Alterations in Skeletal Muscle Gene Expression of *oblob* Mice by mRNA Differential Display

David Vicent, Megan Piper†, Steen Gammeltoft, Eleftheria Maratos-Flier, and C. Ronald Kahn

To identify molecules that contribute to insulin resistance, we compared the patterns of gene expression in skeletal muscle of the obese *oblob* mouse, a genetic model of obesity and severe insulin resistance, with that of its thin littermate (*ob*/+) using the mRNA differential display method. From about 9,000 cDNAs displayed, we found 12 differentially expressed in *oblob* mice skeletal muscle that could be recovered from the differential display gels and confirmed by Northern blot analysis and sequenced. Eight mRNAs were overexpressed in *oblob* muscle: Id2 (a negative regulator of the basic helix-loop-helix family of transcription factors), fast skeletal muscle troponin T, ribosomal protein L3, the integral protein of the peroxisomal membrane 22PMP, the mammalian homolog of geranylgeranyl pyrophosphate synthase, an mRNA related to phosphatidylinositol-glycan–specific phospholipase D, and two unknown mRNAs. The level of overexpression of these mRNAs in skeletal muscle varied from a 500% increase to as little as a 25% increase. Two mRNAs were underexpressed 20–35%, including the *f*-subunit of mitochondrial ATP synthase and a retrovirus-related DNA. Two proteins with multiple transcripts, skeletal muscle α-tropomyosin and one for a repetitive sequence, showed a change in mRNA pattern of expression in the muscle of the *oblob* mouse. Because the primary genetic defect in the *oblob* mouse is known to be in the leptin gene, these data indicate how acquired alterations in gene expression of multiple classes of proteins may play a role in the complex pathogenesis of insulin resistance in obesity and diabetes. *Diabetes* 47:1451–1458, 1998

Type 2 diabetes is a common disease with a strong but complex genetic pattern. The development of insulin resistance is an early event in the disease and has been shown to be determined by genetic factors (1,2). In many type 2 diabetic patients, this insulin resistance is exacerbated by obesity, which may also have important genetic determinants (3). In addition to these primary genetic defects, alterations in gene expression in muscle, fat, and liver may play a profound role in the pathogenesis of type 2 diabetes (4). A number of investigators have screened genes encoding proteins involved in insulin action as possible candidates for type 2 diabetes (5–9); however, thus far there have been limited studies characterizing the expression of genes that might influence the pathogenesis of this disease. Several studies have focused on the expression of specific candidate genes at the mRNA level (10).

We previously reported a strategy for finding candidate molecules by using subtraction cloning to screen for differences in mRNA expression in tissues of diabetic and nondiabetic humans and have identified several changes in gene expression with this technique (11,12). However the technique is both difficult and cumbersome.

Using the mRNA differential display method described by Liang and Pardee (13), we previously found that the neuropeptide melanin-concentrating hormone is overexpressed in the hypothalamus of *ob*/*ob* mice (14). In the present study, we investigated the potential of this method for identifying changes in gene expression related to insulin resistance. We used the *ob*/*ob* mouse, which represents one of the most studied animal models of obesity-linked type 2 diabetes, and focused on expression in skeletal muscle, one of the key tissues in insulin resistance. With this approach, we found 12 changes in gene expression in the skeletal muscle that might affect insulin sensitivity as well as a wide variety of pathways in this animal model. Among the differentially regulated genes, we identified the transcription regulator factor Id2, the mammalian version of geranylgeranyl pyrophosphate synthase, and a homolog of phosphatidylinositol-glycan–specific phospholipase D (PIG-PLD). We used this information to study the expression of some of these mRNAs in liver and white adipose tissue as a preliminary characterization of their alterations in this animal model of obesity-linked type 2 diabetes.

RESEARCH DESIGN AND METHODS

Mice. Male *ob*/*ob* and *ob*/+ mice age 6 weeks were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed at least 4 days after arrival before being used in experiments. All animals received ad libitum diets. Tissues (brain, gastrocnemius muscle, liver, and epididymal fat pad) were removed from the fed animals. Mice were anesthetized with sodium amytal (100 mg/kg) and decapitated; the indicated tissues were immediately frozen in liquid nitrogen and kept at –80°C until the RNA was extracted.

Total RNA preparation. To obtain total RNA for the mRNA differential display, the muscles from four mice were homogenized in Ultraspec RNA (Biotecx, Houston, TX) (50 mg/ml) on ice for 30 sec at maximum speed using a Polytron homogenizer (Kinematika, Switzerland). Next 0.2 ml of chloroform was added per ml of homogenate and, after being mixed, was centrifuged at 12,000*g* at 4°C for 15 min. Total RNA was precipitated from the aqueous phase with isopropanol and centrifugation at 12,000*g* at 4°C for 10 min. The pellet was washed with 75% ethanol, air dried, and dissolved in diethyl pyrocarbonate–treated water. DNA contamination was removed by digestion with DNase I (Boehringer Mannheim, Indi-

From the Research Division, Joslin Diabetes Center, and Department of Medicine, Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Dr. C. Ronald Kahn, Joslin Diabetes Center, One Joslin Pl., Boston, MA 02215. E-mail: kahnr@joslab.harvard.edu.

Received for publication 1 October 1997 and accepted in revised form 20 May 1998.

†Deceased 1995.

bHLH, basic helix-loop-helix protein; dNTP, deoxynucleotide triphosphate; EST, expressed sequence tag; GPI, glycosyl-phosphatidylinositol anchor; ORF, open reading frame; PCR, polymerase chain reaction; PIG-PLD, phosphatidylinositol-glycan–specific phospholipase D; RT, reverse transcription; UTR, untranslated region.

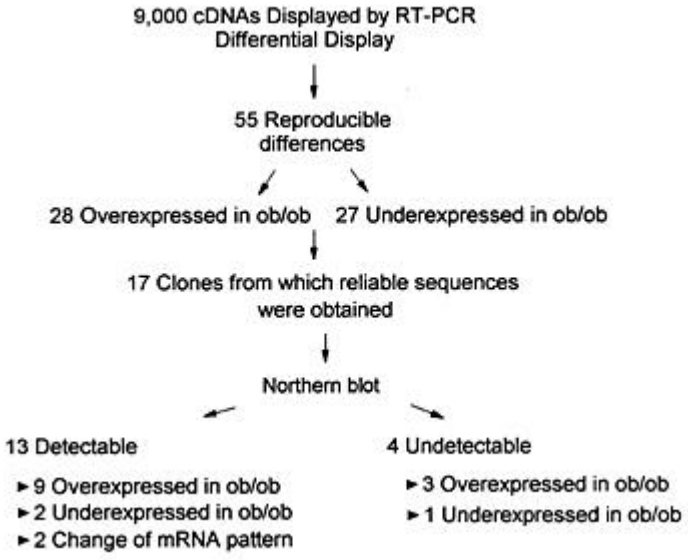

FIG. 1. Schematic diagram indicating the results of the study of mRNA differential display in skeletal muscle of *ob*/+ and *ob/ob* mice.

