Metabolic regulation in the lactating mammary gland: a lipid synthesizing machine

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THE MOUSE MAMMARY GLAND OFFERS an outstanding model system for examining developmental regulation of metabolic processes. Pregnancy in this species lasts ~19 days including an intensive proliferative phase followed by a differentiation phase marked by an increase in milk protein gene expression, lipid droplet formation, and stromal adaptations (42, 43). A fall in progesterone ~day 18 initiates secretory activation, a programmed series of changes in the epithelium that leads to the copious secretion of very rich milk consisting of ~12% protein, 30% lipid, and 5% lactose. Lipid synthesis is particularly remarkable: The mammary gland of the lactating mouse must synthesize an amount of triacylglycerol (TAG) equivalent to the entire weight of the mouse during a 20-day lactation, generally while the mouse is eating a diet containing <8% of the calories as fat (37).

Coordinate transcriptional regulation of many of the lipid synthesis enzymes occurs in other organs such as the liver (12, 17) and adipose tissue (39) as well as pancreatic β-cells (2). We hypothesized that coordinate regulation of the same pathways might be integral to the remarkable increase in lipid and lactose synthesis during the activation of secretion (sometimes called lactogenesis II) in the mammary gland. We wished to identify both the key enzymes that change during the initiation of this program and the transcriptional regulators involved.

Our strategy was first to utilize microarray technology to analyze gene expression in the mammary glands of FVB mice during secretory activation. Having organized the metabolic genes by functional category, we identified genes whose expression changed significantly between the critical time points of pregnancy day 17 (P17) and lactation day 2 (L2). One advantage of these time points is the adipocyte compartment is quite small (41), so that we have some confidence that changes seen occur in the epithelial compartment. In addition, we have previously shown that adipocyte genes have a specific signature (42) that allows them to be identified as such.

As a reference we compared expression levels in the mammary gland to those in the liver at mature lactation (day 9). The synthetic picture that emerged from this analysis was then amplified by quantifying relevant metabolic intermediates using nuclear magnetic resonance spectroscopy (MRS). Finally, we identified potential regulatory genes by further mining both the original data and data from an experiment in which dietary lipid was switched from 8 to 40% (in kcal) in lactating mice. This approach provided a global picture of the multiple points at which the transcriptome of the enzymes of lipid synthesis is regulated and produced several candidate regulatory molecules. We found that mammary gene expression is altered in a unique manner to provide a framework for a balanced upregulation of lactose and lipid synthesis at secretory activation.

EXPERIMENTAL PROCEDURES

Animals and Tissue Preparation

FVB mice were obtained from Taconic (Germantown, NY). All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado Health Sciences Center, and animals were maintained in the institutional animal facility. P1 was identified as the first day a postcoital plug was observed; similarly, L1 was identified as the first day litters were present. Mammary glands from four mice were taken for the time-series microarray study at P12 and P17 and L1, L2, and L9. For the analysis presented in this paper we focused on data from P17 and L2.
However, data from all five time points are given in the supplementary tables. (The online version of this article contains supplementary material.) Three mice were taken at P17 and six each at P18 and L2 for real-time RT-PCR. A different set of 5–12 mice was taken at P17 and L2 for MRS. To study the effects of dietary fat, three mice each were fed diets containing 8 or 40% kcal as fat from L5 to L10 and killed. Both mammary glands and livers were analyzed by microarray in this experiment. The composition of the diets is available as Supplementary Table S6. For all studies upper inquinal mammary glands (usually called 4th gland) were dissected, and the lymph nodes were removed. For RNA analysis samples were stored in RNaLater stabilization buffer (Qiagen, Valencia, CA). For MRS, mice were anesthetized with Avertin IP, the mammary glands exposed and removed rapidly after the blood supply was clamped. The excised gland was sandwiched with aluminum foil and snap frozen between aluminum blocks in liquid nitrogen. Stage of development was verified histologically for each mouse (data not shown) by techniques used previously (42).

Isolation and Validation of Total RNA

Total RNA was isolated and purified following the Qiagen RNA extraction/cleanup protocol (including DNase treatment). Purity, concentration, and integrity of total RNA from each sample were verified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA).

Microarray Analysis

Total RNA was acquired, verified as above, amplified, biotin labeled (Enzo, Farmingdale, NY), and fragmented following the 2002 protocol for eukaryotic target preparation (Affymetrix, Santa Clara, CA). Acceptable samples were hybridized to Affymetrix Mu74AV2 microarray chips. Raw data were gathered from scanned array chips using Affymetrix Microarray Suite (MAS) version 5.0. Quality control chip statistics were evaluated from each array, including background intensity, noise, scaling factor, 3′–5′ ratios of the “housekeeping” genes, % distribution of calls, and presence of “spiked” control genes. Compiled data in the form of 28 individual CEL files, the primary output of scanned Mu74AV2 microarray chips using the contemporary GeneChip Operating Software v1.1 (Affymetrix, Santa Clara, CA), were imported to GeneSpring (Silicon Genetics, Redwood City, CA) for analysis using the native probe level GC-Robust Multi-array Average (GC-RMA) algorithm. The complete results of this analysis are available at http://www.ncbi.nlm.nih.gov/geo under the series record GSE4222.

