Quantitative Expression Analysis of Genes Regulated by Both Obesity and Leptin Reveals a Regulatory Loop between Leptin and Pituitary-derived ACTH*

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Absence of the hormone leptin leads to dramatic increases in appetite, food intake, and adiposity. The primary site of action, at least with respect to appetite, is the hypothalamus. Leptin also has significant effects on the function(s) of peripheral organs involved in maintaining body composition. Some of these effects are mediated through direct interaction of leptin with its receptor on the target tissue, and some effects are indirectly mediated through secondary hormonal and neural pathways. Few of the genes that are responsible for regulating body composition and the peripheral effects of leptin are known. We have used a new gene profiling technology to characterize gene expression changes that occur in the pituitary, hypothalamus, fat, muscle, and liver in response to both obesity and treatment with exogenous leptin. These differences were then overlaid to allow the identification of genes that are regulated by obesity and at least partially normalized by leptin treatment. By using this process we have identified five genes (POMC, PC2, prolactin, HSGP25L2G, and one novel) that are both abnormally expressed in the pituitaries of obese mice and are sensitive to the effects of leptin. We also show that adrenocorticotropic hormone appears to be involved in a regulatory loop involving leptin.

Leptin deficiency in humans (1) and rodents (2) leading to hyperphagia and massive obesity demonstrates that leptin plays a central role in the regulation of food intake and body composition. Leptin acts on the central mediators of appetite control. Thus, acute ventricular administration of leptin activates the STAT1 signaling pathway within relevant areas of the hypothalamus, induces expression of anorexigenic hormones, and suppresses food intake (for reviews on the central effects of leptin see Refs. 3 and 4). In addition to direct central effects, leptin has peripheral effects that appear to be independent of the reduction in food intake. One of the first indications of this came from the observation that weight loss in leptin-treated obese rodents was in excess of that seen in mice that ate the same amount of an identical diet as the leptin-treated group (5). The peripheral effects of leptin appear to include both indirect effects mediated through hypothalamic changes and direct actions on the target tissues. In addition to the widely appreciated metabolic effects of leptin, this hormone also appears to impact fertility, angiogenesis, and the immune response. For recent reviews on the peripheral effects of leptin see Refs. 6 and 7.

The pituitary is clearly implicated in regulating whole body metabolism. Hypopituitary adults and children have decreased lean body mass and an increase in total body fat (8, 9). Although at least some of this effect of the pituitary appears to be the result of growth hormone deficiency, it may be that other pituitary-derived or -influenced hormones are relevant. For example, adrenalectomy in mice leads to resistance to the development of obesity (10), and patients with Cushings disease have an increased body mass index (11, 12). These data suggest that pituitary-derived factors such as ACTH could also be relevant to the increase in body mass index seen in hypopituitary patients. Prolactin has also been implicated in maintaining body composition (13). Although there are several compelling candidates for pituitary-derived factors that could impact body composition, it may be that not all of these have been identified.

Gene expression changes have been used widely in the last decade to identify genes that may be relevant to physiological processes. These have included differential display (14), representational difference analysis (15), serial analysis of gene expression (16), and more recently array-based technologies (17); for a recent review of these approaches see Carullie et al. (18). All of these have proved useful but have limitations in terms of the ability to detect reproducibly small differences between samples, the number of different mRNA species that can be sampled, and the ability to make multiple independent comparisons.

We report here on the use of quantitative expression analysis (QEA®) (19) to identify genes that may be of relevance to obesity and the mechanisms by which leptin regulates body composition. QEA relies on digestion of the cDNA genome into multiple fragments with pairs of restriction enzymes prior to a minimal number of amplification cycles and computer-assisted mass comparisons on high resolution sequencing acrylamide gels. This process allows an analysis with high sensitivity and analytical depth. Furthermore as the data are captured, analyzed, and stored electronically, multiple independent comparisons can be made. We have used this technology to examine gene expression changes that are relevant to obesity in five different organs (pituitary, hypothalamus, liver, muscle, and fat). Specifically we have quantitated transcripts that are expressed in these tissues and are differentially expressed in a
comparison between lean and obese mice. A further set of transcripts was identified as being differentially expressed in a comparison between mice (lean and obese) treated with leptin or vehicle. We have also identified transcripts that are common to these comparisons. We have further characterized a set of pituitary-expressed genes that are altered by obesity and are also at least partially normalized by leptin treatment.

