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Sexual Dimorphism of Rat Liver Gene Expression: Regulatory Role of Growth Hormone Revealed by Deoxyribonucleic Acid Microarray Analysis

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GH has diverse physiological actions and regulates the tissue-specific expression of numerous genes involved in growth, metabolism, and differentiation. Several of the effects of GH on somatic growth and gene expression are sex dependent and are regulated by pituitary GH secretory patterns, which are sexually differentiated. The resultant sex differences in plasma GH profiles are particularly striking in rodents and are the major determinant of sex differences in pubertal body growth rates and the expression in liver of several cytochrome P450 (CYP) enzymes that metabolize steroids, drugs, and environmental chemicals of importance to endocrinology, pharmacology, and toxicology. DNA microarray analysis was used to identify rat liver-expressed genes that show sexual dimorphism, and to ascertain the role of GH as a regulator of their sexually dimorphic expression. Adult male and female rats were untreated or were treated with GH by 7-d continuous infusion using an Alzet osmotic minipump. Poly(A) RNA was purified from individual livers and Cy3- and Cy5-labeled cDNA probes cohybridized to Pan Rat Liver and 5K Rat Oligonucleotide microarrays representing 5889 unique rat genes. Analysis of differential gene expression profiles identified 37 liver-expressed, female-predominant genes; of these, 27 (73%) were induced by continuous GH treatment of male rats. Moreover, only three of 30 genes up-regulated in male rat liver by continuous GH treatment did not display female-dominant expression. Further analysis revealed that 44 of 49 male-predominant genes (90%) were down-regulated in the livers of continuous GH-treated male rats compared with untreated male rats, whereas only five of 49 genes that were down-regulated in male rats by continuous GH treatment were not male dominant in their expression. Real-time PCR analysis applied to a sampling of 10 of the sexually dimorphic genes identified in the microarray analysis verified their sex- and GH-dependent patterns of regulation. Taken together, these studies establish that GH-regulated gene expression is the major mechanistic determinant of sexually dimorphic gene expression in the rat liver model. (*Molecular Endocrinology* 18: 747–760, 2004)

THE TRADITIONAL GENE-BY-GENE approach to understanding hormone action cannot provide a full understanding of the complex regulatory processes that occur in higher eukoryotes, which may contain upwards of 35,000–40,000 individual genes, of which 10,000 or more may be expressed in any given tissue or cell type. To obtain a global view of biological processes, it is essential to obtain simultaneous readouts from a large number, if not all of its components. Methods for detecting and quantitating gene expression include Northern blots (1), differential display (2), sequencing of cDNA libraries (3, 4), and serial analysis of gene expression (5, 6). Advances in genomics, including the availability of DNA sequences covering large segments of the human and rodent genomes and the development of methods for parallel, high throughput detection and quantitation of gene expression levels, have made it possible to obtain a more global picture of cell-type and tissue-specific responses to hormones. In particular, cDNA and oligonucleotide microarrays (7–9) can be used to simultaneously monitor the expression patterns of thousands of genes in a single experiment using a systematic global strategy (10).

Using the power of microarray technology, we have undertaken a global analysis of the role of GH in the sexual dimorphism of rat liver gene expression. GH is secreted by the pituitary gland in a sex-dependent manner in both rodents (11) and humans (12). In male rats, GH is released into circulation approximately every 3.5 h, giving a repeated, pulsatile plasma hormone pattern (13) that contrasts with the more frequent, nearly continuous profile of plasma GH exposure that occurs in adult females. The resultant sex differences in plasma GH profiles are particularly striking in rodents, where they serve as a major determinant of the observed sex differences in body growth rates and the expression in liver of several cytochrome P450 (CYP) enzymes that metabolize steroids, drugs, and environmental chemicals of importance to endocrinology,

Abbreviations: CoA, Coenzyme A; CYP, cytochrome P450; dUTP, deoxyuridine triphosphate; QPCR, quantitative PCR.

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pharmacology, and toxicology (14, 15). Well-studied examples of sex-specific, plasma GH pattern-regulated liver genes include the male-specific androgen 2α- and 16α-hydroxylase CYP2C11, which is strongly induced at puberty in male but not female rat liver, and the steroid sulfate 15β-hydroxylase CYP2C12, which is exclusively expressed in adult female rat liver (16, 17). The sexual dimorphism of liver with respect to expression of these and other liver CYPs is regulated by GH at the level of transcription initiation (18, 19). Sex-dependent expression and GH regulation also characterizes other families of genes involved in steroid or foreign compound metabolism, such as the sulfotransferases (20) and class α and class μ glutathione-*S*-transferases (21).

