Individual variation of adipose gene expression and identification of covariated genes by cDNA microarrays

STÉPHANE BOEUF,1 JAAP KEIJER,2 NICOLE L. W. FRANSSEN-VAN HAL,2 AND SUSANNE KLAUS1
1German Institute of Human Nutrition in Potsdam, 14558 Bergholz-Rehbrücke, Germany; and 2RIKILT, 6700 AE Wageningen, The Netherlands

Received 30 April 2002; accepted in final form 5 July 2002

Boeuf, Stéphane, Jaap Keijer, Nicole L. W. Franssen-van Hal, and Susanne Klaus. Individual variation of adipose gene expression and identification of covariated genes by cDNA microarrays. Physiol Genomics 11: 31–36, 2002.—Gene expression profiling through the application of microarrays provides comprehensive assessment of gene expression levels in a given tissue or cell population, as well as information on changes of gene expression in altered physiological or pathological situations. Microarrays are particularly suited to study interactions in the regulation of large numbers of different genes, since their expression is analyzed simultaneously. For improved understanding of the physiology of adipose tissue, and consequently obesity and diabetes, identification of covariability in gene expression was attempted by analysis of the individual variability of gene expression in subcutaneous white and brown fat of the Siberian dwarf hamster using microarrays containing ~300 cDNA fragments of adipose genes. No sex-dependant variability in gene expression could be found, and overall individual variability was rather low, with more than 80% of clones showing a coefficient of variation lower than 30%. Uncoupling protein 1 (UCP1) displayed a high variability of gene expression in brown fat, which was negatively correlated with the gene expression of complement factor B (FactB), implying a possible functional relationship.

Adipose tissue is actively involved in energy homeostasis, playing a role not only in development of obesity but also insulin resistance and diabetes (15). The two types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT), represent counter-actors in energy partitioning, channeling lipid energy either to accumulation (WAT) or to oxidation and dissipation (BAT) (11). Recently, in two studies microarrays were used to investigate changes in gene expression in WAT or adipocytes associated with obesity, diabetes, and adipocyte differentiation (16, 17). Additionally, we have employed microarrays to identify genes of differential expression in white and brown preadipocytes (2). Gene expression analysis of adipose tissue using microarrays is considered to generate new insights into the regulatory mechanisms involved in obesity and diabetes (15).

Microarrays have opened the possibility for large-scale gene expression analysis (8, 7, 18). Investigations that were previously possible only on single genes can now be done on the scale of whole genomes. Microarrays are used to assess changes in gene expression after the treatment of cells, during differentiation or other processes, or to identify differentially expressed genes between cell types or between populations. Regarding the latter application, some problems were encountered to define statistical criteria for the identification of significant differences in gene expression (13, 20). The assessment of gene expression in populations showed that large differences could be found between individuals, even for genes previously considered as control or housekeeping genes. The existence of housekeeping genes defined as genes with ubiquitous and stable expression has been found to be highly questionable (9, 12; also, our unpublished observations). Also, different genes were found to have different levels of interindividual variability. This implicates the necessity to use different scales of measurement for the different genes when comparing individual samples (20). Currently only few variability data from microarray studies are available. With the growing amount of expression data available, catalogs of gene expression levels including indications on variability will be compiled. This kind of database is being set up for example at http://www.hugeindex.org (9). Analysis of variability in gene expression can also be used to identify covariability in the expression of specific genes, which could be indicative of a possible functional relationship.

For adipose tissue, so far no such data on individual variability in gene expression are available. Therefore, we investigated the variability of gene expression in white and BAT from the Djungarian (also termed Siberian) dwarf hamster Phodopus sungorus, using previously established microarrays containing ~300 adipose-derived cDNA fragments (2). P. sungorus is a widely used animal model in the study of brown fat thermogenesis because it shows very high cold-in-

1 Address for reprint requests and other correspondence: S. Klaus, Deutsches Institut für Ernährungsforschung (DIfE), Arthur-Scheunert-Allee 114-116, 14558 Bergholz-Rehbrücke, Germany (E-mail: Klaus @www.dife.de)
duced, adaptive increase in brown fat thermogenesis and abundant brown fat. Subcutaneous WAT and BAT depots of six animals, three males and three females, were hybridized to the arrays. The aim of these hybridizations was to evaluate the expression levels of adipose tissue genes in vivo, as well as individual and sex-dependent differences of gene expression and to identify possible covariability of gene expression.