**EXPERIMENTAL PROCEDURES**

**Animals**—Mice (C57Bl/6J obese (ob/ob) and lean (ob/+), +/+ litter-mates; 8 weeks of age; females) were purchased from The Jackson Laboratory. They were acclimated for 10 days prior to the beginning of the experiment. The mice were maintained on a 12-h (6:00 p.m. to 6:00 a.m.) darklight cycle, and food and water were provided *ad libitum*. Each mouse was weighed and dosed at 1 mg/kg with a leptin-IgG fusion protein that has been previously described (5). The mice were injected daily, and weights and food intake were measured. Weight gain over the 7 days was as follows: obese/PBS, 2.7 ± 0.3 g; obese/leptin, −3.5 ± 0.2 g; lean/PBS, 0.6 ± 0.1 g; lean/leptin, −0.4 ± 0.1 g. Food intake (per mouse over 7 days) was as follows: obese/PBS, 35 ± 0.8 g; obese/leptin, 20 ± 0.5 g; lean/PBS 23 ± 0.8 g; lean/leptin, 18.5 ± 0.5 g.

After seven daily injections the mice were sacrificed, and muscle (gastrocnemius), liver, fat (pooled peri-reinal and ovarian), pituitaries, and hypothalami were removed and snap-frozen on dry ice. RNA was extracted from the tissues as described below. A total of 240 mice (120 obese and 120 lean) in four groups were used. For each tissue three pools were prepared with each pool containing tissue from between 5 (liver) and 20 (pituitaries and hypothalamus) mice. RNA was prepared from each pool of tissue and analyzed as described below.

**Differential Gene Expression Analysis—**GeneCalling™ reactions were performed essentially as described (19). In brief, total cellular RNA was isolated with Trizol (Life Technologies, Inc.), and poly(A)+ RNA was prepared from 50 μg of total RNA using oligo(dT) magnetic beads (PerSeptive Diagnostics, Cambridge, MA), and first-strand cDNA was prepared from 1.0 μg of poly(A)+ using Superscript II reverse transcriptase (Life Technologies, Inc.). Subsequent to cDNA fragmentation, tagging and amplification steps, samples were loaded onto 5% polyacrylamide, 6 M urea, 0.5× TBE ultrathin gels and electrophoresed on a Niagara instrument. PCR products are visualized by virtue of the nylon membrane. The filter was hybridized with a 32P-labeled 36-base nuclease probe corresponding to bases 551–586 of the i0 m0-307 transcript. The size of the fragments that map in end sequence and length to known mouse genes were used as templates for the design of unlabeled oligonucleotide primers. An unlabeled oligonucleotide designed against one end of the restriction fragments was added in excess to the original reaction re-amplified by PCR. This new reaction with the competing PCR primer was then electrophoresed and compared with a control reaction reamplified without the unlabeled oligonucleotide to evaluate the selective diminution of the peak of interest (19).

**Real Time Quantitative PCR—**RTQ-PCR was performed using an ABI7700 Sequence Detection System instrument and software (PE Applied Biosystems, Inc., Foster City, CA) as described (20, 21) using the primers described in Table I.