DNA microarrays have been employed to determine the expression profiles of key liver-specific genes in rodents as modulated by insulin signaling (22) and small molecules such as leptin (23). Microarrays have also been used to investigate the impact of hormonal insufficiency (hypophysectomy), GH replacement therapy, and GH overexpression on liver gene expression (24–28). The impact of aging on gene expression and the normalizing effects of GH treatment have also been investigated (29). In one study, the effects of hypophysectomy and GH treatment on the gene expression patterns in heart, liver, and kidney were evaluated using cDNA microarrays containing 3000 different rat genes (24). cDNA microarrays have also been used to identify genes that are responsive to GH in the GH-deficient dwarf rat model (25). In another study, subtractive hybridization was used to identify 173 potential GH target genes; however, only 41 of those genes could be confirmed in subsequent cDNA array experiments (26). Of note, these studies of GH-regulated gene expression employed cDNA arrays, whose ability to discriminate between closely related DNA sequences may be limited by the cross-hybridization that invariably occurs when a single set of hybridization conditions is applied to the thousands of genes represented on the array. These ambiguities can be avoided by using microarrays constructed with oligonucleotides that are chosen to be gene specific and matched with respect to length, GC content, and melting temperature.

The present study was undertaken to investigate the role of GH in the sexual dimorphism of rat liver gene expression. Using two commercial oligonucleotide microarrays, we have identified 86 genes that show sexual dimorphism in their patterns of expression in rat liver. The large majority of these genes (72 of 86) were also found to be subject to GH regulation, demonstrating that GH is a major determinant of sexually dimorphic liver gene expression.

RESULTS

Oligonucleotide-based microarray analysis was applied to Poly(A)$^+$ mRNA isolated from livers of untreated adult male, untreated adult female, and continuous GH-treated adult male rats (n = 4 rats/group). Two commercial microarrays representing a total of 5889 unique rat genes were used, one containing 1353 liver-expressed genes (Pan Rat Liver array) and the second containing 5535 genes expressed in various tissues (5K Rat array). Microarray expression data were obtained for four pairs of male and female rat liver cDNA samples, and for four pairs of male and GH-treated male samples, with each of the hybridization pairs (male *vs.* female and male *vs.* GH-treated male) analyzed both on the Pan Rat Liver array and the 5K Rat array. Given the uniform length and GC content of each oligonucleotide spotted on the array, the fluorescence hybridization intensity of each microarray spot provides an indication of the relative level of gene expression. Analysis of the frequency distributions of average spot intensities after background subtraction revealed high expression (>5000 fluorescence units) in male rat liver for 7% and approximately 4% of the genes represented on the Pan Rat Liver and 5K Rat arrays, respectively. Intermediate expression (500–5000 fluorescence units) was obtained for approximately 51% and 28% of the genes, and low but still significant expression compared with background (40–500 fluorescence units) for approximately 40% and approximately 63% of the genes on the two respective arrays. Less than 2% of the Pan Rat Liver array genes and less than 6% of the 5K Rat array genes had average spot intensities below twice the average background level.

Fold increases or decreases in gene expression compared with the untreated male group were scored as male:female and male:male + GH ratios. Genes found to be reproducibly expressed in either a sex-dependent manner (male:female ≥1.5 or ≤0.66) or a GH-regulated manner (male:male + GH ≥1.5 or ≤0.66) are listed in Tables 1–4. These regulated genes varied approximately 900-fold in their relative levels of expression after background correction, with the urinary pheromone-binding protein α-2u globulin being the most highly expressed gene in male liver (averaged normalized fluorescence intensity = 45,540), and heme-oxygenase 3 being the least expressed (average normalized fluorescence intensity = 51) (*c.f.* Table 1, *right column*). The log distributions of the male:female and male:male + GH ratios were compared with 0 on a per-gene basis to examine their statistical significance. Genes identified as sex-dependent and/or GH-regulated and whose distributions surpassed the significance level of $P < 0.05$ are shown in bold in the tables. A summary of the numbers of genes identified in each category of regulation is presented in Table 5.