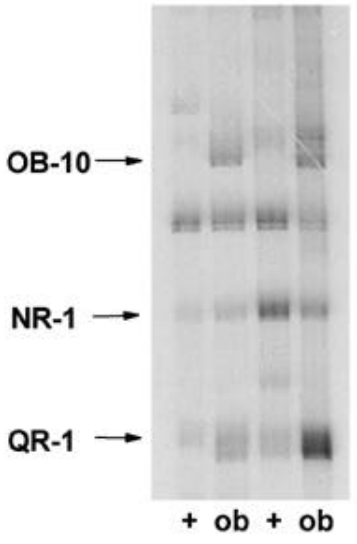

FIG. 2. Typical differential display gel. Duplicate differential display RT-PCR reactions were conducted using RNA from lean (+) and obese (ob) mice; the reaction products were separated side by side on sequencing gels. The autoradiography shown corresponds to the pair of primers T12AC (downstream) and UP-27 (upstream). The band labeled OB-10 was reproducibly overexpressed in ob-RNA samples. This band was cut from the gel and subsequently identified as the mouse homolog of *N. crassa* geranylgeranyl pyrophosphate synthesis by sequence analysis. The other bands are examples of a nonreproducible change (NR-1) and questionably reproducible differences (QR-1). These bands were not subjected to further analysis.

anapolis, IN), and the RNA was re-extracted with phenol/chloroform and ethanol precipitated. Total RNA for Northern blot analysis was obtained from 0.5 g of muscle following a similar procedure. To explore the tissue distribution of low abundance RNAs, polyA RNA was extracted from 1 g of tissue using the Poly (A) Pure Kit (Ambion, Austin, TX).

mRNA differential display. mRNA differential display between *ob*/+ and *ob/ob* muscle RNA was performed in duplicate following the method described by Liang and Pardee (13). In brief, the total RNA was divided in aliquots used to duplicate all the subsequent reactions. cDNA was synthesized using Maloney murine leukemia virus reverse transcriptase (RT) (Superscript RNase H-Reverse Transcriptase; Gibco, Grand Island, NY). We used 0.5 µg of total RNA in nine different RT reactions primed by nine different anchored primers (see below). The cDNA generated was used in polymerase chain reaction (PCR) differential display.

We used 9 downstream anchored primers with the sequence T12VV (where V can be A, C, or G) in conjunction with 20 upstream random, arbitrary primers for PCR display. The arbitrary upstream primers were 10 bases long, did not contain more than 50% guanine and cytosine nucleotides, and had no internal homology. Next 2.5 µl of the RT reaction were amplified by PCR (0.05 U/µl AmpliTaq DNA polymerase [Perkin Elmer/Cetus, Norwalk, CT], 2.0 µmol/l deoxynucleotide triphosphate (dNTP), 0.5 µCi/µl α-[^{35}S]dATP [>1,000 Ci/mmol], 0.2 µmol/l upstream primer, and 0.1 µmol/l downstream primer) for 40 cycles (denaturation 94°C $\times$ 30 s; annealing 40°C $\times$ 2 min; extension 72°C $\times$ 30 s) in a Perkin Elmer Thermal Cycler (Perkin Elmer/Cetus). The duplicate PCR products were separated on sequencing gels under denaturing conditions. Dried gels were exposed to Kodak X-OMAT AR film (Eastman-Kodak, Rochester, NY) for 24–48 h. Bands that were unique to the *ob*/+ or *ob/ob* mouse and were present in both duplicate reactions were excised from the dried gel, extracted by being boiled in 100 µl of Tris-EDTA buffer, and precipitated with ethanol in the presence of mussel glycogen (Boehringer Mannheim). DNA was further amplified using the original set of primers and the same thermal conditions used to generate the particular band, but with increased concentrations of dNTPs (50 µmol/l), upstream primer (0.32 µmol/l), and downstream primer (0.16 µmol/l). Reaction products were run on a 1.7% agarose gel and stained with ethidium bromide to check the purity and compare the band size with the size of the band excised from the sequencing gel. Bands successfully amplified were ligated in PCR Vector and cloned in TA Cloning One Shot competent cells using the TA Cloning Kit (Invitrogen, San Diego, CA).

Sequence of the cloned inserts and searches for homologies. Miniprep cultures were grown from each clone, and the plasmids were purified using QIAprep columns (Qiagen, Santa Clarita, CA). The inserts were sequenced in both directions using an ABI-373 automated sequencer (Foster City, CA) and M13R and M13(–40)F oligonucleotides as primers. Analyses of the sequences were done using the computing facilities of the Molecular Biology Computing Research Resource (Dana Farber Cancer Institute and Harvard School of Public Heath, Boston, MA). Searches of DNA and protein databases for homologies with the cloned sequences were performed with the Blast and Fasta programs (public domain), and comparisons and alignments were performed with the Genetic Computer Group Sequence Analyses Software Package (Genetics Computer Group, Madison, WI).

Northern blots and quantitative analysis of mRNA signal. Plasmids were digested with *Eco*RI, and inserts were agarose purified and extracted from the gel with QIAquick columns (Qiagen). Next 25 µg of DNA were random labeled (Megaprime; Amersham, Arlington Heights, IL) with α-[^{32}P]deoxycytidine 5′-triphosphate and purified with Nick Columns (Pharmacia, Piscataway, NJ). Total RNA (25 µg) from *ob*/+ and *ob/ob* was subjected to formaldehyde-agarose gel electrophoresis. Equal loading of samples was confirmed by ethidium bromide staining of ribosomal RNA bands. The RNA was transferred for 16 h to a nylon membrane, UV cross-linked (UV Stratalinker 2400; Stratagene, La Jolla, CA), prehybridized for 1 h at 42°C, and hybridized with α-[^{32}P]dCTP-labeled probes at 42°C for 16 h. Blots were washed under different degrees of stringency, beginning with the less stringent 2 $\times$ sodium chloride–sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), 42°C, and ending with the more stringent 0.1 $\times$ sodium chloride–sodium citrate, 0.1% sodium dodecyl sulfate, 60°C. Membranes were either exposed to X-OMAT film (Eastman-Kodak) and their signals quantified by densitometry or analyzed using the Molecular Dynamics PhosphoroImager (Sunnyvale, CA).Quantitative results are reported as the difference (in percentage of the value for the control group) between the value, in arbitrary units, of *ob*/+ and *ob/ob* mice. The same procedure was followed for Northern blots with polyA RNA. Possible variation of mRNA loading was tested by reprobing some of the blots for 36B4, a gene whose expression does not change under a variety of conditions (15). Differences in 36B4 levels between *ob*/+ and *ob/ob* in six separate blots varied from –16 to +11%. Therefore only changes in expression >20% were considered significant; all those <50% were reproduced in at least two independent blots.