**Adipocyte Cultures—**Adipocytes were prepared from ovarian fat pads of 8-week-old fasted (2 h) female C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME) (22). Fat pads were minced in Krebs-Ringer HEPES buffer (pH 7.4, containing 200 mM adenosine, 5 mM glucose, 3% fraction V bovine serum albumin, 155 mM NaCl, 2.2 mM CaCl2, 1.25 mM MgSO4, 0.45 mM KH2PO4, 2.17 mM Na2HPO4, and 10 mM HEPES). Adipose tissue samples were digested in the same buffer in the presence of type I collagenase (1 mg/ml; Worthington) at 37 °C with gentle shaking (100 rpm) for 30 min. Isolated adipocytes were separated from undigested tissue by filtration through a 250 μm polypropylene mesh and washed three times. For washing, cells were centrifuged at 500 rpm for 3 min. Each time the infranatant was discarded, and cells were resuspended in Krebs-Ringer HEPES buffer with the final wash being in 5.5 mM glucose Dulbecco's modified Eagle's medium, with 5% bovine serum albumin, 20 mM HEPES, 1 unit/ml adenosine deaminase, and 10 μM (−)-N2-(2-phenylisopropyl)adenosine. Adipocytes were cultured in 48-well plates, 5 × 104 cells per 500 μl per well. Cells were cultured in quadruplicate with rat ACTH (100, 10, 1, and 0.1 nM; Sigma) or rat prolactin (100, 10, 1, and 0.1 nM; Accurate Chemical). Recombinant human insulin (1, 0.1 and 0.01 nM; Genentech) and isoproterenol (30, 10, 3 and 1 nM; Sigma) were added to separate wells as positive controls. Cells were incubated at 37 °C, 5% CO2 50 μl was sampled at 4 h and again at 16 h. Media glucose were measured by a hexokinase colorimetric assay (Sigma); media glycerol were measured by a glycerol kinase/oxidase colorimetric assay (Sigma), and media leptin were measured by enzyme-linked immunosorbent assay (Chemikamin). RNA was prepared from the adipocytes using commercially available material and protocols (RNA-STAT; Tel-Test, Inc., Friendswood, TX).

**Pituitary Cultures—**Pituitary cell cultures were prepared from whole pituitaries of 8-week-old female C57Bl/6J mice. Pituitaries were finely minced and then digested with 0.25% trypsin and 10 μM (−)-N2-(2-phenylisopropyl)adenosine. Digested pituitary samples were washed twice in low glucose Dulbecco’s modified Eagle's medium with 10% fetal bovine serum and then plated 300,000 cells per well in laminin-coated 6-well plates. Cells were incubated at 37 °C, 5% CO2 overnight before being treated with leptin. Murine leptin (Biomol) was added in quadruplicate with rat ACTH (100, 10, 1, and 1 nM) RNA was prepared from the pituitary using commercially available material and protocols (RNA-STAT).

**Northern Analysis—**Total RNA from liver, pituitary, and muscle from lean C57Bl/6J mice was prepared using Trizol (Life Technologies, Inc.), separated on a 1% agarose/formaldehyde gel, and transferred to a nylon membrane. The filter was hybridized with a 32P-labeled 36-base nuclease probe corresponding to bases 551–586 of the 10 m0-307 sequence. The filter was washed at 0.1% SSC at 42 °C and exposed to film for 18 h.

**RESULTS AND DISCUSSION**

**Gene Expression Differences in Response to Obesity and Leptin—**Gene expression profiling is becoming an increasingly important technique in biology, but currently used approaches are limited with respect to sensitivity, depth of analysis, or an inability to perform nested or multiple comparisons (for a recent review see Carulli et al. (18)). At least some of these limitations have been utilized to approach questions relevant to diabetes (23, 24) or obesity (25–27). For example Vincent et al. (27) were able to identify 12 genes differentially expressed in the muscles of ob/ob as compared with the muscles of lean mice. However, this binary comparison does not address whether these changes are causal components of the development of obesity in these mice or are correlates of increased adiposity. In