Forty-nine of the approximately 6000 genes examined were found to be up-regulated in adult male as compared with adult female liver. The expression of 44 of these genes (90%) was down-regulated in male liver by continuous GH treatment (Table 1), whereas five of the 49 male-predominant genes were apparently not responsive to GH treatment (Tables 3A and 5). Four

Table 1. Male-Dominant Genes Down-Regulated in Male Rat Liver by Continuous GH Treatment

Gene Designation	Common Name	Accession No.	Category/Function	Male:Female Ratio	Male:Male + GH Ratio	Average Male Intensity
Bcl2l11	Bcl-2-like 11 apoptosis facilitator; BOD-L; BOD-M; Bim; BimL	AF065433	Apoptosis	**2.92 ± 0.35**	**6.82 ± 2.70**	1694
Tfpt; Amida	TCF3 (E2A) fusion partner (in childhood leukemia)	AB029495	Apoptosis; nuclear factor	2.35 ± 0.64	**3.82 ± 0.57**	406
Ces1	Carboxylesterase 1, kidney microsomal	U10697	Enzyme (esterase)	1.57 ± 0.22	**1.79 ± 0.04**	2079
–	Carboxylesterase (EC 3.1.1.1) ES-4-like; similar to pir‖S62788	Unknown 191	Enzyme (esterase)	**1.57 ± 0.12**	**1.61 ± 0.05**	2227
Phkg2	Phosphorylase kinase γ-2, catalytic subunit	M73808	Enzyme (glycogen metabolism; Ser/Thr protein kinase)	**2.38 ± 0.28**	**2.00 ± 0.18**	1320
–	Heme oxygenase-3 (HO-3)	AF058787	Enzyme (heme metabolism)	2.44 ± 0.45	**1.77 ± 0.09**	51
Hao3	(S)-2-Hydroxy acid oxidase, peroxisomal; glycolate oxidase	X67156	Enzyme (hydroxy acid oxidation)	**6.72 ± 1.06**	**5.77 ± 0.99**	1268
Nox4	NADPH oxidase 4	AY027527	Enzyme (oxidative burst); signal transduction	2.88 ± 1.53	**3.42 ± 0.48**	98
Ca3	Carbonic anhydrase III	AB030829	Enzyme (oxidative stress)	**15.48 ± 4.78**	**7.58 ± 1.49**	6281
Enpp2	Phosphodiesterase I/nucleotide pyrophosphatase 2; autotaxin	D28560	Enzyme (phosphodiesterase; phospholipase)	**2.01 ± 0.14**	**2.44 ± 0.20**	889
Ptgds2	Prostaglandin D2 synthase 2	AF021882	Enzyme (prostaglandin biosynthesis)	**3.73 ± 1.34**	**2.95 ± 0.91**	282
Tnc	Tenascin c	U09361	Extracellular matrix (anti-adhesive protein)	**3.69 ± 1.15**	**2.61 ± 0.46**	265
Stx1a	Syntaxin 1A; synaptic vesicle docking protein (SNARE)	AF217191	Membrane trafficking; neurotransmitter transport	**1.57 ± 0.07**	**1.93 ± 0.20**	834
Slc26a4; Pds	Pendrin	AF167412	Membrane transport (anion exchange)	**1.92 ± 0.22**	**1.83 ± 0.19**	943
Nxph1	Neuroexophilin 1	L27867	Neuropeptide-like secretory factor	**2.11 ± 0.35**	**1.50 ± 0.32**	398
–	Hypothetical protein C01F1.2-like; similar to pir‖T15371, *C. elegans*	Unknown 195	Other	**2.12 ± 0.29**	**1.86 ± 0.20**	719
–	Intraacrosomal protein SP-10-like; similar to pir‖I77964, western baboon	Unknown 51	Other	24.03 ± 20.60	**2.97 ± 0.35**	14664
–	α-2u globulin	AB039829	Pheromone-binding urinary protein	**58.41 ± 50.95**	**7.31 ± 1.97**	45540
Tcn2p; Tc2	Transcobalamin II precursor (TCII)	AF054810	Plasma binding protein (Cobalamin, B12)	**2.16 ± 0.17**	**2.12 ± 0.23**	2437
PstI	Pancreatic secretory trypsin inhibitor	D11325	Plasma protein	**3.60 ± 0.75**	**2.48 ± 0.60**	2384
Ela1	Elastase 1	L00117	Plasma protein (protease)	3.21 ± 1.28	**1.77 ± 0.11**	356
Rpe65	Retinal pigment epithelium-specific protein 65 kDa; plasma retinal-binding protein receptor	AF035673	Receptor	2.76 ± 0.72	1.98 ± 0.41	1198
Chrm2	Muscarinic acetylcholine receptor m2	AB017655	Receptor, G protein-coupled	**5.86 ± 0.92**	**6.28 ± 1.08**	7366
Gpr37; Ednrbl	G protein-coupled receptor CNS1; endothelin receptor type B-like	AF087946	Receptor, G protein-coupled	**1.76 ± 0.23**	**2.37 ± 0.29**	999
Gpr10	G protein-coupled receptor 10; prolactin-releasing peptide receptor	S77867	Receptor, G protein-coupled	**2.70 ± 0.25**	1.55 ± 0.24	152
NILR-1; Pirb	Ig-like transmembrane receptor NILR-1; paired Ig-like receptor B	AF082534	Receptor, transmembrane	**2.64 ± 0.27**	**3.03 ± 0.09**	8383
Gig2	G protein-coupled receptor induced protein	AF205438	Signal transduction (protein kinase)	**2.04 ± 0.08**	**1.85 ± 0.27**	196
Tgfb1i4	TGFβ-stimulated clone 22; TSC-22	L25785	Signal transduction (transcriptional repressor)	**1.73 ± 0.16**	**1.58 ± 0.16**	422
Crem	cAMP responsive element modulator	S66024	Signal transduction (transcriptional repressor)	**2.03 ± 0.23**	**1.64 ± 0.12**	249
Ptk2b; CAKβ; Pyk2	Protein tyrosine kinase 2 β; cell adhesion kinase β	D45854	Signal transduction (tyrosine kinase)	**3.85 ± 0.51**	**5.32 ± 2.48**	322
–	20 α-hydroxysteroid dehydrogenase; LOC171516	D14424/ L32601	Steroid metabolism	**4.52 ± 0.73**	**2.92 ± 0.39**	94