RESULTS

Analysis of initial PCR display of *ob/ob* mouse muscle.

To identify alterations in gene expression associated with insulin resistance and type 2 diabetes, 180 differential display reactions were conducted using skeletal muscle of the *ob/ob* mouse and its lean *ob*/+ control (Fig. 1). From a total number of roughly 9,000 bands that could be visualized, there were 55 differences that were reproducible on duplicate determinations: 27 were visible in only the *ob*/+ and 28 were uniquely visible in only the *ob/ob* sample (Fig. 2). To identify these differentially displayed cDNAs, each band was cut from the gel,

TABLE 1
mRNAs overexpressed in *ob/ob* mouse skeletal muscle

OB number (size of cloned PCR product)	mRNA size (kb)	% change	Best match in GenBank
OB-10 (222 bp)	4.3	500	Geranylgeranyl pyrophosphate synthase:
	1.7	700	*N. crassa*, H (P: 1; 41 a; 60%; C-term)
OB-12 (279 bp)	1.1	35	PIG-PLD: human. H (N: 1; 102 bp; 83%; CDS–3′UTR). Bovine. H (P: 1; 39 a; 71%; C-term)
OB-13 (262 bp)	1.2	25	ID-2 protein: mouse I (N: 3′UTR)
OB-24 (380 bp)	3.5	60	No match
OB-29 (362 bp)	Smear 0.9–1.8	250	No match
OB-30 (301 bp)	1.3	30	22-kDa integral peroxisomal membrane protein: rat. H (N: 1; 274 bp; ~85%; CDS-3′UTR)
OB-48 (167 bp)	7.0	25	Fast skeletal muscle troponin T:
	3.4	30	rat. H (N: 1; 166 bp; ~95%; CDS/P: 1; 54 a; 96%)
	1.3	53	
OB-52 (205 bp)	1.4	60	Ribosomal protein L3: mouse. I (N: CDS-3′UTR/P: C-term)

H, homology; I, identity; N: *n*, homology at the nucleotide sequence and number of matched regions; bp, number of matched base pairs; *n*%, homology; CDS, coding DNA sequence; P: *n*, homology at the protein level and number of matched regions; a, number of matched amino acids; C-term, COOH-terminal.

reamplified by PCR, cloned into the PCR II Vector (Invitrogen), and used for sequence determination and Northern blot analysis. This was successfully achieved for 17 bands. In all of these, the sequence of the primers used for the differential display was present. In five cases, the sequence matched exactly those of mouse cDNAs already cloned (Tables 1–3). In four cases (OB-10, -12, -17, and -30), the sequences either matched cDNA sequences or contained open reading frames (ORFs) that were homologous to proteins cloned in other organisms. The remaining eight sequences (OB-1, -2, -11, -24, -26, -29, -42, and -49) did not match any of those present in the cDNA databases.

Characterization of clones overexpressed in *ob/ob* mouse muscle (Table 1)

OB-10 is the mouse homolog of geranylgeranyl pyrophosphate synthase. OB-10 was overexpressed in the muscle of *ob/ob* mouse in the differential display (Fig. 2). Northern blot of 2 µg polyA RNA from liver and skeletal muscle showed two mRNA species of 4.3 and 1.7 kb in both tissues, but the level of expression varied (Fig. 4). The 4.3-kb band was the main message in muscle, whereas the 1.7-kb band was more abundant in liver. The expression of OB-10 was increased in skeletal muscle and liver of *ob* mice by 5- to 20-fold. The OB-10 sequence encoded an ORF of 67 amino acids with a homology of 60 and 50% to the COOH-terminals of geranylgeranyl pyrophosphate synthase from *Neurospora crassa* (16) and *Saccharomyces cerevisiae* (17). This enzyme catalyzes the synthesis of geranylgeranyl pyrophosphate, the isoprenoid moiety required in the post-translational modification of a large number of *ras*-related proteins, such as Rho, Rab and Rac. The OB-10 sequence also matched the 3′ end of a mouse expressed sequence tag (EST) (accession number D19344). By combining the sequence of OB-10 with this EST, an ORF of 130 amino acids homologous to the fungal and yeast geranylgeranyl pyrophosphate synthase could be obtained.

OB-13 corresponds to mouse Id2. Using the OB-13 probe and a Northern blot of polyA RNA from brain, liver, fat, and muscle, a single band of 1.2 kb in muscle and 1.4 kb in brain, liver, and fat was detected (Fig. 5). The message was upregulated by only 23% in muscle, but was increased by 2- and 11-fold in liver and fat, respectively. Although the increase in muscle was small, this was probably not due simply to fat contamination, since on Northern blots the isoform of Id2 mRNA expressed in these tissues is different. The expression of OB-13 in brain was decreased by 50%. The sequence of OB-13 matched with the 3′ untranslated region (UTR) of the mouse Id2 cDNA (18). Id2 is a member of a family of helix-loop-helix

TABLE 2
mRNAs underexpressed in *ob/ob* mouse skeletal muscle

OB number (size of cloned PCR product)	mRNA size (kb)	% change	Best match in GenBank
OB-11 (277 bp)	1.9	35	Retrovirus-related DNA (MuRRS): mouse. H (N: 8; ~250 bp; ~85%)
OB-17 (375 bp)	0.4	20	F_1F_0-ATP synthase (subunit *f*):cattle. H (N: 2; 297 bp; ~85%; CDS-3′UTR / P: 2; 67 a; ~80%; C-term)

H, homology; N: *n*, homology at the nucleotide sequence and number of matched regions; bp, number of matched base pairs; *n*%, homology; CDS, coding DNA sequence; P: *n*, homology at the protein level and number of matched regions; a, number of matched amino acids; C-term, COOH-terminal.

TABLE 3
mRNAs that change the expression pattern in *ob/ob* mouse skeletal muscle

OB number (size of cloned PCR product)	mRNA size (kb)	% change	Best match in GenBank
OB-15/25 (206/351 bp)	3.4	–75	Skeletal muscle α-tropomyosine:
	2.6	–30	mouse. I (N: CDS-3′UTR/P: C-term)
	1.5	+65	
OB-49 (190 bp)	1.4	+25	L1Md-14LH repetitive sequence:
	1.1	–25	mouse. H (N: 3; 182 bp; ~80%)

H, homology; N: *n*, homology at the nucleotide sequence and number of matched regions; bp, number of matched base pairs; *n*%, homology; CDS, coding DNA sequence; P: *n*, homology at the protein level and number of matched regions; C-term, COOH-terminal.

proteins (Id1 to 4), termed "Id" for inhibitors of differentiation. The Id proteins are negative regulators of the basic helix-loop-helix (bHLH) family of transcription factors that have been shown to play a key role in the differentiation of a number of eukaryotic cell lineages, including muscle (19). Id proteins bind to bHLH transcription factors forming heterodimers that are unable to bind DNA, in this way inhibiting their transcriptional activity (20).