Data Analysis

Genes were removed that scored below 15.0 RAW intensity data units in four or more of the conditions analyzed. Genes that were expressed within 15% of their mean across all time points were also removed giving a filtered list of 6,560 genes. To define genes that vary significantly over either the time course or the diet experiment, the Kruskal–Wallis nonparametric statistical test was run on the 6,560 gene list using a false discovery rate of 0.05.

Quantitative Real-time RT-PCR

We utilized real-time RT-PCR (QRT) to amplify the RNA for 15 lipid synthesis genes as well as forms of the sterol regulatory element binding proteins not available on the chip and four genes commonly used as references. In some samples a multiplexing strategy was used with MHC gene β2-microglobulin. Copy number per initial 1 μg of RNA was calculated from the concentration of the amplicons used to generate the standard curve. Because the differences between results obtained at P17 and P18 were, in general, not significant (see Table S3A), the data were combined for comparison with P17 data from array analysis.

Primer and Probe Design

Commercial software and web-based algorithms were used in the design of all primer and probe sets, TaqMan Primer Express Applied Biosystems software (http://www.appliedbiosystems.com/) and Integrated DNA Technologies (http://biotools.idtdna.com/primersquest/).respectively. Generally, we constructed a primer/probe construct to target a splice boundary within a message sequence so that genomic DNA was not amplified. All primers, labeled probes, and synthetic amplicon sequences were purchased from either Applied Biosystems or Integrated DNA Technologies. Sequences are given in Supplementary Table S3B. The primers and probes for independent targets were combined at proper concentrations prior to addition of the PCR master mix for both the gene investigated and β2-microglobulin. For each investigated gene in the 25-μl total volume reaction, a 20-μl master mix was prepared, including 12.5 μl PCR mix, 2.5 μl GeneA primer/probe solution (900 nM forward, 200 nM probe, 900 nM reverse), 0.25 μl of 100 units/μl AmpErase-UNG (a uracil-N-glyco- sylase that prevents carry-over amplification from templates containing deoxyuridine, i.e., RNA), 0.125 μl of 50 units/μl AmpliTagGold polymerase. This mix with 1.25 μl of nuclease-free water was loaded to the 96-well plate. To the 20 μl of master mix, 5 μl of 1:10 diluted cDNA template was added. cDNA was synthesized in a 20-μl reaction volume using 1.0 μg of total RNA in 8.5 μl of nuclease free water, 2.5 μl of random hexamers (50 μM), 4 μl of 5× 1st-strand buffer, 2 μl DT T (0.1 M), 1 μl dNTP blend (10 mM), 1 μl RNase inhibitor (40 U/μl), and 1 μl superscript III (200 u/μl); reagents were incubated at 42 degrees for 60 min and enzymes were inactivated at 75°C for 5 min. All real-time PCR data were captured using an Opticon Monitor II real-time PCR detection system plus the accompanying software version 2.02.24 (MJ Research, Bio-Rad Laboratories, Hercules, CA). Copy numbers of the investigated genes were calculated from the respective standard curves using the formula 10^[((Ct minus Y intercept)/(slope)), where Ct is cycle time. Finally, for each sample the ratio of the copy number of the investigated gene to the copy number of the 18S RNA times 10^6 produced normalized data.

Mammary Gland Extraction for Metabolomic Analysis

For MRS analysis, snap-frozen mammary glands (~0.2 g) were extracted using the dual-phase methanol-chloroform procedure. Briefly, frozen tissues were weighed, powdered in the presence of liquid nitrogen, and homogenized in 4 ml of ice-cold methanol. Ice-cold chloroform (4 ml) was added, samples vortexed, and an additional 4 ml of ice-cold water were added. After storage at 4°C overnight, they were centrifuged for 30 min at 1,400 g at 4°C. The upper methanol phase (water-soluble extract) was transferred into a lyophilizing glass and freeze-dried overnight then dissolved in 0.5 ml of deuterium oxide (D2O) prior to [1H]MRS analysis.

Quantitative MRS Metabolic Analysis

All one- and two-dimensional [1H]MR spectra were obtained on a Bruker 500 MHz DRX spectrometer (Bruker Bispin, Fremont, CA) using an inverse 5-mm TXI probe. For metabolite identification in water-soluble mammary gland extracts, a two-dimensional 2D-1H, C-heteronuclear single quantum correlation technique was used. The experiments were acquired with 512 increments and 160 scans per increment, using 90° pulse and a recovery delay of 0.5 s. The spectral width was 10 ppm in the proton dimension and 140 ppm in the 13C dimension. Lactate (Lac3, CH3) was used as a chemical shift reference for both carbon (21 ppm) and proton (1.32 ppm) axes. Tissue metabolites were identified based on the results from our chemical shift data base. For metabolite quantification, a standard water pre-saturation pulse program “zzpr” was used to suppress water residue
signal (44). The total number of acquisitions was 40, the pulse delay of 12.8 s was applied between acquisitions for fully relaxed \(^1\)H]NMR spectra (calculated as 5°T1). An external standard, trimethylsilylpro-pionic acid (TMSP-d\(_4\), 0.6 mmol/l) was used for metabolite quantification and as a chemical shift reference (0 ppm). After performing Fourier transformation and making phase and baseline corrections, we integrated each identified \(^1\)H peak using 1D WINNMR program (Bruker Biospin). The absolute concentrations of single metabolites were then referred to the TMSP integral and calculated according to the Eq. 1:

\[
C_x = \frac{I_x N_x C}{1.9} \times V M
\]

where \(C_x\) = metabolite concentration, \(I_x\) = integral of metabolite \(^1\)H peak, \(N_x\) = number of protons in metabolite \(^1\)H peak (from CH, CH\(_2\), CH\(_3\), etc.), \(C = \) TMSP concentration, \(I = \) integral of TMSP \(^1\)H peak at 0 ppm (9 since TMSP has 9 protons), \(V = \) volume of the extract, and \(M = \) weight of mammary gland tissue sample.