Table 1. *Continued*

Gene Designation	Common Name	Accession No.	Category/Function	Male:Female Ratio	Male:Male + GH Ratio	Average Male Intensity
Hsd11b1	Corticosteroid 11-β-dehydrogenase	J05107	Steroid metabolism	**4.87 ± 0.89**	**7.25 ± 2.25**	1153
Hsd3b; Hsd1	3-β-Hydroxy-Δ(5)-steroid dehydrogenase	M67465	Steroid metabolism	**2.95 ± 0.67**	**2.94 ± 0.44**	358
Ste; Estsul	Sulfotransferase ST1E2; estrogen sulfotransferase form 3	S76489	Steroid metabolism	**37.64 ± 24.74**	**74.26 ± 41.94**	9039
Akra	Aldo-keto reductase A-like; similar to gbd‖AF177041, Mus musculus	Unknown 22	Steroid metabolism	**1.73 ± 0.06**	1.99 ± 0.35	1573
Cyp2c11	Cytochrome P450 2C11	J02657	Steroid/foreign compound metabolism	**21.12 ± 8.34**	**7.64 ± 2.64**	5484
Cyp2c13	Cytochrome P450 2C13	J02861	Steroid/foreign compound metabolism	**14.20 ± 10.19**	**7.80 ± 1.61**	3081
Sult1a2; Stp2; St1c1	Sulfotransferase ST1C1, N-hydroxy-arylamine-bioactivating	L22339	Steroid/foreign compound metabolism	2.18 ± 0.36	**2.05 ± 0.23**	1320
Cyp3a2	Cytochrome P450 3A2	M13646	Steroid/foreign compound metabolism	**4.69 ± 2.11**	**7.70 ± 2.03**	1184
Cyp3a18	Cytochrome P450 3A18	X79991	Steroid/foreign compound metabolism	**3.07 ± 0.37**	**3.71 ± 0.36**	757
Cyp4a2/ Cyp4a3	Cytochrome P450 4A2/P450 4A3 (genes not distinguished)	M33936	Steroid/foreign compound metabolism	**2.43 ± 0.34**	**1.81 ± 0.08**	2969
Gjb6	Gap junction protein β 6; Connexin 30	AF170284	Transmembrane channel	2.52 ± 0.72	**3.58 ± 0.40**	494
–	MIC2 like 1; Mic2l1; vms-tm2; Rhombex-40	AB031014	Transmembrane protein	**3.22 ± 0.43**	**2.65 ± 0.38**	1441
Asct2	Sodium-dependent neutral amino acid transporter, ASCT2	AJ132846	Transporter (amino acids)	**2.41 ± 0.23**	**2.20 ± 0.22**	420

Shown are genes expressed at a higher level in male compared with female and continuous GH-treated male rat liver (mean ± SE, n = 4 or 8 individual livers/group). All genes shown met the criteria for inclusion detailed in *Materials and Methods*. *Bold* ratios were found to be statistically significant ($P < 0.05$). –, No gene designation is available.

other genes were down-regulated by continuous GH treatment but did not display a detectable sex-difference in expression (Tables 4B and 5).