OB-12 encodes a protein related to the phosphoinositol-glycan–specific phospholipase D. Two independent Northern blots analyses of 25 µg total RNA showed that the message level of OB-12 was increased by ~30–40% (Fig. 6*A*). The OB-12 sequence had no significant matches in DNA databases in its 5′ to 3′ direction of translation, but its complementary sequence showed high homology with the end of the coding region and beginning of 3′ UTR of two PIG-PLD cDNAs cloned from human and bovine liver (21). This was due to the fact that instead of annealing with the polyA tail of the sense strand, the anchored primer annealed with an internal region reach in adenines on the antisense strand in an inverted position, thereby allowing for amplification of an inverted sequence. The complementary sequence of OB-12 encodes the COOH-terminal 63 amino acids of a protein, the last 39 of which were 71% homologous to the COOH-terminal of the bovine and human PIG-PLD. The remaining 24 amino acids encoded in this cDNA had no homology with the GenBank sequence, suggesting that it is either a different isoform or that there are significant species differences.

PIG-PLDs are enzymes that can specifically cleave the glycosyl phosphatidylinositol anchor that maintains many proteins associated with the external face of the cell membrane (22). Although the enzyme has been purified from serum and two cDNAs have been cloned from human and bovine tissues, the physiological function of this protein is uncertain (23). However, phosphatidylinositol-glycan–specific phospholipases have been suggested to play a role in generation of some mediators of insulin action (24).

OB-30 corresponds to the mouse 22-kDa integral peroxisomal membrane protein mRNA. OB-30 was overexpressed in *ob/ob* mouse on duplicate Northern blots by ~30% (Fig. 6*A*). The DNA sequence of OB-30 had an 85% homology with the 3′ end of the coding region and the 3′ UTR of a cDNA encoding the rat 22-kDa integral peroxisomal membrane protein (25) and the 3′ end of the mouse coding sequence (26). This protein is the major component of liver peroxisomes and is thought to be responsible for the permeability of the peroxisomal membrane (27).

OB-52 corresponds to the mouse ribosomal protein L3. OB-52 showed a perfect match with the end of the coding region (last 48 amino acids) and the 3′ UTR of the mouse ribosomal protein L3 (28), and was overexpressed on Northern blots of 25 µg total RNA by 60% (Fig. 6*A*). L3 is the largest protein of the 60S ribosomal subunit. It is at the center of the channel through which the nascent peptide emerges from the ribosome (29), and has been reported to be part of the peptidyltransferase complex (30). The functional relevance of L3 is underscored by the fact that in yeast a mutation on this protein is able to confer resistance to antibiotics (e.g., trichodermin, anisomicin) that inhibit protein synthesis in eukaryotic cells (31).

OB-48 is the mouse fast skeletal muscle troponin T. Northern blot analysis using OB-48 as probe revealed three bands at 7.0, 3.4, and 1.3 kb, the latter being the dominant message. All of these mRNA species were overexpressed in muscle of the *ob/ob* mouse by 25–53% (Fig. 6*C*). The sequence of OB-48 encodes an ORF of 55 amino acids with a 95% match to the DNA of rat fast skeletal muscle troponin T cDNA. The

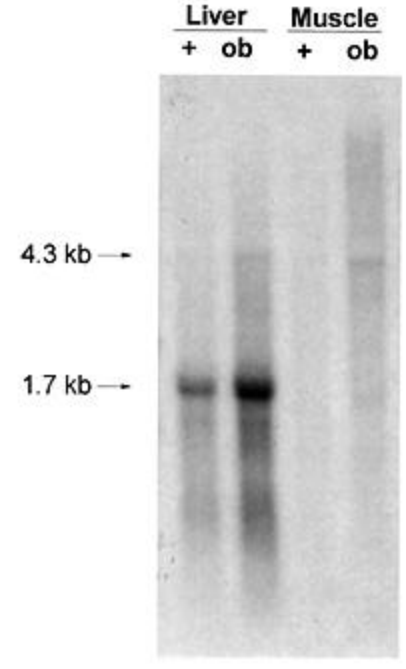

FIG. 3. Northern blot analysis for OB-10 mRNA expression. Shown are 2 µg of PolyA RNA from skeletal muscle and liver of lean (+) and obese (ob) mice used in a Northern blot and probed with a random primed probe made to the mRNA (i.e., mouse geranylgeranyl pyrophosphate synthase OB-10).

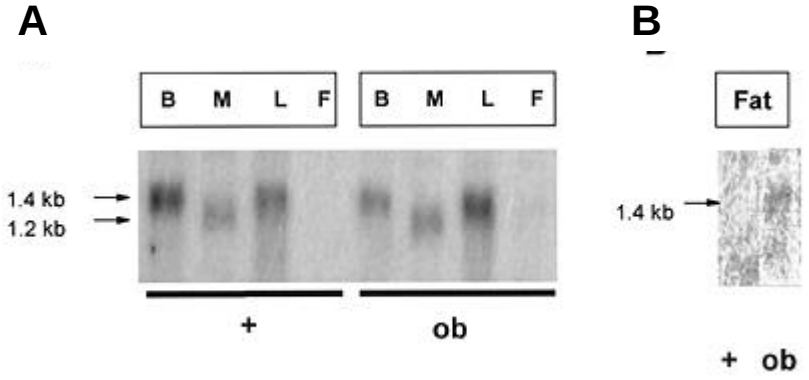

FIG. 4. Northern blot analysis for OB-13 (Id2 protein) mRNA expression. *A*: There were 2 µg of polyA RNA from brain (B), skeletal muscle (M), liver (L), and white adipose tissue (F) of lean (+) and obese (ob) mice used in a Northern blot probed with a random primed probe to OB-13 mRNA. *B*: Analysis of the same experiment as in *A*, except that exposure has been increased to make visible the bands in adipose tissue.

encoded region corresponds to exons 11 and 12 of the rat gene, which are represented in all described isoforms of the protein (32). Troponin T is a sarcomeric protein that binds tropomyosin and, together with troponin I and C, controls the calcium-dependent interaction of actin and myosin that results in muscle contraction.