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**Table I**

| Gene         | Direction | Sequence                      |
|--------------|-----------|-------------------------------|
| RPL19        | Forward   | 5’ ATGTATCCACAGCGTCTAGTG     |
|              | Reverse   | 3’ TTTAGTTCAAAGGGAAGCCTCA    |
| POMC         | Forward   | 5’ AGCAACCGGCGCCAAGG         |
|              | Reverse   | 3’ GCCGTCGTCCTGCTCCTCGG      |
| PC2          | Forward   | 5’ CAGCCCGCGATATAACAAAGGCG  |
|              | Reverse   | 3’ GAAACCAGGGGCTGGTGTTG      |
| Prolactin    | Forward   | 5’ TGACCTGCAGAATGCTGACAGAC  |
|              | Reverse   | 3’ CGGAGAAGAACTCCTGCGACT    |
| Leptin receptor | Forward  | 5’ TGTGTTGTCGGAGAACCCGAA   |
|              | Reverse   | 5’ TCAAAACCGGCGATTGTTT      |
| HSGP25L2G    | Forward   | 5’ AACCTGTGTCCTTGGAGAATAAT  |
|              | Reverse   | 5’ GGTGATGTGGCGTTCAAATGTG   |
| Probe        | Forward   | 5’ AGGGTTGAGGGCGTCCTCCCTTTAA |

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*Gene Calling reactions were compared pairwise (i.e. ob/ob pituitary vs. lean pituitary) using software designed to detect the difference over certain threshold limits. Data base queries were performed using the information inherent to the sized fragments with ends defined by restriction digestion fragmentation.

**Gene Confirmation by Oligonucleotide Poisoning—**Restriction fragments that map in end sequence and length to known mouse genes were used as templates for the design of unlabeled oligonucleotide primers. An unlabeled oligonucleotide designed against one end of the restriction fragments was added in excess to the original reaction re-amplified by PCR. This new reaction with the competing PCR primer was then electrophoresed and compared with a control reaction reamplified without the unlabeled oligonucleotide to evaluate the selective diminution of the peak of interest (19).
Gene expression differences in response to obesity and leptin treatment

The indicated comparisons were made for each of the tissues, and the numbers of gene fragments that are different (at least 2-fold; \( p < 0.05 \)) in peak height between the two sets of cDNAs are reported. A secondary search was carried out to find those gene fragments that were altered by obesity and at least partially normalized by leptin. Note that for adipose tissue the number of approximately half the number of subsequent analyses was performed on the fat as compared with the other tissues leading to a proportional reduction in the number of gene fragments detected.

| Tissue (total no. gene fragments analyzed) | No. gene fragments that are different in the indicated comparisons | Gene fragments altered by obesity and leptin |
|------------------------------------------|---------------------------------------------------------------|------------------------------------------|
|                                          | Obese vs. lean | Leptin vs. vehicle (lean) | Leptin vs. vehicle (obese) |
| Pituitary (22,000)                       | 117 10 23 14   | 196 22 80 12         |
| Fat (12,250)                             | 158 29 44 18   | 196 22 80 12         |
| Muscle (26,300)                          | 587 32 110 58  | 82 32 73 5           |
| Liver (25,520)                           | 196 22 80 12   | 196 22 80 12         |
| Hypothalamus (27,000)                    | 117 10 23 14   | 196 22 80 12         |

In this report we describe the use of a novel gene profiling technology that allows an analysis at high sensitivity and increased depth of analysis. We are also able to use the electronic data capture capabilities to search for differences that are common to different experimental manipulations. This ability to search for differences that are present in biologically independent comparisons increases our ability to focus on those gene expression changes that may be more relevant to the underlying biology.

The effect of obesity and leptin administration on gene expression differences was examined using QEA. Five of the major tissues implicated in metabolic control (pituitary, hypothalamus, muscle, liver, and fat) were analyzed from each of four groups of female mice as follows: obese (ob/ob) treated with leptin; obese treated with vehicle (PBS); lean (ob/+ or +/+ ) treated with leptin, and lean treated with vehicle. For each tissue three pools were prepared with each pool containing tissue from between 5 (liver) and 20 (pituitary and hypothalamus) mice. RNA was prepared from each pool of tissue, and the cDNA derived from the RNA was analyzed using 96 pairs of restriction enzymes. Previous results indicate that this will allow the analysis of greater than 90% of the expressed genome with a sensitivity of detection greater than 1:100,000 (19).