RESULTS AND DISCUSSION

Analysis of Metabolic Pathways During Secretory Activation

To examine the expression of genes contributing to the synthesis of lactose and lipid from glucose, we performed microarray analysis of total RNA from FVB mice using Affymetrix Mu74Av2 probe arrays. We examined the expression ratio between P17 and L2 for 102 genes involved in glucose metabolism (Fig. 1; Supplementary Table S1, A and B). Ratios that differed significantly from one are shown in colored blocks (shades of blue for ratios less than one and shades of red for ratios greater than one). A metabolic map depicting the position of the regulated genes in the lipid and lactose metabolism pathways is shown in Fig. 2.

Verification of data. We used two approaches to verify the changes in gene expression. We compared the ratios we obtained with data from a similar study using the same microarray chips with a different strain of mice (45, 48). Raw data files kindly provided by T. Stein were subjected to the same GeneChip analysis as used here. We found that the ratios obtained from a comparison of data obtained at P16.5 and L3 differed from a comparison of data obtained at P16.5 and L3 differed from our ratios by \(<\)20% of the mean value 62% of the time and that the directional change was consistent for 93% of the genes examined in our study (Table S3C). In addition we examined the expression level of 15 of these genes, as well as four regulatory genes and four reference genes, by QRT (Fig. 3, Table S3A). For all but one of these genes the fold changes obtained by the array and QRT methods were in the same direction, and for all but five the difference in ratios was not statistically significant. For most genes the fold change tended to be smaller with the QRT, averaging \(\sim 62\%\) of the values obtained with microarray probably due to differences in normalization. We obtained expression levels of three genes that are often used for normalizing RNA data in the mammary gland, GAPDH, cytokeratin 18, \(\beta\)-2-macroglobulin, as well as the 18S ribosomal RNA (see Table S3A). Because the 18S ribosomal RNA varied the least we used it for normalization, slightly reducing the variance between individual samples. The dilution of the mRNA by milk protein mRNA is likely to further dilute the values obtained from the lactating gland, probably explaining at least part of the observed decrease in GAPDH, keratin 18, and \(\beta\)-2-macroglobulin at lactation when normalized to S18. In the following discussion, we make a notation where marked differences between either the Stein data or the QRT data were found.

Expression of genes for plasma membrane substrate transporters. Milk lipid and lactose synthesis begin with transport of substrate from the plasma in the form of glucose, amino acids, and fatty acids. Although data for \(\sim 160\) transporters for these substances are available on the Affymetrix chip used, we catalogued only transporters for those substances with reasonably high levels of mammary expression (raw expression values \(>40\)) in Fig. 1. Glucose transporters that are present from the expression data are GLUT-1 (SLC2a1), GLUT-4 (SLC2a4), GLUT-8 (SLC2a8), and the sodium-dependent glucose transporter SLC5a1. GLUT-1 and SLC5a1 mRNAs were present at much higher levels in the mammary gland than the liver, and GLUT-1 gene expression increased at least 1.7-fold after parturition by both array and real-time RT-PCR (Fig. 3, Table S3C). GLUT-4 (SLC2a4) mRNA is also expressed in the mammary gland, but it is localized in the adipose compartment and shows the decrease during pregnancy and lactation expected of adipose genes (data not shown and Ref. 41). GLUT-12, known to be expressed in the mammary gland (30), is not present on the Mu74Av2 chip.

Transcripts for four genes associated with amino acid transport were identified from the array data. SLC1a4, a system alanine-serine cysteine (ASC) transporter, is a sodium-coupled transporter with specificity for alanine, serine, and cysteine capable of producing high gradients of these amino acids between the cell and the interstitial fluid (24). The L-system neutral amino acid transporter consists of a heteromer of SLC3a2 (4F2hc) and SLC7a5 (LAT1) with a broad specificity for neutral amino acids. It is thought to operate in the exchange mode only (36), possibly using amino acid gradients produced by the ASC and other transporters to increase cellular concentrations of such amino acids as valine, serine, and threonine. SLC1a4 and SLC7a5 mRNAs were upregulated 1.5-fold or more at the initiation of lactation and are expressed at levels many times higher than the liver, suggesting mammary specificity.

Genes for three members of the SLC27 family of fatty acid transporters are expressed in the mammary gland: SLC27a1, SLC27a3, and SLC27a4. SLC27a1 shows a temporal expression pattern similar to adipose tissue genes (data not shown) (42). SLC27a3 is expressed at a level about eight times that in the liver and, as we shall see, is subject to dietary regulation. SLC27a4 is expressed at a level similar to that of the liver, but its mRNA is upregulated at least 1.5-fold at the onset of lactation. CD36 is a multifunctional scavenger receptor that is also thought to transport fatty acids (8); although not upregulated at the onset of lactation, it is expressed at a level much higher than the liver.