OB-29 contains a B1 element. The last 100 bases of OB-29 contain a B1 sequence that produced 428 matches in a DNA search with the Blast program. The remaining 264 bases had no match in DNA databases. On Northern blot analysis, OB-29 gave a smeary signal between 1.8 and 0.9 kb; the main component at 1.8 kb was increased by 2.5-fold in the *ob*/*ob* mouse (Fig. 6*D*). B1 elements are the murine homolog of human *Alu* sequences. They are found in the UTRs of many mRNAs and as small cytoplasmic transcripts (33).

Characterization of clones underexpressed in *ob*/*ob* mouse (Table 2)

OB-17 encodes subunit f of mouse F_1F_0-ATP synthase. OB-17 showed only a 25% reduction on duplicate Northern blot analysis (Fig. 6*B*). OB-17 encoded an ORF of 67 amino acids that is 82% identical with the COOH-terminal of the *f*-subunit of mitochondrial F_1F_0-ATP synthase from cattle (34). The 5′ end of the DNA sequence also overlapped with the 3′ 91 bases of a 144-base mouse EST (GenBank accession number z31049). By extending our sequence with this tag sequence, an ORF of 86 amino acids could be identified that was 94% homologous with bovine F_1F_0-ATP synthase.

Mitochondrial ATP synthase is the central enzymatic complex of mitochondrial oxidative phosphorylation. Subunit *f* is a transmembrane protein of the internal membrane of the mitochondria and is part of the membrane-associated part of the complex F_0 (34,35). Mitochondrial ATP synthase has also been shown to play a central role in coupling fuel metabolism to insulin secretion in the β-cell, and its activity is decreased in diabetic rats. For these reasons it has been considered as a candidate molecule for diabetes (36).

OB-11 contains a long-terminal repeat sequence, but does not match with known genes or proteins. OB-11 was underexpressed on repeated Northern blots by 30–40% (Fig. 6*B*). OB-11 did not match any known genes. There is a polyadenylation signal 18 bases upstream of the polyA tail, suggesting that it corresponds to the 3′ end of an mRNA mol-

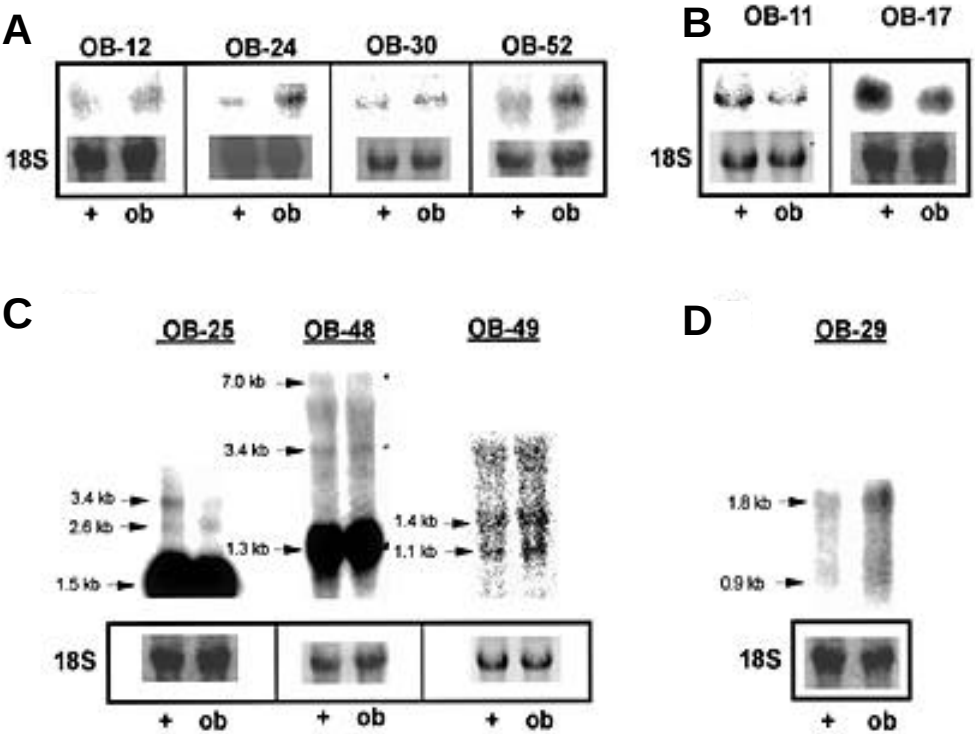

FIG. 5. Northern blot analysis of differentially expressed cDNAs. We analyzed 25 µg of total RNA from skeletal muscle of lean (+) and obese (ob) mice by Northern blot using random primed probes to each of the indicated clones. The ethidium bromide–stained bands for the 18S ribosomal RNAs are shown under the band or bands detected on the autoradiography. *A*: Example of OB mRNAs that were overexpressed in *ob* mouse muscle and detected as a single band. *B*: Examples of OB mRNAs that were underexpressed in *ob* mouse muscle and detected as a single band. *C*: Examples of OB mRNAs detected as multiple bands. In the case of OB-48 (skeletal muscle troponin), all three bands were increased in *ob* mouse, whereas OB-25 (α-tropomyosin) and OB-49 (a novel gene) showed a change in the pattern of expression. *D*: A smeary signal was detected for OB-29 (new gene), which was clearly overexpressed in the *ob* mouse.

ecule. There was no obvious ORF encoded in its sequence. OB-11 sequence matched with genes that contained long-terminal repeat-insertion sequence elements.

Characterization of clones associated with changes in mRNA expression pattern in the *ob/ob* mouse (Table 3)

OB-15 and 25 were alternatively spliced transcripts of the α-tropomyosin gene. The OB-25 sequence matched with the coding region (last 48 amino acids) and the 3′ UTR of an isoform of mouse skeletal muscle α-tropomyosin. A polyadenylation signal was present 22 bases upstream of the 3′ end. The cDNA sequence reported in GenBank for this isoform has a 3′ extension of 67 bases, with a second polyadenylation signal 17 bases upstream of the 3′ end (37). OB-15 corresponds to the last 206 bases of OB-25, encoded in exon 12. OB-15 was overexpressed but OB-25 was underexpressed in the differential display. On Northern blot analysis using OB-25 as a probe, three bands of 3.4, 2.6, and 1.5 kb were detected (Fig. 6*C*). A 1.5-kb band, which is the main component, was overexpressed by about 66% in the *ob* mouse, whereas the 2.6 and 3.4 bands were underexpressed by 73 and 29%, respectively. This complex pattern of expression is most likely related to the way α-tropomyosin isoforms are generated.

α-Tropomyosins are a group of actin-binding proteins encoded by a single gene that is expressed in muscle and non-muscle cells. In skeletal muscle, tropomyosins are part of the sarcomere, the fibrillar structure that performs skeletal muscle contraction. In association with the troponin complex, tropomyosin regulates the calcium-sensitive interaction of actin and myosin. The mouse and rat α-tropomyosin genes have 13 exons (37,38). At least nine isoforms are derived from the rat α-tropomyosin gene by use of alternative promoters, splicing, and polyadenylation sites (38,39). OB-25 corresponds to part of exon 10 and exons 11 and 12, and OB-15 corresponds to exon 12.