Three binary comparisons were initially made, obese versus lean mice (both vehicle-treated), lean mice, vehicle versus leptin-treated and obese mice leptin versus vehicle-treated. A difference was called if the peak heights differed by more than 2-fold (\( p < 0.05 \)). The results of these comparisons are shown in Table II. As described under “Experimental Procedures” each gene fragment represents part of a gene and any one gene has the potential for generating multiple independent gene fragments; some genes will give rise to only one that is detectable, whereas others can give rise to 5–10. Results presented below, from Shimkets et al. (19), indicate that there is an approximate three to one ratio between gene fragments and represented genes. That the differences in peak height reflect a difference in expression of the underlying mRNA has been previously validated (19) and is further supported by data presented below.

RNA from each of the five tissues was analyzed by QEA, and the number of detectable gene fragments was determined (Table II). As described above, each gene fragment represents part of a particular cDNA, and so the number of gene fragments can be used as a measure of the number of genes expressed within the tissue. There is a large difference in the number of gene fragments detected in the fat as compared with the other tissue due to approximately half the number of QEA reactions having been performed for adipose compared with the other tissues. Given this, it is reasonable to expect a comparable number of gene fragments, and a corresponding increase in the number of differentially expressed bands to be observed for adipose in this experiment. By comparing peak heights for each gene fragment, it is also apparent that there are large differences in the number of genes that are responding to either obesity or leptin in the different tissues. Thus the liver is very sensitive to obesity with 587 gene fragments (2.3% of the total) changing more than 2-fold relative to the expression in lean liver. In contrast only 82 (0.3%) differences were detected in the hypothalamus. The other tissues are intermediate between these two, with 117 (0.5%) gene fragments changing in the pituitary, 158 (0.6%) in muscle, and 196 (1.6%) in fat. It should be noted that these assessments are drawn from the expression of the total tissue. As the liver is more homogenous than the hypothalamus in terms of cell type, the numbers of gene expression differences in the hypothalamus could be an underestimate. Fat and liver were the most responsive tissues to leptin treatment with approximately 0.6 and 0.4% of the genes changing more than 2-fold in response to a 1-week treatment. As described below this analysis does not address whether these are direct effects of leptin on the fat or liver as compared with leptin altering liver gene expression indirectly via, for example, alterations in proteins being delivered by the pituitary. There are more differences detected in response to leptin in the obese mice as compared with lean mice. This is comparable to what is seen with respect to the physiological responses (food intake and fat loss) in the same sets of mice (see “Experimental Procedures”).

Our primary goal for this experiment was the identification of those genes that are relevant to the development of obesity. The simple two-way comparisons described above will identify not only these more relevant genes but also those that are altered as a compensatory response to obesity and those that are altered by leptin but may be related to the reproductive effects of leptin (28). Thus we further analyzed the gene expression changes by searching for those genes for which expression was altered by obesity, and at the same time expression was returned toward the lean pattern of expression by a 1-week course of treatment with leptin. Only the obese mice treated with leptin were used for this comparison as they are more sensitive to the effect of leptin.

Between 6 and 12% of the gene fragments that differ between obese and lean mice are at least partially normalized by leptin treatment (Table II). Because of uncertainties relating to cellular heterogeneity and the relationship between gene fragment number and gene number, it is unclear if the differences between the tissues are significant. The converse of this analysis indicates that approximately 90% of the obesity-related differences are not significantly normalized by a 1-week course of treatment with leptin and points to the dangers inherent in a simple binary comparison for the detection of leptin-responsive genes. Possible reasons for the failure of 90% of the differences to normalize would include the length of treatment and irreversible alterations established by 8 weeks of leptin deficiency.