**MATERIALS AND METHODS**

*Animals, cell cultures, and mRNA isolation.* Djungarian dwarf hamsters (*P. sungorus*) were obtained from our own breeding colony. Animals were kept at 23°C (thermoneutrality) in long photoperiod (18:6 h, light:dark). Tissue samples were obtained from six hamsters, three males and three females, aged 11 wk. Average body weight of Siberian hamsters is around 45 g under these housing conditions. For tissue preparation animals were anesthetized with CO₂ and killed by cardiac puncture. The inguinal WAT and several subcutaneous depots of BAT (axillar, suprasternal, interscapular, dorsal-cervical) were isolated. The BAT depots were pooled for each animal, amounting to around 1 g per animal. Total RNA from tissue samples was extracted using the single step acid phenol-guanidine protocol (4). For the synthesis of the reference cDNA, RNA from the WAT of 16 animals and from the BAT of 18 animals aged 11–30 wk was pooled. The primary cell cultures of white and brown preadipocytes were described in Ref. 2. Mature adipocytes were obtained after 10 days of cell culture and induction of differentiation at day 3 of culture. For β-adrenergic stimulation, cells were treated chronically with either 0.1 μM CL-316.243 (a β3-adrenoreceptor-specific agonist) or 10 μM isoproterenol from day 3 on. mRNA was isolated using a mRNA purification kit (Amersham Pharmacia).

*cDNA microarrays.* The microarrays used in this study were described previously (2). They contain 256 clones obtained by representational difference analysis of cDNA from cultured white and brown preadipocytes and 47 reference clones with established functional relevance to adipocyte development. The clones were printed in duplicate onto the arrays.

*Sample preparation.* One microgram of sample mRNA was labeled by incorporation of either Cy3-dCTP or Cy5-dCTP during a reverse transcription as described (2). The individual tissue samples were labeled with Cy5, the reference mRNA with Cy3. After purification of the obtained labeled cDNAs, they were dissolved in 20 μl hybridization buffer [5 × SSC, 0.2% SDS, 5 × Denhardt’s solution, 50% (vol/vol) formamide, 0.2 mg/ml denatured herring sperm DNA] and denatured.

*Hybridizations.* The microarrays were hybridized with a 1:1 (vol/vol) mixture of Cy5-labeled tissue cDNA and Cy3-labeled reference cDNA. Twelve hybridizations were performed corresponding to the WAT and BAT of six animals, as well as six replicated hybridizations with the cell culture probes (i.e., a total of 12 hybridizations for cell culture probes). After prehybridization, arrays were hybridized overnight at 42°C using a Geneframe (1 × 1 cm², 25 μl hybridization volume; Westburg, Leusden, The Netherlands). They were washed and scanned using a confocal laser scanner ScanArray 3000 (General Scanning). Scans were made with a pixel resolution of 10 μm, a laser power of 90%, and a PMT voltage of 55%. The software package ArrayVision (Imaging Research, Ontario, Canada) was used for image analysis of the TIFFF files as generated by the scanner. Average spot intensities were collected for each individual spot and stored for further data processing in Microsoft Excel.

*Data analysis.* Prior to the normalization of data, we compared the Cy3 reference hybridizations on the 12 arrays to assess the methodological variability (Fig. 1). Only at low signal intensities, the coefficient of variation (CV) is dependent on the mean signal. For the spots with higher signals, the levels of variation found were low. The median CV for all clones is 12.5%; 78.1% of them show a CV lower than 20%. The noise due to methodological errors is thus rather low. Data points with too low signal intensity, i.e., with a signal intensity lower than twice the background level, as well as data points with poor reproducibility on duplicate spots, i.e., with a difference between duplicate spots higher than one-half the mean of their signals, were excluded from data analysis. Yet these represented few clones. More than 90% of the clones on the arrays gave signals higher than twice the background.