Together these data suggest that expression of the RNA for three classes of transporters is significantly increased at the onset of lactation, glucose, amino acids, and fatty acids. Since substrate transport into the cell likely represents a potent regulation point for synthesis of milk components, this increase in gene expression is appropriate.

Lactose synthesis. Lactose synthesis from glucose and UDP-galactose takes place in the trans-Golgi catalyzed by lactose synthase, an enzyme complex made of up the 1, 4 β-galactosyl transferase (β1,4 GaIT) and the mammary-specific protein α-lactalbumin. The presence of the cofactor, α-lactalbumin, is
rate limiting for lactose synthesis (47), and its gene expression increases 5- to 15-fold between P17 and L2 (Figs. 1, 3; Table S. 3).

Glycolysis. Surprisingly, mRNA levels for the enzymes conducting the initial two steps of glycolysis, hexokinase and glucose phosphate isomerase (GPI), were significantly downregulated at the initiation of lactation and GPI was expressed at levels significantly lower than the liver (Figs. 1 and 2). Because the $K_m$ of lactose synthase for glucose is in the millimolar range (26), a decrease in hexokinase activity would effectively partition glucose toward lactose production. A decrease in GPI would help shunt glucose-6-PO₄ to the pentose phosphate shunt (see below; Fig. 2). Phosphofructokinase, aldolase C, and pyruvate kinase were expressed at levels much higher than their liver counterparts, probably because the isoforms present in the mammary gland differ from those in the liver. Aldolase C is expressed mainly in the brain where it likely contributes to myelin synthesis (1); its substantial upregulation by both array

| Table 1. Properties of metabolic genes in the mammary gland. Data for selected transporters and metabolic enzymes taken from temporal and diet experiments. L2/P17 is the ratio of gene expression at lactation day 2 (L2) to pregnancy day 17 (P17). MG/Liver is the ratio of mammary to liver gene expression. Figure continued on next page. |
|-----------------|-----------------|-----------------|
| Glycolysis      |                 |                 |
| Hk1             | Hexokinase 1    | 0.61 ± 0.06     | 1.85 ± 0.08 |
| Gpi1            | Phosphoglucomutase 1 | 0.43 ± 0.08     | 0.42 ± 0.09 |
| Pfk1            | Phosphofructokinase, I, B-type | 1.15 ± 0.07 | 7.06 ± 0.05 |
| Aldolase C      | Aldolase C      | 0.34 ± 0.12     | 45.09 ± 10  |
| Glucose-phosphate dehydrogenase 2 | 2.88 ± 0.07 | 3.29 ± 0.09 |
| Pdc1            | Phosphoglucose dehydrogenase | 0.65 ± 0.09 | 0.75 ± 0.07 |
| Pgl1            | Phosphoglucose dehydrogenase | 1.59 ± 0.05 | 2.68 ± 0.09 |
| Riba            | Ribose-5-phosphate isomerase A | 1.58 ± 0.19 | 0.93 ± 0.11 |
| Tkt1            | Transketolase   | 1.51 ± 0.06     | 3.11 ± 0.10 |
| Tald1           | Transaldolase 1 | 0.77 ± 0.08     | 2.43 ± 0.05 |
| Gluconeogenesis |                 |                 |
| Fbp1            | Fructose biphosphatase 3 | 1.15 ± 0.03 | 1.60 ± 0.20 |
| Pepck           | Phosphoenolpyruvate carboxykinase (PEPCK) | 0.85 ± 0.09 | 0.92 ± 0.11 |
| G6pc3           | Glucose 6-phosphate, catalytic, 3 | 0.69 ± 0.11 | 1.48 ± 0.14 |
| Citric Acid Cycle |                 |                 |
| Pck1            | Pyruvate Carboxylase | 2.00 ± 0.07 | 2.25 ± 0.11 |
| Psala1          | Pyruvate dehydrogenase E1 alpha 1 | 0.65 ± 0.04 | 3.61 ± 0.04 |
| Dihidrolipoamide branched chain transacylase E2 | 1.51 ± 0.09 | 0.39 ± 0.07 |
| Dihidrolipoamide S-acetyltransferase (E2) | 1.43 ± 0.09 | 4.52 ± 0.10 |
| Dihidrolipoamide dehydrogenase (E3) | 0.90 ± 0.15 | 0.54 ± 0.07 |
| Cac          | Citrate synthase | 2.17 ± 0.07 | 13.95 ± 0.78 |
| Citc1          | Mitochondrial tricarboxylic acid transporter | 4.16 ± 0.13 | 7.65 ± 0.14 |
| Acn1            | Aconitase 1     | 1.06 ± 0.03     | 1.06 ± 0.05 |
| Isocitrate dehydrogenase | 0.77 ± 0.10 | 0.38 ± 0.06 |
| Succinate-Coenzyme A ligase, | 1.00 ± 0.20 | 1.00 ± 0.03 |
| Succinate-Coenzyme A ligase, | 1.10 ± 0.08 | 0.28 ± 0.06 |
| Succinate dehydrogenase complex, subunit C | 0.60 ± 0.06 | 0.40 ± 0.08 |
| Succinate dehydrogenase complex, subunit D, | 0.84 ± 0.06 | 0.52 ± 0.03 |
| Malate dehydrogenase | 0.83 ± 0.06 | 0.93 ± 0.05 |

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Fig. 1. Properties of metabolic genes in the mammary gland. Data for selected transporters and metabolic enzymes taken from temporal and diet experiments. L2/P17 is the ratio of gene expression at lactation day 2 (L2) to pregnancy day 17 (P17). MG/Liver is the ratio of mammary to liver gene expression. Figure continued on next page.
and PCR analysis at the onset of lactation is consistent with previous observations that the enzyme localizes to mammary alveoli and is responsive to prolactin (31). It is likely important in generation of the glycerol-phosphate backbone for TAG synthesis.