**Pituitary Genes That Respond to Obesity and Leptin**—In this study we have further analyzed the gene expression differences detected in the pituitary. Subsequent experiments and reports will describe findings in the other tissues. Gene profiling allowed the identification of 117 gene fragments that were differentially expressed in the pituitaries of lean in comparison to

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2 M. Renz, E. Tomlinson, B. Hultgren, N. Levin, Q. Gu, R. A. Shimkets, D. A. Lewin, and T. A. Stewart, unpublished observations.
obese mice. The minimum expression difference is 2-fold; the maximal differences cannot be accurately estimated owing to the low level of expression of some genes. Based on the ratio of gene fragments identified to genes represented (Table III and data not shown), it is estimated that the 117 gene fragments represent approximately 40 different genes. A comparable assessment indicates that we could detect the expression of approximately seven pituitary-expressed genes that are regulated by leptin (in obese mice). Fourteen of the 117 gene fragments altered by obesity were at least partially normalized by leptin. These 14 gene fragments are listed in Table III according to a nomenclature that is derived from the restriction fragments that were used to fragment the DNA and the size (in base pairs) of the fragment.

Gene identification on the 14 pituitary-derived gene fragments altered by both obesity and leptin treatment was carried out using a combination of oligonucleotide poisoning and the cloning and sequencing of gene fragments. By these approaches, the following 14 gene fragments corresponding to each of PC2 and POMC are shown in Fig. 1. Each of the four panels represents a comparison between either obese mice treated with leptin or PBS (upper panels) or between obese and lean mice treated with PBS (lower panels). Each data set is shown in a separate window and contains two or three traces. In some sets only two traces are shown as not all cDNA was successfully analyzed for each pair of restriction fragments, a minimum of two traces was required for subsequent analysis. Each trace is derived from one of the three independent pools of pituitaries. Each cDNA sample was analyzed in triplicate, and each trace represents the average of this triplicate.

Identification of the pituitary-expressed genes that respond to both obesity and leptin

QEA fragments and pituitary transcripts that change at least 2-fold with obesity and are at least partially normalized by leptin treatment. For three fragments (marked as unknown), repeated attempts at cloning failed.

| Gene | Gene fragment ID | -Fold change in expression | -Fold change induced by leptin |
|------|------------------|---------------------------|-------------------------------|
| PC2  | d0l0–154         | 7.8                       | 0.62                          |
|      | 0e1–277          | 2.1                       | 0.71                          |
|      | i0p0–185         | 4.2                       | 0.59                          |
|      | i0u0–303         | 3.7                       | 0.55                          |
| Unknown | g1n0–246     | 0.09                      | 2.68                          |
| Novel | i0n0–307         | 2.6                       | 0.75                          |
| Unknown | y0k0–253       | 2.8                       | 0.59                          |
| Unknown | i0u0–172       | 0.21                      | 2.15                          |
| Prolactin | m1n0–118      | 0.31                      | 1.73                          |
|      | r0v0–237         | 0.17                      | 2.08                          |
|      | m1s0–329         | 0.35                      | 1.41                          |
|      | r0v0–158         | 0.16                      | 3.42                          |
| POMC | m0r0–191         | 5.9                       | 0.5                           |
| HSGP25L2G | d0l0–136     | 6.5                       | 0.58                          |

**FIG. 1.** Pituitary expression of POMC and PC2 are regulated by obesity and by leptin. Shown are the QEA traces for RNA extracted from pools of pituitaries taken from obese animals treated with either leptin or PBS (upper panels) or PBS-treated obese and lean animals (lower panels). The left set of panels show the traces that surround the gene fragment d0l0-154; this gene fragment was subsequently shown to be derived from the cDNA encoding PC2. The right set of panels show traces that surround the gene fragment m0r0-191; this gene fragment was subsequently shown to be derived from the cDNA encoding POMC. Each trace shown is the mean of three analytical runs and each trace is derived from one of three independent RNA samples.
The reliability of QEA to detect gene expression differences has been previously documented (19). To increase confidence further in the data set, we have used real time quantitative PCR to characterize transcript levels for a representative set of the genes (PC2 and POMC). Again three pools of pituitaries were used (completely independent of the original experiment and containing five pituitaries/pool); the extracted RNA was analyzed using real time quantitative PCR. The results shown in Fig. 2 confirm that obesity increases the expression of both PC2 and POMC. The ΔCT values are given in Fig. 2A, and these data are used to generate the average relative expression (Fig. 2B).