OB-49 does not correspond to any known gene. OB-49 does not have a polyadenylation signal nor does it encode an obvious ORF. Its sequence contains an L1Md-14LH element that produces a number of matches in DNA searches. OB-49 was underexpressed in *ob/ob* mouse by differential display and gave two bands in the Northern blot: one at 1.5 kb that was overexpressed in the *ob* mouse by 25% and another at 1.1 kb that was underexpressed by 26% (Fig. 6*C*). In this case, as for α-tropomyosin, this different regulation may be due to post-transcriptional modifications that change the pattern.

DISCUSSION

The identification of molecules that contribute to the insulin resistance seen with obesity is an important step in the elucidation of the pathogenesis of type 2 diabetes. Although insulin resistance is one of the earliest detectable defects in longitudinal studies of type 2 diabetes and appears to be genetically linked, thus far no single gene has been shown to play a determinant role in insulin resistance of a significant number of diabetic patients. The results obtained in the current study using mRNA differential display and the best characterized rodent model of obesity-related type 2 diabetes, the *ob/ob* mouse, indicate the broad range of secondary modifications in gene expression that occur in muscle.

Twelve changes in gene expression were found in the ~9,000 mRNAs examined. The messages detected by this technique represent genes with every level of expression, from very high (skeletal muscle troponin T) to very low (Id2). The ability of the differential-display method to detect rare messages implies that the amplification of cDNAs is dependent mainly on the primers sequence and not on the template concentration (40). There was no redundancy among the identified genes, with the exception of OB-15 and OB-25, which both correspond to transcripts from the α-tropomyosin gene but appear to correspond to different messengers from this gene, one of which is overexpressed and the other of which is underexpressed.

One of the most interesting of the overexpressed genes is Id2, a gene product that regulates the activity of a set of bHLH transcription factors, including ubiquitously expressed factors such as E12/47 and tissue specific factors such as MyoD, the latter of which controls differentiation of a number of cells, including skeletal muscle (18). Our results showed that Id2 is overexpressed in fat, liver, and muscle and underexpressed in brain in *ob/ob* mice. The difference of expression in muscle was small in comparison with the difference found in fat, but was not due to contamination of muscle with adipose tissue, since the size of the message in both tissues is different. Preliminary experiments also suggest that the isoform of the protein that is expressed may be different.

Two of the mRNAs found in this differential display, fast skeletal muscle troponin T and skeletal muscle α-tropomyosin, are developmentally regulated genes that are constitutively expressed in the terminally differentiated muscle cells (41). The tissue specificity of fast skeletal muscle troponin T expression is determined at the level of transcription and by alternative splicing during development (32). Cell type–specific regulation of expression for α-tropomyosin takes place at multiple levels by use of alternative promoters, splicing, and polyadenylation sites (38,39). In the case of skeletal muscle troponin T, the increase in all the messages detected by Northern blot is compatible with an increased transcription in the *ob* mouse. For α-tropomyosin, three independent facts suggest that in the *ob* mouse there are post-transcriptional changes: *1*) the expression of OB-15 (exon 12) in the differential display was increased, whereas expression of OB-25 (exons 10, 11 and 12) was decreased; *2*) the sequences of both cDNAs show a processing of the 3′ end that corresponds to the brain isoform-2 instead of the skeletal muscle isoform, suggesting that the transcripts amplified by the PCR and differentially displayed do not correspond to the major isoform in skeletal muscle; and 3) in the *ob* mouse, the pattern of transcripts that hybridize in the Northern blot with a probe specific for the skeletal muscle isoform was changed, with a decrease in bands corresponding to minor mRNA species, whereas the major form was overexpressed.

We also found that subunit *f* of mitochondrial ATP synthase is underexpressed in skeletal muscle of the *ob* mouse. F_1F_0-ATP synthase is the central enzyme complex of mitochondrial oxidative phosphorylation and contains subunits encoded in nuclear DNA, such as subunit *f*, as well as subunits encoded in mitochondrial DNA. The expression of these needs to be coordinated to produce a functional enzyme. Although the mitochondrial genes are considered housekeeping genes, their transcription is regulated during differentiation and in response to specific stimuli, such as continuous muscle contraction. Previous results from our group have shown that in the diabetic state, including

the *ob* mouse, the coordination between nuclear and mitochondrial expression is lost (11). The expression of some mitochondrial-encoded components of the oxidative phosphorylation chain is upregulated, whereas the expression of cytochrome oxidase 7a, which is encoded in the nucleus, shows no change or is decreased, as it is the case for subunit *f*.

OB-10 and OB-12 encode two proteins that may be related with insulin resistance. OB-10 (including a sequence from the EST database) contains a characteristic motif for prenyltransferases (42) and corresponds to mouse geranylgeranyl pyrophosphate synthase, a gene previously cloned only in the fungus *N. crassa* and in the yeast *S. cerevisiae* (16,17). Although this protein has been purified from bovine brain, and its enzymatic activity and tissue distribution characterized (43,44), this is the first cloning of the mammalian homologene. The tissue distribution of mRNA expression supports the fact that OB-10 corresponds to the mouse geranylgeranyl pyrophosphate synthase.

Protein isoprenylation (farnesylation and geranylgeranylation) plays a critical role in the function of many proteins that regulate cellular processes, including cell growth, vesicular trafficking, and actin network integrity (45). Among the many protein families that are geranylgeranylated, the Rab and Rho families of small GTPases can be directly related with insulin action through their role in vesicular traffic (46) and effects on the cytoskeleton (47). The overexpression of geranylgeranyl pyrophosphate synthase in muscle and liver of the *ob* mouse would imply alteration of a general regulatory mechanism for the cell in this animal model of insulin resistance.

OB-12 shows a high homology with the bovine PIG-PLD. The homology is limited to the COOH-terminal of the protein, a domain related to the enzymatic activity (48). The phosphatidylinositol-glycan anchors proteins to the external face of the cell membrane and is present in all types of eukaryotic cells. Among the proteins known to be anchored by this glycolipid in mammalian cells are hydrolytic enzymes (e.g., alkaline phosphatase), adhesion molecules, lymphocyte differentiation antigens, and transforming growth factor-β2 receptor (49). The biological function of PIG-PLD is unclear because the purified enzyme is unable to hydrolyze the glycosyl-phosphatidylinositol anchor (GPI) in vivo (50). Nevertheless the existence of an anchor-specific phospholipase has been inferred because some of the anchored proteins are present in soluble form; in addition, there is experimental evidence to support the existence of a cell-associated PIG-PLD activity (51). Thus far no such protein has been purified or cloned. The high homology at a protein level of OB-12 with the COOH-terminal of both bovine and human PIG-PLD makes it a suitable candidate for a cell-associated phospholipase specific of the GPI anchor. Apart from its function as a protein anchor, GPI has been implicated in signal transduction as a substrate for the generation of putative mediators of signaling produced in response to insulin (24), nerve growth factor (52), and interleukin-2 (53).