Through the use of a unique reference mRNA it is possible to compare multiple mRNA samples. The mean of the Cy5/Cy3 ratio was calculated for each clone on the array and normalized with the median Cy5/Cy3 ratio of all spots on the array. We have shown the validity of this method previously (2). By the comparison of microarrays hybridized to probes obtained from identical mRNA samples, it could be shown that the expression ratios thus calculated are highly reproducible. No more than 2% of the clones on the arrays were found to have more than twofold differences between replicated hybridizations. We compared expression levels between WAT and BAT and found 24 clones with higher expression levels in WAT and 28 in BAT. As the majority of the clones are equally expressed in WAT and BAT and there are approximately as much clones higher expressed in WAT than in BAT, our normalization procedure with the median introduces no bias.

The “expression ratios” thus calculated reflect the expression level of the corresponding gene in one tissue sample relative to the expression in the reference tissue pool. They are not linked to the initial signal intensity of the corresponding spots. For graphical representations, these ratios were rescaled by multiplication with the mean corresponding reference (Cy3) signal from all hybridizations, so that clones with low-intensity signals are represented by low “corrected expression ratios” in the graphs. This correction does not affect the expression ratios thus calculated accurately.

![Fig. 1. Variation of the normalized Cy3 signal intensities for the 12 microarrays hybridized to tissue probes. The Cy3 signals were normalized on each array prior to comparison to avoid bias due to different hybridization efficiencies on the arrays. For each clone on the arrays, the coefficient of variation (CV = mean signal ± SD) for each of the 12 signal intensities is % is plotted against the mean Cy3 signal.](http://physiolgenomics.physiology.org/Downloaded-from)
AFFECT THE COMPARISON BETWEEN ARRAYS. STATISTICAL ANALYSIS OF
EXPRESSION DATA WAS PERFORMED USING SPSS FOR WINDOWS 8.0.

RESULTS

COMPARISON OF FEMALE AND MALE HAMSTERS. WE COMPARED THE GENE
EXPRESSION IN ADIPOSE TISSUES OF THREE FEMALE AND THREE MALE
HAMSTERS. MEAN CORRECTED EXPRESSION RATIOS FROM FEMALES AND Males
WERE CALCULATED AND COMPARED. FIGURE 2 IS A SCATTER PLOT REPRESENTING
THIS COMPARISON IN WAT (Fig. 2A) AND IN BAT (B). THE THICK SOLID LINE IS THE
X = Y AXIS; THE THIN SOLID LINES ARE THE X = 2Y AND THE X = Y/2 AXIS.

As shown in Fig. 1, methodological variability is higher for these clones, so that these differences cannot be
considered as significant.

GLOBAL INTRINDIVIDUAL VARIABILITY OF GENE EXPRESSION. NO SEX-SPECIFIC
DIFFERENCES BEING FOUND IN OUR DATASET, WE WERE INTERESTED IN THE GLOBAL
HOMOGENEITY OF THE GENE EXPRESSION LEVELS BETWEEN THE ANIMALS. FOR EACH
CLONE ON THE ARRAYS, THE CV OF THE EXPRESSION RATIOS IN THE SIX ANIMALS WAS CALCULATED. THE DISTRIBUTION OF THE
CV VALUES IN WAT AND BAT IS SHOWN IN THE HISTOGRAMS IN FIG. 3. GLOBALLY, THE INTERINDIVIDUAL VARIABILITY OF
EXPRESSION WAS RATHER LOW: MOST OF THE CLONES (86% IN WAT AND 83% IN BAT) HAVE A CV LOWER THAN 30%. THE
MEDIAN VALUE OF THE CV OF EXPRESSION RATIOS LIES AT 18.5% IN WAT AND AT 17.5% IN BAT, AND THE DISTRIBUTION
IN WAT AND BAT IS RATHER SIMILAR. THE ONLY NOTICEABLE DIFFERENCE IS THE PRESENCE OF MORE CLONES
WITH CVs BETWEEN 40 AND 60% IN BAT THAN IN WAT.