We report here that the mRNA levels of both PC2 and POMC are increased in obese mice and decreased by leptin treatment. That peptides derived from the POMC precursor are important in maintaining body composition has been re-inforced by the observation that both humans (29) and mice (30) deficient in POMC develop obesity. This gene inactivation experiments do not, however, address the relative importance of either different peptides derived from the POMC gene or the different tissues that express POMC. PC2 (31) is one of the two major proteases that appear to be involved in processing the POMC precursor to smaller bioactive peptide hormones. Interestingly the two related proteases (PC1 and PC2) appear to have different substrate specificities and are expressed in different pituitary cell types (for review see Bertagna (32)). We do not yet know whether the effect of leptin on PC2 expression is limited to particular cell types. With the exception of ACTH the physiological function of the other POMC-derived peptides is still unclear. The possibility that a leptin-mediated differential processing of the POMC precursor could be physiologically relevant remains to be addressed. Whereas hypothalamic changes in POMC expression in response to leptin have been extensively investigated (33–35), there has been significantly less attention paid to the effect of leptin on POMC expression by the pituitary. It has been reported that both leptin and ACTH appear in the circulation in a negatively correlated pulsatile fashion (36). In contrast to this negative correlation between leptin and ACTH and the results described here, it has been reported that leptin induces release of ACTH from pituitary fragments in culture (37). There may be differences between the relatively acute effect of leptin as detected in the study by Raber et al. (37) and the more chronic effects reported here.

**Prolactin**—Four gene fragments derived from the prolactin cDNA were altered by both obesity and by leptin treatment. Thus obesity suppressed prolactin mRNA levels by 3–5-fold, and leptin increased prolactin mRNA levels by 2–3-fold in the obese mice (Table III). Previous results have correlated diminished circulating prolactin with obesity both in humans (13, 38–41) and in rodent models (42–44). One previous report indicated that leptin is able to induce prolactin secretion from cultured pituitaries (45) although this only occurred at high concentrations of leptin. That prolactin expression is both reduced by obesity and induced by leptin as reported here is consistent with a causal role for diminished prolactin in the development or maintenance of obesity. This possibility is also consistent with the observation that the anorexigenic fenfluramine increased prolactin release (46) and restored the abnormally low arginine-stimulated prolactin response to normal (47). The mechanisms by which lower prolactin levels could contribute to the development of obesity are not clear. Paradoxically, bromocriptine reduces both prolactin and body weight in obese patients (48). As bromocriptine also modulates serotonin and norepinephrine within the hypothalamus (49), the effects on obesity may be mediated through central rather than peripheral mechanisms.

**HSGP25L2G**—The fourth gene identified is the mouse homologue of *HSGP25L2G*. The expression of this gene was increased approximately 6-fold in obese mice and suppressed by 50% with leptin treatment. The encoded protein is one member of a family of proteins that appears to reside within the endoplasmic reticulum but has an unknown function (50). Interestingly the mRNA encoding one member of this family is coordinately expressed with POMC in Xenopus (51).

The gene fragment designated i0 m-0-307 was cloned and sequenced. The 307-base pair sequence obtained did not correspond to any sequence in the public data bases and did not contain any significant open reading frames. cDNA clones were obtained using standard hybridization protocols, and three independent cDNAs of approximately 1200 base pairs were sequenced. This sequence does not appear in public data bases and does not have any significant open reading frames. Northern hybridization analysis was used to estimate the size of the corresponding mRNA and to examine the tissue pattern of expression. As can be seen in Fig. 3a a transcript of approximately 1200 base pairs that hybridizes with the i0 m-0-307 sequence is expressed in liver and weakly in muscle as well as...
in the pituitary. Although it is possible that there are longer protein-encoding transcripts present at levels below the detection limit, it appears that this gene may not encode a protein. There is a precedent for this. The \( H19 \) gene also does not encode a protein but appears to contribute to the regulation of the closely linked gene encoding insulin-like growth factor-2 (52). Whether there is also a gene close to and regulated by \( m0-307 \) is under investigation. We have also investigated whether the \( m0-307 \) transcript is regulated by obesity in tissues other than the pituitary. Re-examination of the QEA traces found no evidence for regulation of \( m0-307 \) in any of the other tissues examined (data not shown). In a direct analysis by RTQ-PCR on an independent set of RNA samples, there was no significant difference in the expression of \( m0-307 \) between lean and obese mice in fat, liver, or muscle (Fig. 3B).