In summary, using the mRNA differential display method, we identified 12 changes in gene expression in skeletal muscle of the *ob*/*ob* mouse model of type 2 diabetes. This animal model of diabetes is clearly linked to obesity with a primary genetic defect in leptin synthesis (54), but is characterized by severe insulin resistance. The results of the present study indicate the extensive nature of secondary acquired changes in gene expression that may develop in the pathogenesis of this disease. Although none of the identified genes is known to be directly involved in insulin signaling, several of them affect a variety of important cellular functions that could affect the insulin responsiveness of the cell. These include major regulators of transcriptional activity, protein prenylation, and membrane anchoring. Future studies should determine not only the impact of these changes on metabolic pathways, but also the possibility of reversing these alterations in the *ob*/*ob* mouse with dietary restriction or leptin treatment to explore the mechanisms involved in producing the changes described herein and their physiological relevance.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant DK-45935 (C.R.K.), an American Diabetes Association Research Grant (E.M.-F.), and Joslin's Diabetes and Endocrinology Research Center Grant P30-DK-36836. D.V. was the recipient of a fellowship from the Spanish Ministerio de Educacion y Ciencia.

The authors wish to thank Dr. Daquing Qu for helpful suggestions and useful discussions, Roger McCarrick and Jennifer Gillette from the Joslin Diabetes Center DNA Core Facility, the Joslin's Animal Facility, and Terri-Lyn Bellman and Natalie Zahr for secretarial assistance.

REFERENCES

1. Warram JH, Martin BC, Krolewski AS, Soeldner JS, Kahn CR: Slow glucose removal rate and hyperinsulinemia are predictors of the risk of type II diabetes in offspring of diabetic parents. *Ann Intern Med* 113:909–915, 1990
2. Lillioja S, Mott DM, Spraul M, Ferraro R, Foley JE, Ravussin E, Knowler WC, Bennett PH, Bogardus C: Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus: prospective studies of Pima Indians. *N Engl J Med* 329:1988–1992, 1993
3. Bouchard C: Genetics of obesity in humans: current issues. *Ciba Found Symp* 201:108–115, 1996
4. Granner DK, O'Brien RM: Molecular physiology and genetics of NIDDM: importance of metabolic staging. *Diabetes Care* 15:369–395, 1992
5. Urhammer SA, Rasmussen SK, Kaisaki PJ, Oda N, Yamagata K, Moller AM, Fridberg M, Hansen L, Hansen T, Bell GI, Pedersen O: Genetic variation in the hepatocyte nuclear factor-1 alpha gene in Danish Caucasians with late-onset NIDDM. *Diabetologia* 40:473–475, 1997
6. O'Rahilly S, Choi WH, Patel P, Turner RC, Flier JS, Moller DE: Detection of mutation in insulin-receptor gene in NIDDM by analysis of single-stranded conformation polymorphisms. *Diabetes* 40:777–782, 1991
7. Almind K, Bjorbaek C, Vestergaard H, Hansen T, Echwald SM, Pedersen O: Amino acid polymorphisms of insulin receptor substrate-1 in non-insulin-dependent diabetes mellitus. *Lancet* 342:828–832, 1993
8. Ludwig DS, Vidal-Puig A, O'Brien RM, Printz RL, Granner DK, Moller DE, Flier JS: Examination of the phosphoenolpyruvate carboxykinase gene promoter in patients with noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 81:503–506, 1996
9. Bjorbaek C, Echwald SM, Hubricht P, Vestergaard H, Hansen T, Zierath J, Pedersen O: Genetic variants in promoters and coding regions of the muscle glycogen synthase and the insulin-responsive GLUT4 genes in NIDDM. *Diabetes* 43:976–983, 1994
10. Garvey WT, Maianu L, Hancock JA, Golichowski AM, Baron AD: Gene expression of GLUT4 in skeletal muscle from insulin-resistant patients with obesity, IGT, GDM, and NIDDM. *Diabetes* 41:715–722, 1992
11. Antonetti DA, Reynet C, Kahn CR: Increased expression of mitochondrial-encoded genes in skeletal muscle of humans with diabetes mellitus. *J Clin Invest* 95:1383–1388, 1995
12. Reynet C, Kahn CR: Rad: A member of the ras family overexpressed in muscle of type II diabetic humans. *Science* 262:1441–1444, 1993
13. Liang P, Pardee AB: Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967–971, 1992
14. Qu D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, Cullen MJ, Mathes WF, Przypek J, Kanarek R, Maratos-Flier E: A role for melanin-concentrating hormone in the central regulation of feeding behavior. *Nature* 380:243–247, 1996
15. Aiello LP, Robinson GS, Lin YW, Nishio Y, King GL: Identification of multiple