Thirty-seven genes were found to be expressed in a female-predominant fashion. Of these, 27 genes (73%) were both female predominant and GH regulated, as demonstrated by their up-regulation in continuous GH-treated male liver as compared with untreated male liver (Table 2). Three other genes were induced in male rat liver by GH treatment, but in contrast to the other continuous GH-inducible genes, these three genes were not expressed at a significantly higher level in female compared with male liver (Table 4A). Nine other genes were expressed in a female-predominant fashion but were not induced in male liver by continuous GH treatment (Table 2B), whereas one female-predominant gene was suppressed after GH treatment (Tables 3C and 5).

The data presented in Table 5, which plots sex specificity *vs.* GH regulation, lie heavily on the diagonal, indicating that GH is an important determinant of the sex dependence of liver gene expression. Analysis of these data showed the distribution to be highly significant ($P \ll 0.001$ by Pearson's χ^2 analysis), supporting the conclusion that GH is a major regulatory determinant for both the male-predominant and the female-predominant genes. Furthermore, analysis of the quantitative relationship between sex specificity and GH regulation revealed a linear correlation ($r = 0.814$) when male:female and the corresponding male: male + GH expression ratios were plotted for a group of 278 genes selected on the basis of their highly reproducible ratios between the different liver pairs ($P < 0.1$ for n = 4 or n = 8) (Fig. 1). A similar correlation was obtained by analysis of male:female and male: male + GH ratio distributions for approximately all 6000 genes represented on the arrays (data not shown).

QPCR analysis was carried out to quantitate the relative expression levels of 17 genes selected from genes represented on the microarrays (see QPCR primer sequences published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). These analyses used RNA samples prepared from a set of male, female, and continuous GH-treated male rat livers (n = 4–8 livers/group) separate from the set used in the microarray studies. Expression patterns determined by QPCR for 10 of the genes shown in Tables 1 and 2 are presented in Table 6, A and B. In all 10 cases, the overall pattern of expression revealed by QPCR was in good agreement with the results of the microarray experiments. Moreover, for seven of the 10 genes, male:female and/or male:male + GH expression ratios determined by QPCR were substantially higher (Table 1 genes) or substantially lower (Table 2 genes) than the corresponding ratios determined by microarray analysis (Table 6, A and B). In the case of one of the genes, CYP4A2, the higher expression ratio obtained by QPCR reflects

Table 2. Female-Dominant Genes Up-Regulated in Male Rat Liver by Continuous GH Treatment

