. This procedure has two major advantages: 1) it leads to a high enrichment in preadipocytes and a virtual elimination of other cell types present in the stromal vascular fraction of adipose tissues. Preliminary experiments have shown that under our culture conditions almost 100% of adipose cells were adipocytes and a virtual elimination of other cell types, since so far no molecular markers or distinguishing phenotypes had been established.

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identification of few exclusively expressed genes but at least theoretically to an enrichment of cDNAs with predominant expression in one cell population. Because we observed quite different patterns in DP2 and DP3, we decided to analyze cDNA fragments from both. However, this yielded a high number of clones, which made it necessary to apply a rather large-scale screening method, i.e., microarray screening.

In comparing expression levels we used a twofold and higher expression difference as cutoff because our test of reproducibility showed an error of only 2% at this threshold (Fig. 1). A total of 21 different genes were identified meeting this criteria in at least one of the two different hybridizations. In our analysis we focused only on the 11 genes with higher than twofold expression levels in both experiments. Normalization of the microarray data to a reference probe from pooled BAT and WAT tissue allowed us not only to compare gene expression of white and brown preadipocytes but also during adipocyte differentiation and under different culture conditions, i.e., comparison of multiple samples. Insulin has been shown to be essential for lipid accumulation, i.e., adipogenesis of Djungarian hamster preadipocytes (24, 21). We therefore compared not only cells at different time of culture (i.e., different differentiation levels) but also preadipocytes at confluence, treated with and without insulin (Ad 6 and pAd 6, respectively). Also included were cells treated chronically with isoproterenol, a general β-adrenergic agonist. This treatment has been shown to induce proliferation of white and brown preadipocytes, to decrease lipid content by increasing lipolysis, and to increase UCP1 gene expression (25).

The suitability of the microarray screening was confirmed using control genes with an established expression pattern. An obvious control is UCP1, whose expression pattern as shown in Fig. 4A corresponds quite well to previous findings. The residual expression of UCP1 in WAT cultures is most probably due to the presence of brown preadipocytes within the WAT de-

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**Fig. 4.** Expression pattern of standard genes: uncoupling protein 1 (UCP1, A), cytochrome c-oxidase (B), and peroxisome proliferator-activated receptor-γ (PPARγ, C), in various cell types and in vivo. The abbreviations are explained in Table 1. The mean corrected ratios Cy5/Cy3 (±SD) from several spots and from two hybridizations are plotted (except for the “tissue,” where only one hybridization was performed). As UCP1 and PPARγ were spotted only once on the array, no error bars can be shown.

**Fig. 5.** Expression pattern of genes with a higher expression in white preadipocytes: complement factor B (A) and δ-6 fatty acid desaturase (B), in various cell types and in vivo. The abbreviations are explained in Table 1. The mean corrected ratios Cy5/Cy3 (±SD) from several spots and from two hybridizations are plotted (except for the “tissue,” where only one hybridization was performed). As δ-6 fatty acid desaturase was spotted only once on the array, no error bars can be shown.
pots, which are able to differentiate into brown adipocytes (25). We have shown previously that about 10–15% of adipocytes in WAT cultures express UCP1 (21). The more than 10-fold increase of UCP1 expression by β-adrenergic stimulation also corresponds very well to previous results using Northern blot analysis (24, 25). Another cDNA related to thermogenic capacity is COI, a mitochondrially encoded subunit of cytochrome c oxidase, which can be used as a marker for mitochondrial transcription activity. Accordingly, COI expression was very low in WAT compared with BAT and significantly higher in mature adipocytes than in preadipocytes (Fig. 4B), confirming previous findings (24). However, the β-adrenergic increase in COI was similar in white and brown adipocyte cultures. As another control we spotted PPARγ, which plays a key role in adipose conversion and is strongly increased during adipocyte differentiation (10, 52). This was also confirmed by our findings (Fig. 4C). Its expression in both white and brown preadipocytes during proliferation was at the same low level and increased more than twofold in mature adipocytes. In tissue expression was higher in BAT than in WAT, also confirming previous findings (20).