Pentose phosphate shunt. In lipid synthesizing tissues, the pentose phosphate shunt provides a substantial fraction of the reducing equivalents needed for NADPH synthesis. From our data this increase in activity would be accomplished both by downregulating GPI (see above) and possibly increasing expression of the gene for glucose-6-phosphate dehydrogenase (Figs. 1 and 2), although a large increase was not confirmed in either the Stein data set or by QRT; mRNA expression for other enzymes in this pathway, phosphogluconate dehydrogenase was increased significantly in both arrays, and this and other enzymes and this as well as transketolase and transaldolase were expressed at levels three times those in the liver. At secretory activation in the rat mammary gland the activity of this pathway was increased 22 times (9). The fact that none of the increases in expression for the enzymes in this pathway was close to 22-fold, suggests that the increased flux is likely also due increased substrate availability or other modes of regulation.

Gluconeogenesis. The data in Fig. 1 confirm the expectation that gluconeogenesis is not a function of the murine mammary epithelium. Mammary expression of two of the key enzymes of gluconeogenesis, PEPCK and fructose-bis-phosphatase, is 2% of that of the liver, suggesting that carbon backbones transported into the mammary gland in the form of amino acids are not significantly converted into glucose in this organ, a point of importance when we consider citrate synthesis.

### METABOLIC REGULATION OF MILK LIPID SYNTHESIS

**Fig. 1—Continued**

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**Fig. 1—Continued**

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**Fig. 1—Continued**

![Image](http://physiolgenomics.physiology.org/)
Citric acid cycle. Gene expression for many enzymes of the citric acid cycle is significantly downregulated at the onset of lactation; these genes are also expressed at levels significantly lower than the liver (Fig. 1). The exceptions are pyruvate carboxylase, some components of pyruvate dehydrogenase, and citrate synthase. All are expressed at levels at least threefold higher than the liver, and most are significantly upregulated at secretory activation. In addition, there is an impressive increase in the tricarboxylic acid (citrate) transporter, three- to fourfold, by both microarray and real-time RT-PCR (Figs. 1 and 3). If these adaptations are translated to the protein level they would serve to focus mitochondrial activity on the generation and export of citrate to the cytoplasm where it would be converted to substrates for fatty acid and cholesterol synthesis as well as for the generation of NADPH via the malate shuttle.

Management of plasma fatty acids. Plasma TAG are hydrolyzed in the capillary by lipoprotein lipase, and their products are transferred across the cell membrane by fatty acid and glycerol transporters, thought to be specific aquaporins. Unfortunately these aquaporins were not represented on the chip. Lipoprotein lipase has long been known to be expressed at very high levels in the lactating mammary gland (15), and the array results are consistent with this finding. Once hydrolysis is complete, lipid transporters, described above, transfer the fatty acids to the cytoplasm where they must be bound to fatty acid binding proteins (FABP) or acylated and bound to fatty acyl binding proteins. As shown in Fig. 1, the mRNA for FABP3, the heart-type binding protein, is expressed at very high levels in the mammary gland (4).

Acylated fatty acids can be metabolized by the enzymes of the β-oxidation pathway or incorporated into TAG for secretion into milk. In the mammary gland the mRNA for most enzymes of the β-oxidation pathway falls two- to threefold at secretory activation (Fig. 1, Table S3) directing fatty acids to TAG synthesis. One unexpected finding was that cytoplasmic thioesterase 1 (Cte-1), an epithelial enzyme that specifically hydrolyzes long-chain saturated acyl-CoAs (20), is expressed at very high levels in pregnancy (see Table S1), falling nearly 26-fold at the onset of lactation. In the liver acyl-CoA thioesterases are thought to be critical for maintaining the balance between energy storage (TAG synthesis) or energy utilization (β-oxidation) (21). The fall in the mRNA for Cte-1 suggests at least a gate-keeping function of this enzyme for entry of fatty acids into the β-oxidation pathway; in addition the fall would prevent hydrolysis of acyl-CoAs, which are then available for TAG synthesis.
**Fatty acid synthesis.** Citrate, generated in the mitochondria and transported to the cytoplasmic compartment, is hydrolyzed by ATP citrate lyase to provide substrate for both NADPH and malonyl CoA synthesis (3). As shown in Figs. 1 and 2 and Table S3C, the mRNA of most of the enzymes involved in fatty acid synthesis is upregulated at secretory activation, and the final expression level of most of them is two- to threefold above the liver. ATP citrate lyase in particular was upregulated at least 2.7-fold by microarray and QRT, suggesting that its activity is a major metabolic control point. Acetyl-CoA carboxylase has been identified as a major regulator of fatty acid synthesis in other species, but it was not present on the MU74AV2A chip used in these experiments; preliminary assays using real-time QRT (not shown) showed a 1.5-fold change in gene expression in the murine mammary gland. However, the existence of several uncharacterized isoforms in