Gene Designation	Common Name	Accession No.	Category/Function	Male:Female Ratio	Male:Male + GH Ratio	Average Female Intensity
Tat	Tyrosine aminotransferase	AJ010709	Amino acid metabolism	**0.61 ± 0.07**	**0.45 ± 0.06**	799
Hal	Histidine ammonia lyase	M58308	Amino acid metabolism	**0.67 ± 0.07**	**0.52 ± 0.07**	402
Lal	Lysosomal acid lipase	S81497	Cholesterol metabolism	**0.62 ± 0.06**	**0.58 ± 0.05**	623
Adh1	Alcohol dehydrogenase 1	M15327	Enzyme (alcohol metabolism)	**0.48 ± 0.07**	**0.46 ± 0.04**	3407
Facl5; Acs5	Acyl-CoA synthetase 5; fatty acid CoA ligase, long chain 5	AB012933	Fatty acid synthesis	**0.57 ± 0.03**	**0.58 ± 0.06**	1081
Cd36	Fatty acid translocase; CD36 antigen	AF072411	Fatty acid synthesis	0.31 ± 0.12	**0.26 ± 0.04**	724
Facl4	Acyl-CoA synthetase 4; fatty acid CoA ligase, long chain 4	D85189	Fatty acid synthesis	**0.61 ± 0.08**	**0.43 ± 0.05**	178
Fmo5	Similar to flavin-containing monooxygenase 5, mouse	Unknown 184	Foreign compound metabolism	**0.54 ± 0.05**	0.51 ± 0.12	102
Fgf5	Fibroblast growth factor 5	D64086	Growth factor; signal transduction	**0.44 ± 0.01**	0.53 ± 0.11	730
Nsf	N-ethylmaleimide sensitive fusion protein	AF189019	Membrane fusion	0.50 ± 0.18	0.57 ± 0.25	1263
Syt8	Synaptotagmin 8, Ca+2/phospholipid-binding	U20110	Membrane fusion	0.40 ± 0.12	**0.39 ± 0.05**	1497
5E5 antigen	Intranuclear neuronal antigen 5E5, DNA-binding	D37934	Nuclear protein	**0.39 ± 0.04**	**0.48 ± 0.05**	1148
A1bg	α-1-B glycoprotein, liver regeneration-related and GH-regulated	AJ302031	Plasma glycoprotein	**0.13 ± 0.03**	**0.15 ± 0.02**	1897
Kng_v1	K-kininogen, bradykinin-containing	M11884	Plasma protein	**0.42 ± 0.03**	**0.42 ± 0.07**	2562
–	K-kininogen, LMW I precursor-like; similar to pir‖A28055, rat	Unknown 121	Plasma protein	**0.51 ± 0.07**	**0.57 ± 0.10**	2532
Lifr	Leukemia inhibitor factor receptor α-chain (LIF-Rα)	D86345	Receptor, cytokine	**0.35 ± 0.06**	0.36 ± 0.13	218
Htr	Serotonin (5-hydroxytryptamine) receptor	M21410	Receptor, G protein-coupled	0.52 ± 0.13	**0.40 ± 0.05**	653
Prlr	Prolactin receptor	M95683	Receptor, hormone	**0.24 ± 0.07**	**0.33 ± 0.02**	365
Hrasls3	H-ras-like tumor suppressor H-rev107	X76453	Signal transduction	**0.51 ± 0.10**	**0.53 ± 0.06**	936
Avdp; Akr1b7	Aldo/keto reductase-related, androgen-regulated vas deferens protein	AF182168	Steroid metabolism	0.31 ± 0.15	**0.28 ± 0.10**	674
Sult2a1	Sulfotransferase SULT2A1, hydroxysteroid; alcohol sulfotransferase	D14989	Steroid/foreign compound metabolism	**0.16 ± 0.03**	**0.40 ± 0.05**	16793
Cyp2c12	Cytochrome P450 2C12; steroid sulfate 15β hydroxylase	J03786	Steroid/foreign compound metabolism	**0.21 ± 0.04**	**0.21 ± 0.06**	11187
Cyp2c7	Cytochrome P450 2C7	M18335	Steroid/foreign compound metabolism	**0.46 ± 0.06**	**0.63 ± 0.05**	9207
Cyp2c23	Cytochrome P450 2C23; arachidonic acid epoxygenase	S67064/U04733	Steroid/foreign compound metabolism	**0.51 ± 0.04**	**0.52 ± 0.05**	3016
Gsta5	Glutathione-*S*-transferase Yc2	S82820	Steroid/foreign compound metabolism	**0.46 ± 0.04**	**0.59 ± 0.03**	3853
Sth2	Sulfotransferase gene 2, hydroxysteroid	XM_214755	Steroid/foreign compound metabolism	**0.19 ± 0.04**	**0.41 ± 0.03**	15505
Shank1	Shank1, postsynaptic density scaffold protein, SAPAP-interacting	AF102855	Synapse formation; signal transduction	0.38 ± 0.13	**0.31 ± 0.06**	911

Shown are genes expressed at a lower level in male compared with female and continuous GH-treated male rat liver (mean ± SE, n = 4 or 8 individual livers/group). All genes shown met the criteria for inclusion detailed in *Materials and Methods*. *Bold* ratios were found to be statistically significant ($P < 0.05$). –, No gene designation is available.