One confounder in the analysis of differential gene expression in preadipocytes could be that the cultured white and brown stromal vascular cells at semiconfluence represent different developmental stages. This would lead to the identification of genes regulated during preadipocyte differentiation rather than real differences between white and brown preadipocytes. Although we cannot completely rule out this possibility, the expression pattern of the control genes like COI and PPARγ indicates that this is not the case, because they show essentially the same expression pattern in white and brown preadipocyte cultures.

Less than 30% of cloned RDA products did show differential expression using microarray screening. This might seem not very efficient; however, it should be noted that RDA can also pick up splice variants that cannot be detected using DNA microarrays. Using Northern blot analysis in at least one case, we observed different-sized messengers in the different cell cultures, which is supportive of this possibility (data not shown). Furthermore, we have excluded spots with low signal intensities, because they showed low reproducibility (about 5 to 10% of spots). Therefore, we might have missed low-abundance clones by the way DNA microarray screening was applied.

The majority of differentially expressed genes surprisingly showed only two- to threefold differences in expression between white and brown preadipocytes. This could be due to the method of analysis. In microarray experiments, expression differences can be underestimated compared with other methods of analysis, such as real-time quantitative PCR (42). However, the expression levels observed here seem real because during preadipocyte differentiation as well as in tissue, much higher expression differences could be observed by application of microarrays. For example, the expression of UCP1 was almost 50-fold higher in isoproterenol-treated brown adipocytes than in mature white adipocytes (Fig. 5A). Northern blot analysis also confirmed the expression differences found by microarray analysis.

In our experiments, we identified only a limited number of differentially expressed genes. This seems in contrast with the 300 expressed sequence tags (ESTs) that were found to be differentially expressed, induced, or switched off, during 3T3-L1 preadipocyte cell differentiation (16). However, our objective was to identify genes differentially expressed between two different types of preadipocytes. As white and brown preadipocytes in cell culture do not display any known morphological or metabolic differences (in contrast to...
differentiated white and brown adipocytes), it seems reasonable that the number of differentially expressed genes is much lower. Our data also suggest that there are rather subtle but relevant differences in expression levels of these genes.

Of the four clones with higher expression in white preadipocytes, three represent homologies to members of the complement system. The complement system, consisting of about 20 proteins, plays an essential role in nonspecific and immunologically induced host defense. Complement factors C3 and B belong to the alternative pathway, and C2 belongs to the classic pathway. Other studies with microarrays also found abundant expression of C2 in human and rodent adipose tissue (13, 34). It is long known that adipocytes are able to secrete the essential components of the alternative pathway, i.e., adipsin (complement factor D), C3, and B (8). C3adesArg (acylation-stimulating protein) stimulates triglyceride synthesis in adipocytes (reviewed in Ref. 11). Here we found a consistently lower expression of complement factors C3 and B in brown preadipocytes than in white preadipocytes; only in fully differentiated adipocytes were expression levels similar in both cell types. Interestingly, in both WAT and BAT cultures β-adrenergic stimulation significantly decreased C3 and B expression, suggesting a sympathetic regulation of components of the alternative pathway in adipose tissue.

The only other clone with significantly higher expression in white preadipocytes was a homolog of the δ-6 fatty acid desaturase (FADSD6). This enzyme catalyzes the rate-limiting step in the conversion of linoleic acid and linolenic acid to arachidonic acid and eicosapentaenoic acid, respectively, which are precursors for various hormones, prostaglandins, etc., and thus are involved in large number of biological functions. Although δ-6 desaturase activity is normally considered to be very low in nonhepatic tissues, the mRNA was recently reported to be similarly expressed in human liver, lung, and heart and even higher in brain (6).

The clones with higher expression in brown preadipocyte (Table 2) code for proteins that can roughly be divided into two functional categories: three structural proteins that are implicated in cell adhesion and cytoskeleton organization (fibronectin, actinin-4, metargardin), and four nuclear proteins that might function in gene transcription and protein synthesis (vigilin, HCAP, necdin, small nuclear ribonucleoprotein polypeptide A (Snrpa)).