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<td>2.42 ± 0.24</td>
<td>2.40 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>92592_at</td>
<td>Glycerol-3-P-DH</td>
<td>Gpd1</td>
<td>1.05 ± 0.15</td>
<td>2.25 ± 0.13</td>
<td>*</td>
</tr>
</tbody>
</table>

* *P < 0.05

**Fig. 3.** Real-time RT-PCR summary. Real-time RT-PCR was performed as in EXPERIMENTAL PROCEDURES, and the ratio of late pregnancy to lactation was compared with the ratio from the microarray analysis over that same time period. Colored as in Fig. 1.
the mouse make this conclusion unreliable. Fatty acid synthase gene expression was increased at least 1.3-fold by microarray and real-time RT-PCR at the initiation of lactation (Figs. 1 and 3).

The mRNAs for SCD2 and Evolv 1 are expressed at much higher levels in the mammary gland than the liver as is the Δ5 fatty acid desaturase 1. These enzymes are responsible in part for formation of very-long-chain polyunsaturated fatty acids; the high expression of their mRNAs, along with the higher expression of mRNA for other enzymes for fatty acid synthesis, suggests most of the long-chain polyunsaturated fatty acids present in mouse milk are formed in the mammary gland and not transported from the liver.

TAG synthesis. To synthesize TAGs, both fatty acyl-CoAs and glycerol 3-phosphate must be available. Glycerol can enter the mammary alveolar cell from the plasma to be phosphorylated by glycerol kinase, expressed at a level three times that of the liver and significantly upregulated at secretory activation in the Stein data set (Table S3C). Prior to TAG synthesis, fatty acids must be acetylated; the most highly expressed of the enzymes that do this is long-chain acyl-CoA synthetase 1, moderately upregulated at the onset of lactation. Finally the acyl-CoA groups must be added to glycerol phosphate. In the mammary gland the most highly expressed enzyme in this process is 1-acylglycerol-3-phosphate O-acyltransferase (AgPAT).

Cholesterol synthesis. Many of the enzymes of cholesterol synthesis are present on the microarray chip, and a good number were significantly upregulated between P17 and L2 (Figs. 1 and 2), suggesting a high degree of regulation in the mammary gland. However, many fall by L9 (see Table S1), when they are expressed at high levels in the liver. This finding is consistent with the data from goats that only the high expression of their mRNAs, along with the higher concentrations of the individual sugars to be measured. As expected, the concentrations of glucose, lactose, and galactose were all increased in glands from lactating animals. β-Glucose increased more than twofold between pregnancy and lactation as expected from the increase in transport in the face of a fall in hexokinase. The change is consistent with previous measures of glucose concentration in the lactating gland (27). Both galactose and lactose were nearly undetectable in pregnancy but rose to millimolar levels in the lactating gland. Sialic acid (neuraminic acid or NANA), utilized in the formation of the complex carbohydrates present in milk, was detectable only in the lactating gland. Inositol, which plays a role in the maintenance of osmotic pressure as well as being an important component of phospholipids, also increased significantly.

The second group of metabolites in Fig. 4B contains several amino acids, e.g., alanine, arginine, aspartate, and glutamate, whose concentrations are increased up to twofold or more. All these amino acids can be deaminated and utilized in the citric acid cycle, providing potential substrates for generation of ATP as well as citrate for fatty acid and cholesterol synthesis. Arginine is also an important intermediate in the urea cycle, which provides a sink for the amino nitrogen created by deamination of amino acids from the blood stream. Although the urea content of mouse milk is unknown, the fact that urea is present in human and cow’s milk at concentrations up to 5 mM (33, 50), suggests that it is an important end product of amino acid utilization. Unfortunately urea has no [1H]NMR detectable signal. Putting these observations together it seems likely that, at least in the mouse, amino acids contribute carbon backbones for fatty acid synthesis with urea as a waste product.

The third group of metabolites in Fig. 4B includes citrate, reflecting the increase in citrate synthesis and transport from the mitochondrion; betaine, a choline derivative important in homocysteine metabolism; as well as creatine and hydroxybutyrate. The final group includes choline, precursor to phosphocholine (PC), and glycerolphosphocholine (GPC), the major choline metabolites in rat milk (40). Both are precursors of the major membrane phospholipids, phosphatidylcholine found in the milk fat globule membrane and other membrane fractions of milk. No changes in lactate were observed between pregnancy and lactation, explained by the rapid utilization of pyruvate for citrate formation.