GLOBAL INTERINDIVIDUAL VARIABILITY OF THE EXPRESSION OF SELECTED GENES. A SIMPLE STATISTICAL MODEL (21) SHOWS THAT
TWO COMPONENTS ARE INVOLVED IN THE SD OF EXPRESSION RATIOS: A NOISE COMPONENT DUE TO THE BACKGROUND, WHICH
IS DECREASING WITH INCREASING MEAN SIGNAL INTENSITY, AND THE INTERINDIVIDUAL VARIABILITY OF GENE EXPRESSION.
THE LATTER SHOULD BE INDEPENDENT OF SIGNAL INTENSITY. TO ASSESS THE IMPORTANCE OF THE NOISE COMPONENT IN OUR
DATA, WE LOOKED AT THE DISPERSION OF THE CV VALUES RELATIVE TO THE MEAN EXPRESSION RATIOS OF THE CORRESPONDING CLONES (Fig. 4). THE MAIN CLOUD OF SPOTS CORRESPONDS TO CV VALUES LESS THAN 30%. FOR LOW-INTENSITY SIGNALS ON THE ARRAYS THE CV CAN REACH HIGHER VALUES. THIS DISPERSION OF DATA POINTS AT LOW SIGNAL INTENSITIES
CAN BE ATTRIBUTED TO THE NOISE COMPONENT IN THE SD OF EXPRESSION RATIOS AND THEREFORE REFLECTS MEASUREMENT ERRORS. HOWEVER, SOME CLONES WITH HIGHER EXPRESSION LEVELS ALSO SHOWED A RELATIVELY HIGH CV, ESPECIALLY IN BAT.

WE WERE INTERESTED IN CLONES SHOWING HIGH CVs THAT WERE NOT DUE TO METHODOLOGICAL ERRORS. TO DO SO, WE
CONSIDERED ARBITRARILY ALL THE CLONES WITH A MEAN CORRECTED EXPRESSION RATIO IN THE HIGHEST TERTILE OF ALL
RATIOS. LOW EXPRESSION LEVELS ARE THUS AVOIDED. IN TABLE

Fig. 2. Comparison of gene expression in adipose tissues from male
and from female animals. Shown are scatter plots comparing the
mean corrected expression ratios from males and females in WAT (A)
and in BAT (B). The thick solid line is the x = y axis; the thin solid
lines are the x = 2y and the x = y/2 axis.

Fig. 3. Distribution of coefficients of variation in WAT (A) and BAT (B). For each
corresponding clones (Fig. 4). The main cloud of spots corre-
corresponds to CV values less than 30%. For low-intensity
signals on the arrays the CV can reach higher values.

Physiol Genomics • VOL 11 • www.physiolgenomics.org
1 clones are listed with a variability in the highest 10 percentile of variation, i.e., higher than 25.9% in WAT and 28.9% in BAT. It should be noticed that these levels of variability are much higher than the methodological variability measured on the Cy3 signals (Fig. 1). For WAT, the following genes were found to be represented in this area of the graph, some of them being represented by several clones on the array: cytochrome c oxidase chain I (COI), complement factor B (FactB), and three unsequenced clones; and for BAT, FactB, uncoupling protein 1 (UCP1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), acyl-CoA synthetase II (ACS), stimulatory G protein subunit-α (Gsa), and one unsequenced clone. The two genes with the highest levels of variability in BAT are FactB and UCP1, with a CV close to 40%. The variability of the Gsa protein expression is also higher in BAT compared with WAT (22% vs. 16%). In contrast to this, the variability of the COI expression is much higher in WAT than in BAT (26% and 19%, respectively).

To identify possible covariabilities of gene expression, we analyzed the correlations of all highly variable genes listed in Table 1. Of special interest in this respect is UCP1. UCP1 expression characterizes the brown adipocyte phenotype and reflects the thermogenic capacity of adipocytes (10, 11). Therefore it was interesting to investigate whether the high variability in expression levels of other genes could be linked to UCP1 expression. We found a significant ($P < 0.05$, Pearson) negative correlation between UCP1 and FactB expression levels (Fig. 5). Data points obtained from hybridization of the same arrays to cultured brown adipocytes treated or not with β-agonists were added to the graph, showing the same tendency of a negative link between FactB and UCP1 expression. As a matter of fact, in cell culture, β-agonist treatment induced UCP1 gene expression but repressed the expression of FactB in BAT (2). Between UCP1 and Gsa protein expression levels, a positive but not significant correlation was found. For other genes, no correlation could be detected.