genes in bovine retinal pericytes altered by exposure to elevated levels of glucose by using mRNA differential display. *Proc Natl Acad Sci U S A* 91:6231–6235, 1994
16. Carattoli A, Romano N, Ballario P, Morelli G, Macino G: The *Neurospora crassa* carotenoid biosynthetic gene (albino 3) reveals highly conserved regions among prenyltransferases. *J Biol Chem* 266:5854–5859, 1991
17. Jiang Y, Proteau P, Poulter D, Ferro-Novick S: BTS1 encodes a geranylgeranyl diphosphate synthase in Saccharomyces cerevisiae. *J Biol Chem* 270:21793–21799, 1995
18. Sun XH, Copeland NG, Jenkins NA, Baltimore D: Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. *Mol Cell Biol* 11:5603–5611, 1991
19. Olson EN, Klein WH: bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes Dev* 8:1–8, 1994
20. Benezra R, Davis RL, Lockshorn D, Turner DL, Weintraub H: The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* 61:49–59, 1990
21. Scallon BJ, Fung WJ, Tsang TC, Li S, Kado-Fong H, Huang KS, Kochan JP: Primary structure and functional activity of a phosphatidylinositol-glycan-specific phospholipase D. *Science* 252:446–448, 1991
22. Cross GA: Glycolipid anchoring of plasma membrane proteins. *Annu Rev Cell Biol* 6:1–39, 1990
23. Davitz MA, Hereld D, Shak S, Krakow J, Englund PT, Nussenzweig V: A glycan-phosphatidylinositol-specific phospholipase D in human serum. *Science* 238:81–84, 1987
24. Saltiel AR, Fox JA, Sherline P, Cuatrecasas P: Insulin-stimulated hydrolysis of a novel glycolipid generates modulators of cAMP phosphodiesterase. *Science* 233:967–972, 1986
25. Kaldi K, Diestelkotter P, Stenbeck G, Auerbach S, Jakle U, Magert HJ, Wieland FT, Just WW: Membrane topology of the 22 kDa integral peroxisomal membrane protein. *Growth Regul* 315:217–222, 1993
26. Bryant DD, Wilson GN: Differential evolution and expression of murine peroxisomal membrane protein genes. *Biochem Mol Med* 55:22–30, 1995
27. VanVeldhoven PP, Just WW, Mannaerts GP: Permeability of the peroxisomal membrane to co-factors of beta-oxidation: evidence for the presence of a pore-forming protein. *J Biol Chem* 262:4310–4318, 1987
28. Peckham I, Sobel S, Comer J, Jaenisch R, Barklis E: Retrovirus activation in embryonal carcinoma cells by cellular promoters. *Genes Dev* 3:2062–2071, 1989
29. Kisilevsky R, Gore J: A comparison of accessibility of ribosomal proteins on free and membrane-bound ribosomes: the ribosomal proteins potentially involved in ribosome-membrane binding. *Biochim Biophys Acta* 910:282–291, 1987
30. Fabijanski S, Pellegrini M: Identification of proteins at the peptidyl-tRNA binding site of rat liver ribosomes. *Mol Gen Genet* 184:551–556, 1981
31. Fried HM, Warner JR: Cloning of yeast gene for trichodermin resistance and ribosomal protein L3. *Proc Natl Acad Sci U S A* 78:238–242, 1981
32. Breitbart RE, Nadal-Ginard B: Complete nucleotide sequence of the fast skeletal troponin T gene: alternatively spliced exons exhibit unusual interspecies divergence. *J Mol Biol* 188:313–324, 1986
33. Jelinek WR, Schmid CW: Repetitive sequences in eukaryotic DNA and their expression. *Annu Rev Biochem* 51:813–844, 1982
34. Collinson IR, Runswick MJ, Buchanan SK, Fearnley IM, Skehel JM, Van Raaij MJ, Griffiths DE, Walker JE: F_0 membrane domain of ATP synthase from bovine heart mitochondria: purification, subunit composition, and reconstitution with F_1-ATPase. *Biochemistry* 33:7971–7978, 1994
35. Belogrudov GI, Tomich JM, Hatefi Y: ATP synthase complex: proximities of subunits in bovine submitochondrial particles. *J Biol Chem* 270:2053–2060, 1995
36. Matschinsky FM: Banting Lecture 1995: a lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. *Diabetes* 45:223–241, 1996
37. Schleef M, Zuhlke C, Schoffl F, Jockusch H: Subtractive cDNA cloning as a tool to analyze secondary effects of a muscle disease: characterization of affected genes in the myotonic ADR mouse. *Neuromuscul Disord* 4:205–217, 1994
38. Wieczorek DF, Smith CW, Nadal-Ginard B: The rat alpha-tropomyosin gene generates a minimum of six different mRNAs coding for striated, smooth, and non-muscle isoforms by alternative splicing. *Mol Cell Biol* 8:679–694, 1988
39. Lees-Miller JP, Goodwin LO, Helfman DM: Three novel brain tropomyosin isoforms are expressed from the rat α-tropomyosin gene through the use of alternative promoters and alternative RNA processing. *Mol Cell Biol* 10:1729–1742, 1990
40. Wan JS, Sharp SJ, Poirier GMC, Wagaman PC, Chambers J, Pyati J, Hom YL, Galindo JE, Huvar A, Peterson PA, Jackson MR, Erlander MG: Cloning differentially expressed mRNAs. *Nat Biotechnol* 14:1685–1695, 1996
41. Wade R, Sutherland C, Gahlmann R, Kedes L, Hardeman E, Gunning P: Regulation of contractile protein gene family mRNA pool sizes during myogenesis. *Dev Biol* 142:270–282, 1990
42. Chen A, Kroon PA, Poulter CD: Isoprenyl diphosphate synthases: protein sequence comparisons, a phylogenetic tree, and predictions of secondary structure. *Protein Sci* 3:600–607, 1994
43. Sagami H, Morita Y, Ogura K: Purification and properties of geranylgeranyldiphosphate synthase from bovine brain. *J Biol Chem* 269:20561–20566, 1994
44. Ericsson J, Runquist M, Thelin A, Andersson M, Chojnacki T, Dallner G: Distribution of prenyltransferases in rat tissues: evidence for a cytosolic all-trans-geranylgeranyl diphosphate synthase. *J Biol Chem* 268:832–838, 1993
45. Farnsworth CC, Gelb MH, Glomset JA: Identification of geranylgeranyl-modified proteins in HeLa cells. *Science* 247:320–322, 1990
46. Cormont M, Tanti JF, Zahraoui A, Van Obberghen E, Tavitian A, Le Marchand-Brustel Y: Insulin and okadaic acid induce Rab4 redistribution in adipocytes. *J Biochem* 268:19491–19497, 1994
47. Tapon N, Hall A: Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. *Curr Opin Cell Biol* 9:86–92, 1997
48. Stadelmann B, Butikofer P, Konig A, Brodbeck U: The C-terminus of glycosylphosphatidylinositol-specific phospholipase D is essential for biological activity. *Biochim Biophys Acta* 2:107–113, 1997
49. Hirose S, Knez JJ, Medof ME: Mammalian glycosylphosphatidylinositol-anchored proteins and intracellular precursors. *Methods Enzymol* 250:582–614, 1995
50. Low MG, Huang KS: Factors affecting the ability of glycosylphosphatidylinositol-specific phospholipase D to degrade the membrane anchors of cell surface proteins. *Biochem J* 279:483–493, 1991
51. Metz CN, Brunner G, Choi-Muira NH, Nguyen H, Gabrilove J, Caras IW, Altszuler N, Rifkin DB, Wilson EL, Davitz MA: Release of GPI-anchored membrane proteins by a cell-associated GPI-specific phospholipase D. *EMBO J* 13:1741–1745, 1994
52. Chan BL, Chao MV, Saltiel AR: Nerve growth factor stimulates the hydrolysis of glycosylphosphatidylinositol in PC-12 cells: a mechanism of protein kinase C regulation. *Proc Natl Acad Sci U S A* 86:1756–1760, 1989
53. Merida I, Pratt JC, Gaulton GN: Regulation of interleukin 2–dependent growth responses by glycosylphosphatidylinositol molecules. *Proc Natl Acad Sci U S A* 87:9421–9425, 1990
54. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425–432, 1994