Analysis of Potential Regulatory Genes

Having determined a number of functionally important genes and metabolites that are regulated in a tissue-specific manner at the onset of lactation, we asked the question: How is the expression of these genes coordinately regulated? Here we must examine two types of regulation: 1) positive upregulators of lipid and lactose synthetic processes and 2) negative regulators whose expression is high in pregnancy to inhibit milk synthesis and which decrease at the onset of lactation to release this repression. For the first category we examined the temporal profiles and expression levels of categories of genes known to regulate lipid metabolism in other tissues including the Akts, C/EBPs, IGFs, PPARs, LXRα, SREBPα, and STATs (17, 25, 46) (Fig. 5). Many of these genes were expressed at levels much lower in the mammary gland than in the liver (shown in blue in MG/liver column), suggesting that they may not have a role in the regulation of metabolism in the lactating mammary gland. The exceptions were AKT1, C/EBPδ, LXRα, PPARγ, STAT5α, and STAT5b, all of which were expressed at levels at least threefold above those in the liver. In addition, although the prolactin receptor (PRLR) and steroid regulatory element binding protein-1 (SREBP-1) were expressed at levels approximately equal to those of the liver, these two genes, along with Akt1 and STAT5b, were significantly upregulated at the onset of lactation.
Akt1 was increased nearly threefold at the onset of lactation in the array; previous publications show a 10-fold upregulation by Northern blot at this time and high expression of the protein in the lactating mammary gland (43). Akt1 has been shown to mediate translocation of GLUT1 to the plasma membrane and regulate hexokinase distribution (38) and likely plays a similar role in the lactating mammary gland. Both isoforms of STAT5 have long been known to be involved in the specific regulation of milk protein synthesis in the mammary gland (16); high level expression in the mammary gland is expected. A recent analysis of mice with various prolactin receptor defects, also implicates STAT5 in the regulation of lipid synthesis (33). Expression of the long form of PRLR has previously been shown to be increased several-fold by ovariectomy of the

Fig. 4. Metabolomic analysis of the initiation of lactation. A: representative magnetic resonance spectra of the aqueous fraction isolated from whole mammary tissue at P17 and L2. The methanol residue in the upper spectrum is an artifact of the extraction procedure and was variably present. B: quantification (described in EXPERIMENTAL PROCEDURES) of metabolites showing significant differences in concentration by t-test between P 18 and L 2 (n = 6 to 11 animals at each time point). Data are shown as mean concentrations ± SE.
pregnant mouse, a procedure that leads to secretory activation (32), and PRL signaling is essential for the maintenance of lactation (34). The finding that the mRNA for all these factors is upregulated reinforces the utility of microarray analysis for identification of factors involved in regulation of milk synthesis.

For the SREBPs, however, the microarray analysis was inadequate because it didn’t distinguish between the splice forms SREBP-1a and SREBP-1c, which have different roles in the fatty acid synthesis; furthermore, SREBP-2 was not represented on the chip. We, therefore, used real-time RT-PCR to quantitate the expression of SREBP family members during the initiation of lactation (Fig. 3), finding that SREBP-1c was the most highly expressed and nearly doubled at the onset of lactation. SREBP-1c has been implicated as a metabolic regulator in many organs including the liver (11), adipocytes (28), muscle (14), and pancreas (2), as well as the mammary gland (5, 6). In pregnancy SREBP-1a was expressed at a level about equal to SREBP-1c and the regulator of cholesterol synthesis, SREBP-2, was expressed at a threefold lower level. However, only SREBP1-c increased at the onset of lactation. During secretory activation, expression of 38 of the 102 metabolic genes examined was upregulated >1.4-fold. Of these genes, 17 corresponded to genes whose activity has been suggested to be upregulated by SREBPs on the basis of gene expression studies in the liver (17, 19). This observation plus the increase in SREBP-1c at the initiation of lactation (Fig. 3 and Table S3) suggest that SREBP-1c may be a prominent regulator of lipid synthesis in the mammary gland.

A number of the highly expressed metabolic regulators were significantly downregulated at the onset of lactation including AKT2, C/EBPα, PPARγ, STAT3. Of these C/EBPα and STAT3 have been implicated in the apoptotic processes that follow weaning in the mammary gland (10, 49); a fall in these factors in the context of the initiation of lactation suggests protection from apoptosis. The role of AKT2 and PPARγ in the mammary gland is unknown but they are highly expressed in adipose tissue and show expression patterns characteristic of that compartment (data not shown and Ref. 41).

We have mentioned negative regulators above and have previously implicated transforming growth factor (TGF)-β, TGF-β3, Wnt5b, and IGFBP5 (41). As shown in Fig. 5, all were down-regulated in this analysis; however, none has been linked extensively to regulation of metabolic processes and they will not be discussed further here. On the other hand a role for negative metabolic regulators should also be considered. In the liver, expression of the genes for fatty acid metabolism enzymes appears to be regulated by two reciprocal systems involving PPAR and LXR nuclear hormone receptors. Competitive dimerization of these proteins with RXRs (influenced by binding of their respective ligands) balances the cellular choice between lipogenesis and β-oxidation (22). Possibly the isomers of PPAR (PPARγ) and LXR (LXRβ), present at appreciable levels in the mammary gland (see Table S1) mediate these reactions (23).

**Effect of Changing Dietary Fat Content on Gene Expression**

Because fatty acid synthesis in the mammary gland is known to be regulated by dietary lipid (35), we sought to confirm a role for some of the regulatory molecules described above. We
fed FVB mice diets containing 8 or 40% of their caloric content as lipid, substituting lipid for carbohydrate, from L5 to L10. Gene expression was examined in the mammary gland using the same Affymetrix gene chips. We found that expression of 85 genes was significantly altered by the high fat diet in the mammary gland, compared with 760 in the liver (Table S6), reflecting the much more complex role of the liver in metabolic regulation. By comparison the lactating mammary gland is devoted to one process, the synthesis and secretion of milk. Although the mammary gland is known to decrease fatty acid synthesis in response to a high fat diet (35), there are few other diet-sensitive changes. Because of space limitation we confine
this discussion to the metabolic and regulatory genes previously analyzed in this paper.