**DISCUSSION**

Adipose tissue displays a marked metabolic heterogeneity (3) depending on adipose tissue type (white/brown), sex (male/female), location (subcutaneous/visceral). The mean CVs lying in the highest 10 percentiles of all CVs [25.9% in white adipose tissue (WAT), 28.9% in brown adipose tissue (BAT)], COI, cytochrome c oxidase chain I; FactB, complement factor B; UCP1, uncoupling protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACS, acyl-CoA synthetase II; Gsa, stimulatory G protein subunit-α.

**Table 1. Interindividual coefficients of variation of gene expression in WAT and BAT**

<table>
<thead>
<tr>
<th>Gene</th>
<th>WAT</th>
<th>BAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCP1</td>
<td>29.00*</td>
<td>41.45*</td>
</tr>
<tr>
<td>COI</td>
<td>26.01*</td>
<td>19.00</td>
</tr>
<tr>
<td>FactB</td>
<td>24.45</td>
<td>37.07*</td>
</tr>
<tr>
<td>ACS</td>
<td>12.12</td>
<td>31.43*</td>
</tr>
<tr>
<td>GAPDH</td>
<td>13.18</td>
<td>29.10*</td>
</tr>
<tr>
<td>Gsa</td>
<td>15.78</td>
<td>21.99</td>
</tr>
</tbody>
</table>

*The mean CVs lying in the highest 10 percentiles of all CVs [25.9% in white adipose tissue (WAT), 28.9% in brown adipose tissue (BAT)].

COVARIABILITY OF ADIPOSE GENE EXPRESSION

**Fig. 5.** Expression levels of FactB vs. UCP1 in BAT: the expression ratios of UCP1 in the BAT of 6 animals are plotted against the expression ratios (±SD) of FactB (black squares). The regression line for these 6 data points is shown. Data points corresponding to hybridizations with cultured adipocytes were added to the graph: mature adipocytes (open square), adipocytes treated with the β3-adrenoreceptor-specific agonist CL-316.243 (solid triangle), and adipocytes treated with isoproterenol (open triangle).
ceral), as well as different physiological and metabolic situations (e.g., age, obesity, diabetes, physical fitness). This should be reflected by different patterns of gene expression. Indeed, marked differences in adipose gene expression between lean and obese mice have been described using oligonucleotide microarrays (16, 17). It is conceivable that in the near future such microarrays or DNA chips can be used as a diagnostic tool for specific metabolic or pathophysiological situations through assessment of individual adipose gene expression profiles. Essential for this approach however, is the knowledge of the normal range of individual variabilities of adipose gene expression.

Here we used adipose tissue samples from six non-inbred dwarf hamsters of same age and physiological adaptation for microarray hybridizations. Interestingly, in the comparison of gene expression levels in BAT and WAT of three male and three female animals, no sex-dependent differences could be found (Fig. 2, A and B). For the genes with a high interindividual variability also no sex-depandent distribution of expression levels could be observed. This supports previous observations that specific enzyme activities or gene expressions are not different in female and male hamsters (S. Klaus, unpublished results).

An interesting finding of this study is that the individual variability of gene expression in BAT and WAT was relatively low (Fig. 3). It has been shown recently that high levels of interindividually variability of gene expression can be found even in inbred animals. Miller et al. (13) examined the expression of 153 genes in the liver of four mice and found variability of expression levels higher than 30% for 80% of them. In another study, the hippocampi of inbred rats were compared. The variability of gene expression ranged from 1.6-fold to 20-fold differences between individuals (1). As inbred animals represent the minimal expectable variability, much higher differences might be encountered between animals from outbred colonies or from wild populations. However, with 90% of the clones showing a CV lower than 26% in WAT and 29% in BAT, our gene expression data reveal to be rather homogenous. This might indicate that gene expression in adipose tissue is rather robust and stable under identical environmental conditions.