Fibronectin is an adhesive extracellular matrix (ECM) protein which plays an important role in tumor development (45). It is known to be strongly expressed in preadipocytes and decrease during adipose conversion which it possibly inhibits (4, 38, 43, 48). Fibronectin could be detected immunohistochemically in human BAT and BAT tumors (hibernomas) but not in the ECM of typical WAT cells (17), corroborating our data of increased fibronectin expression in brown preadipocytes. Actn4 (α-actinin-4) is the second non-muscle α-actinin isoform belonging to a family of actin-binding proteins important in cytoskeleton organization and in cell adhesion. Its expression is apparently correlated with substrate adhesion (36). Metargardin (MDC15) is a transmembrane glycoprotein belonging to the metalloprotease disintegrins which is widely expressed in different tissues and cell types and might function in cell-cell adhesion and/or signaling (27, 35, 5).

The function of HCAP (also named breast cancer-associated gene 1) is not known. Its association with tumor cells might imply a role in cell proliferation. Vigilin (or HBP, high-density lipoprotein binding protein) was first identified as a cholesterol responsive protein (33) whose exact physiological function is still unknown (12). It belongs to a highly conserved family of RNA binding proteins present in cytoplasm and nucleus (33, 39, 29) and is thought to be important for cells which are active in protein synthesis (28). The expression pattern of vigilin was very similar to that of Srnpa (3) belonging to the small nuclear ribonucleoproteins (snRNPs), which are essential components of the mRNA splicing machinery.

Necdin was first identified in neurally differentiated embryo carcinoma cells (32) and later found to be ubiquitously expressed in both neuronal and nonneuronal tissues in humans (53). Necdin is functionally similar to retinoblastoma (pRb) protein family, all of which are growth suppressors. It is reported to be exclusively expressed in postmitotic cells and can be a unique growth suppressor. In this respect, it is surprising that we found highest expression of necdin in proliferating brown preadipocytes. Interestingly, we identified also a retinoblastoma binding protein (RbAp46) among the difference products enriched in brown preadipocytes, but its expression difference did not quite meet our significance level. pRb has been implicated in white and brown adipocyte cell differentiation (40), and a pRb knockout in fibroblasts was reported to result in an upregulation of UCP1 (K. Kristiansen, personal communication). Vigilin, Snrpa, and necdin are all nuclear proteins, which certainly warrants further investigation of their function in adipocyte proliferation and differentiation.

Although expression of brown preadipocyte-enriched genes was not dramatically increased compared with white preadipocytes (2- to 3-fold differences), they all showed rather similar expression profiles (Fig. 6): a decrease during brown preadipocyte differentiation and no changes during white adipocyte differentiation. Their expression levels were rather low in BAT, with fibronectin and vigilin showing the highest differences (15-fold and 10-fold higher expression in brown preadipocytes than in tissue, respectively). This might indicate that these genes are indeed specifically expressed in brown preadipocytes rather than in other cell types because preadipocytes are known to represen only around 10% of cells in BAT (14).

Generally, we found that the expression of many genes was rather different in BAT and WAT tissue compared with cell cultures. This is certainly to a large part due to the fact that adipose tissue consists of various cell types like blood cells, pericytes, endothelial...
cells, fibroblasts, adipoblasts, etc. Adipocytes, for example, represent only between 30 and 60% of adipose tissue cells (2), whereas in our primary culture system we have a high enrichment of adipogenic cells. But even in adipogenic cells, gene expression might be very different in vitro and vivo because of differences in for example matrix and hormonal environment.

In conclusion, we demonstrate that RDA in combination with DNA microarray screening is a useful method for identification of genes with different expression levels in different cell populations or different physiological situations. This might be especially useful when studying species for which very few or no EST or cDNA microarrays are available today, i.e., species other than mice, rats, or humans. We have identified several genes that show differential expression in white and brown preadipocyte as well as distinct patterns of expression during adipose conversion. Several complement factors showed higher expression in white preadipocytes. Genes of higher abundance in brown preadipocyte include structural proteins involved in cell adhesion and nuclear proteins possibly involved in cell proliferation and protein synthesis. The function of these genes in determination of preadipocyte remains to be investigated.

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