In the liver four genes for fatty acid degradation were upregulated, but expression in the mammary gland remained low (Fig. 6). Expression of many hepatic cholesterol synthesis genes in the liver was also affected by the increased dietary lipid although the direction of the changes was inconsistent. In the mammary gland the major effects were to increase expression of the mRNA for the major fatty acid transporter FAT 3, and decrease the mRNA for enzymes of the pentose phosphate shunt, the mitochondrial citrate transporter, and fatty acid synthetase. Of the regulatory genes, expression of C/EBPβ and C/EBPα as well as, most significantly, SREBP-1c was decreased. Of the 12 metabolic genes whose mammary expression was significantly altered, eight have been shown in numerous experiments to be regulated by SREBP-1c (18), firmly implicating this transcription factor in regulation of fatty acid synthesis. Of the regulatory genes, expression of C/EBPα and C/EBPβ as well as, most significantly, SREBP-1c was decreased. Of the 12 metabolic genes whose mammary expression was significantly altered, eight have been shown in numerous experiments to be regulated by SREBP-1c (18), firmly implicating this transcription factor in regulation of fatty acid synthesis in the mammary gland. Surprisingly, expression of TGF-β3 and IGFBP5 was increased, possibly suggesting that high-fat, low-carbohydrate diets lead to a degree of apoptosis of the mammary epithelium. Linkages between energy metabolism and cell survival have been made in many systems (7, 38) and possibly warrant additional investigation in the mammary gland. C/EBPβ has been found to be necessary for ductal morphogenesis, lobuloalveolar differentiation, and functional differentiation of the mammary gland (13). We found that it is significantly decreased by diets with fat content >40%, suggesting that it may respond to changes in dietary lipid.

In conclusion, it is clear from the data in Figs. 1 and 2 that multiple control points at the level of mRNA expression exist for lipid and lactose synthesis in the mammary gland. Indeed, of the 102 metabolic genes tabulated in Fig. 1 expression of 19 was up- or downregulated more than twofold at the onset of lactation by array analysis and an additional 19 increased 1.4- to twofold. The data support the following concepts that have previously been put forward.

- Glucose transport into the cell and Golgi is facilitated by upregulation of GLUT1 mRNA. It is also known to be increased by translocation of the protein into the plasma membrane and Golgi (30).
- Lactose synthesis is increased by a massive increase in the cofactor, α-lactalbumin (α-lact) leading to highly increased lactose synthesis in the lactating gland. The high levels of lactose observed by MRS are also consistent.

The data also lead to multiple hypotheses that will need to be tested in subsequent experiments examining both substrate fluxes and enzyme activity. These are:

- Increased NADPH synthesis by the pentose phosphate pathway may be facilitated both by downregulation of phosphoglucoisomerase (GPI) and upregulation of genes of the pentose phosphate shunt.
- Glycerol-3-P synthesis is increased by the upregulation of the mammary- and brain-specific aldolase C (1, 31) as well as glycerol kinase.
- Increased cellular concentrations of key amino acids as shown by metabolomic analysis are brought about by the upregulation of mRNA for amino acid transporters. The data further suggest that amino acids are an important substrate for lipid synthesis at least in species that produce milk with a very high lipid content, such as the mouse.
- Increased citrate production in the mitochondrion is brought about by the upregulation of the E2 component of pyruvate dehydrogenase and the enzyme pyruvate decarboxylase as well as increases in citrate synthase and the citrate transporter. Thus it seems likely that citrate formation and transport is a dominant activity of the mitochondrion in the lactating mammary gland.

- Increased cholesterol production through the upregulation of the mRNA for enzymes of cholesterol synthesis occurs at secretory activation. However, at midlactation expression of the relevant genes was much higher in the liver, suggesting that much of the cholesterol exported into milk is synthesized in the liver and transported through the blood stream.

- Fatty acid synthesis, including shaping of the fatty acid profile of milk by desaturation and elongation of fatty acids, takes place predominantly in the mammary gland, and the expression of an entire suite of enzymes involved is upregulated, possibly by increased activity of SREBP-1c.

- In the mammary gland, unlike other organs, AgPAT appears to be the dominant acyl-transferase upregulated for synthesis of triglyceride.

- Regulatory factors whose expression is consistent with a regulatory role in balanced synthesis of mammary lipid and lactose include AKT1, STAT5a, STAT5b, SREBP-1c, C/EBPβ, with other factors, potentially PPARγ, LXRβ, and fatty acids generated by CTE-1 activity, playing inhibitory roles during pregnancy.

These studies indicate that significant regulation of lactose and lipid synthesis in the mammary gland occurs at the level of mRNA expression. From studies in other tissues we surmise that much of this regulation is at the level of gene transcription and, indeed, a number of activating transcription factors including SREBP-1c, STAT5a and STAT5b, and C/EBPβ, as well as inhibitory factors, PPARγ and LXRβ, are likely involved. This study of mRNA levels and prominent metabolites has allowed us to obtain a global picture of the adjustments in gene expression necessary to turn a quiescent organ into an incredibly efficient machine for the massive synthesis and secretion of a complex mixture that includes highly regulated concentrations of lactose and lipid. These data provide a comprehensive framework for testing of the hypotheses generated, testing that will have to be carried out at the protein and functional levels.

GRANTS

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