**Fig. 3.** Expression of a 1.2-kilobase pair mRNA corresponding to \( m0-307 \). \( A \), a poly(A) plus mRNA filter was purchased from CLONTECH and hybridized to an oligonucleotide (36 bases) derived from the 307-base pair fragment identified in the QEA analysis. \( B \), the expression of \( m0-307 \) was measured by RTQ-PCR in fat, liver, and muscle from obese and lean mice. Data are expressed as \( \Delta C_T \) (\( m0-307, RPL19 \)), mean and S.D.

**Fig. 4.** ACTH suppresses leptin mRNA levels in and release from primary adipocytes. Primary mouse adipocytes were cultured for 24 h in the presence of ACTH. The supernatants were assayed for leptin release, and RNA from the adipocytes was analyzed for leptin expression by RTQ-PCR. The mean (±S.D.) \( \Delta C_T \) values (relative to the ribosomal protein gene \( RPL19 \)) for each experiment is shown. Note that an increase in the \( \Delta C_T \) value is indicative of a decrease in expression and that there is a logarithmic relationship (base 2) between \( C_T \) and mRNA levels. The fold expression difference is calculated from the \( \Delta C_T \) assuming a 2-fold change in expression per unit change in the \( C_T \).
For the two genes tested there was no evidence that leptin directly and acutely altered pituitary gene expression. The mRNA encoding the leptin receptor is present on pituitary cells, although the identity of the relevant cells is not known (37, 53, 54). It is possible that leptin directly alters the expression of the genes identified in this study, but we were not able to detect these changes under the culture and leptin exposure conditions used. As leptin has been shown to alter hypothalamic gene expression and peptides derived from the hypothalamus have been shown to alter pituitary gene expression, it is also possible that the differences seen in this study are secondary to hypothalamic changes.

**Peripherial Consequences of Pituitary Responses to Leptin—**

The POMC prepropeptide is processed by PC2 to several bioactive fragments including ACTH, αMSH, βMSH, and β-lipotropin, and results described above indicate that expression of both POMC and PC2 is altered by leptin. Furthermore, it has been shown that at least some of the POMC-derived peptides have direct effects on adipocytes, the primary if not exclusive site of leptin synthesis. Thus we considered the possibility of a regulatory loop involving both leptin and peptides derived from POMC. Primary mouse adipocytes were prepared and treated with increasing concentrations of ACTH, αMSH, and βMSH and β-endorphin. After 24 h the media were collected and analyzed for both glycerol and leptin. In addition, RNA was prepared from the adipocytes and used for RNA analysis. As shown in Fig. 4 ACTH decreased both leptin release and leptin mRNA levels. This result is consistent with the negative correlation found between ACTH and leptin in vivo (55). These data support the concept of a regulatory loop involving leptin and ACTH, an increase in ACTH will lead to a decrease in leptin and in turn the decrease in leptin will allow hypothalamic changes.

**Conclusions—**We have found that up to 2% of the genes expressed within a particular tissue are altered in response to obesity; however, only approximately 10% of these are returned toward normal by a 1-week treatment with leptin. We have found five pituitary-expressed genes (PC2, POMC, prolaactin, HSGP25L2G, and one novel) that are both altered by obesity and at least partially normalized by leptin. POMC appears to function in a regulatory loop involving ACTH (produced by the pituitary and regulated by leptin) and leptin (produced by adipocytes and regulated by ACTH). In contrast, prolactin does not appear to impact directly on adipocyte biology and may participate in the reproductive aspects of leptin biology.

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![Fig. 5. A model for a regulatory loop involving fat-derived leptin and pituitary-derived ACTH. See text for details.](http://www.jbc.org/Downloaded from 26.7.3.818 by guest on July 26, 2018)
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