Yet some genes were found with high levels of variability. A calculated CV includes methodological and interindividual variabilities. As the methodological component of variability decreases with higher signal intensity, we further considered only clones with high signals on the arrays (Fig. 4). Thus more genes of high individual variabilities were identified in BAT than in WAT (Table 1). We exclude the possibility that the variabilities found could be linked to sampling. The animals used were kept in identical conditions and in all animals identical depots of WAT and BAT were dissected. The high CV value found for UCP1 in BAT (41.5%) could possibly be explained by differences in the cellular composition of BAT because UCP1 is only expressed in differentiated brown adipocytes (10). Adipose tissue containing a higher number of brown adipocytes would result in higher UCP1 expression levels. If this were the case, then a similar behavior would be seen for other genes that are higher expressed in BAT vs. WAT, such as COI, which has a 10 times higher expression in BAT than in WAT (2). However, the variability of expression of COI was found to be rather low in BAT (19%), and no correlation was found between the expression of UCP1 and COI in BAT. It thus seems unlikely that the variability of gene expression levels found reflect different cellular compositions of the adipose tissues. The UCP1 expression levels rather suggest different degrees of activation of BAT, although all animals were of same age and kept at exactly the same environmental conditions.

We also analyzed covariabilities of expression in genes showing high interindividual variabilities in BAT, a significant negative correlation between the expression levels of complement factor B (FactB) and UCP1 could be detected, not only in vivo, but also in primary cell culture (Fig. 5). FactB belongs to the alternative pathway of complement. It is, as well as other complement components, known to be expressed in adipose tissue (2, 5). The activation of the proximal portion of the alternative pathway of complement in adipocytes leads to the production of acylation stimulating protein (ASP) (6), which stimulates triglyceride synthesis in adipocytes. FactB is the rate-limiting enzyme in ASP synthesis. The role of complement components in BAT is unknown. C3-deficient, and thus ASP-deficient, male mice showed almost 90% enlarged BAT depots under a low-fat diet, whereas gonadal and perirenal WAT depots were moderately decreased (14). The authors of this study hypothesized that this disease was due to a lower nonesterified fatty acid (NEFA) uptake in BAT, which lead to the repartitioning of NEFAs into WAT. Our data indicate a more direct role for complement in brown adipocytes and suggest that they could also be able to synthesize ASP, since they express all the complement components necessary for this (2). This indicates a possible function of ASP in BAT, which might be different from its function in WAT. Previously we found a downregulation of the expression of all complement components through β-adrenergic stimulation in cell culture, suggesting a sympathetic regulation in adipose tissue (2). UCP1 expression is stimulated by β-adrenergic activation, supporting the hypothesis that the expression of FactB in brown adipocytes is negatively associated with states characterized by high UCP1 expression. This indicates that there might be a functional link between thermogenesis and FactB in brown adipocytes. Interestingly, von Praun et al. (19) compared hamsters kept at 23°C or at 4°C for 2 or 7 days and found a significant positive correlation between the expression levels of UCP1 in BAT and the serum-free fatty acid (FFA) levels. In cold induction, the higher serum FFA levels are linked to an increased lipid catabolism. This could be directly linked to lower FactB expression and thus lower ASP levels.

In conclusion, our data demonstrate that individual variability of gene expression in WAT and BAT, at
least in dwarf hamsters, is rather low and apparently not sex dependent. The comparison of normal individuals of a homogeneous population can give some indications about possible functional links between different genes or groups of genes. Here we found a possible negative link between FactB and thermogenic function in brown fat. It should thus be worthwhile to elucidate the precise functional role of complement components and ASP in BAT.

We thank Antje Sylvester for excellent technical assistance and Evelien Kramer for assistance with the microarray experiments. This study was supported by a grant from the Deutsche Forschungsgemeinschaft (to S. Klaus) and by the European Commission (COST Action 918).

REFERENCES