Table 3A. Male-Dominant Genes Not Responsive to Continuous GH Treatment in Male Rat Liver (mean ± SE, n = 4 or 8 Individual Livers/Group)[a]

Gene Designation	Common Name	Accession No.	Category/Function	Male:Female Ratio	Male:Male + GH Ratio	Average Male Intensity
Alpi2	Intestinal alkaline phosphatase-II	AF227508	Enzyme (phosphatase)	**1.72 ± 0.22**	1.00 ± 0.11	380
Apoc3	Apolipoprotein C-III	J02596	Plasma lipoprotein	**1.51 ± 0.11**	1.17 ± 0.16	16470
–	Plasma proteinase inhibitor α-1-inhibitor	M22360	Plasma protein	**1.52 ± 0.09**	1.13 ± 0.10	1385
Cntfr	Ciliary neurotrophic factor receptor α	S54212	Receptor, cytokine	**1.81 ± 0.17**	1.11 ± 0.08	270
Cyp2c22	Cytochrome P450 2C22	M58041/X53477	Steroid/foreign compound metabolism	**3.44 ± 0.51**	0.87 ± 0.06	3264

Table 3B. Female-Dominant Genes Not Responsive to Continuous GH Treatment in Male Rat Liver (mean ± SE, n = 4 or 8 Individual Livers/Group)[a]

Gene Designation	Common Name	Accession No.	Category/Function	Male:Female Ratio	Male:Male + GH Ratio	Average Female Intensity
Agm	Heparin sulfate proteoglycan Agrin	S44194	Basement membrane	**0.62 ± 0.10**	0.86 ± 0.05	1183
Sc5d	Sterol-C5-desaturase-like	AB052846	Cholesterol biosynthesis	**0.57 ± 0.07**	0.91 ± 0.06	650
Tpm	β-tropomyosin, skeletal muscle	L00381	Cytoskeleton	**0.44 ± 0.05**	0.88 ± 0.10	90
Amd1	S-adenosylmethionine decarboxylase 1	M34464	Enzyme (polyamine biosynthesis)	**0.42 ± 0.10**	0.88 ± 0.07	350
Csnk1a1	Casein kinase 1, α I	U77583	Enzyme (Ser/Thr protein kinase)	**0.60 ± 0.13**	1.04 ± 0.13	93
–	Histones TH2A and TH2B, testes	X59962	Histones	**0.66 ± 0.06**	1.10 ± 0.13	104
Mt1a	Metallothionein	M11794	Metal detoxification	**0.49 ± 0.08**	0.89 ± 0.10	4794
–	Homeobox protein R3	M37567	Other	**0.55 ± 0.06**	0.88 ± 0.08	840
Por	NADPH-cytochrome P450 oxidoreductase	M10068	Steroid/foreign compound metabolism	**0.54 ± 0.07**	0.94 ± 0.05	634

Table 3C. Female-Dominant Genes Suppressed in Male Rat Liver by Continuous GH Treatment (mean ± SE, n = 4 or 8 Individual Livers/Group[a]

Gene Designation	Common Name	Accession No.	Category/Function	Male:Female Ratio	Male:Male + GH Ratio	Average Female Intensity
Hsd17b2	Hydroxysteroid-17β, dehydrogenase type 2	X91234	Steroid metabolism	0.52 ± 0.09	1.97 ± 0.41	1364

[a]All genes shown met the criteria for inclusion detailed in *Materials and Methods. Bold* ratios were found to be statistically significant ($P < 0.05$). −, No gene designation is available.

the specificity of the PCR primers, in so far as the oligonucleotide used to detect CYP4A2 on the microarray cross-hybridizes with CYP4A3, whose expression in liver is not sex dependent or hormonally regulated (30). However, in the case of the other CYP genes, the microarray primers are gene specific (Fig. 2 and data not shown). Expression ratios determined by microarray analysis may therefore understate the true extent of differential regulation of those genes that are highly regulated, as quantitated by QPCR, in agreement with another report (31).

QPCR analysis was applied to seven other genes represented on the microarray. Five of these genes (Table 6C) exhibited higher expression in female and in GH-treated male liver than in untreated male liver by microarray analysis, but did not make the male: female and male:male +GH threshold ratio of 0.66 for inclusion in Table 2. In all five cases, QPCR verified the overall pattern of expression seen in the microarray analysis. Indeed, QPCR indicated a substantially higher degree of regulation in several cases: most notably, activated leukocyte cell adhesion molecule. QPCR also verified the unique pattern of expression seen for two other genes (Table 6D): hydroxysteroid-17β dehydrogenase type 2, which was female predominant but suppressed by continuous GH treatment (Table 3C), and growth response protein CL-6, which was sex independent and induced by GH but did not meet the criteria for inclusion in Table 4A.

Table 4A. Non-Sex-Specific Genes Induced by Continuous GH Treatment of Male Rats (mean ± SE, n = 4 or 8 Individual Liver Comparisons)

Gene Designation	Common Name	Accession No.	Category/Function	Male:Female Ratio	Male:Male + GH Ratio	Average Male Intensity
Epb4.1l1	Erythrocyte protein band 4.1-like isoform	AB019257	Cytoskeleton	0.87 ± 0.03	**0.63 ± 0.03**	208
Syl	Syncollin	AF008197	Membrane fusion	1.05 ± 0.03	0.63 ± 0.17	66
Hgfac	Hepatocyte growth factor activator	AB013092	Plasma protease	0.92 ± 0.04	**0.66 ± 0.05**	1037

Table 4B. Non-Sex-Specific Genes Suppressed by Continuous GH Treatment of Male Rats (mean ± SE, n = 4 or 8 Individual Liver Comparisons)

Gene Designation	Common Name	Accession No.	Category/Function	Male:Female Ratio	Male:Male + GH Ratio	Average Male Intensity
Cdo1	Cysteine dioxygenase 1, cytosolic	D83481/M35266	Amino acid metabolism	0.97 ± 0.04	**1.59 ± 0.10**	2420
Ces3	Carboxylesterase 3	AF171640	Enzyme (esterase)	1.23 ± 0.08	**1.66 ± 0.23**	1040
Camk2d; CAMK1	Calcium/calmodulin-dependent protein kinase II, δ	X77194	Protein kinase	1.02 ± 0.06	**1.60 ± 0.13**	500
Ugt8	UDP-glucuronosyltransferase 8	L21698	Steroid/Foreign compd metabolism	0.97 ± 0.07	**1.97 ± 0.22**	60

Genes shown met the criteria for inclusion detailed in *Materials and Methods*. *Bold* ratios were found to be statistically significant ($P < 0.05$).

Table 5. Summary of the Gene Lists

Sex Specificity	Response to Continuous GH Treatment		
	Induction	Suppression	Not Responsive
Female-dominant	27	1	9
Male-dominant	0	44	5
Non-sex-specific	3	4	>2475[a]

Shown are the total number of genes that met the criteria described in *Materials and Methods*, sorted by sex specificity and response to continuous GH treatment. Pearson's χ^2 tests displayed a high level of association between the two experimental paradigms.

[a] Estimated based on the number of genes, combined across rat liver and rat 5K arrays, with ratios between 0.8 and 1.2 on at least six of eight arrays.

DISCUSSION

Oligonucleotide-based microarrays were used to characterize the sex dependence of liver gene expression and to identify the role that GH plays in establishing and maintaining this sexual dimorphism. Traditional methods of analyzing GH-regulated genes have employed GH-deficient rodent models, *e.g.* hypophysectomized rats, and have identified genes such as CYPs, sulfotransferases, and glutathione-*S*-transferases as being dependent on GH for expression. Some liver-expressed genes are direct targets of GH action (*e.g.* IGF-I), whereas others, including the GH-dependent liver CYP enzymes, are regulated by GH in a complex fashion and in a manner that depends on the temporal pattern of plasma GH stimulation, which is sexually dimorphic. The major goal of this study was to obtain a global view of the sexual dimorphism of liver gene expression, and in particular, to ascertain whether GH is a major factor regulating this expression. Our findings demonstrate that the large majority of male-predominant rat liver genes identified by microarray analysis (44 of 49 genes identified) are under the control of GH, as shown by their down-regulation in male rat liver after treatment with GH for 7 d in a continuous manner (*i.e.* female-like plasma GH pattern) (Fig. 3 and Table 5). The majority of female-predominant genes identified were also shown to be responsive to the effects of GH (27 of 37 genes), as demonstrated by their up-regulation in male rat liver after continuous GH treatment. However, nine other female-dominant genes did not apparently exhibit this GH response, suggesting that their female-predominant pattern of expression reflects other endocrine regulatory factors. In addition, three genes were expressed at similar levels in male and female liver, yet were induced by continuous GH treatment, and four genes that were not sexually dimorphic were suppressed by GH treatment. These observations indicate that the actions of GH are not restricted to sexually dimorphic liver gene expression and may include regulation of genes that are not gender specific.

Using traditional methods to monitor gene expression, sexual dimorphism and/or GH regulation have been demonstrated for several of the female-predominant genes identified in the present microarray study. These include CYP2C12 (16, 17), 17β-hydroxysteroid dehydrogenase type 2 (32), alcohol dehydrogenase (33), glutathione-*S*-transferase Yc2 (21), α1B-glycoprotein (34), prolactin receptor (35), and several hy-

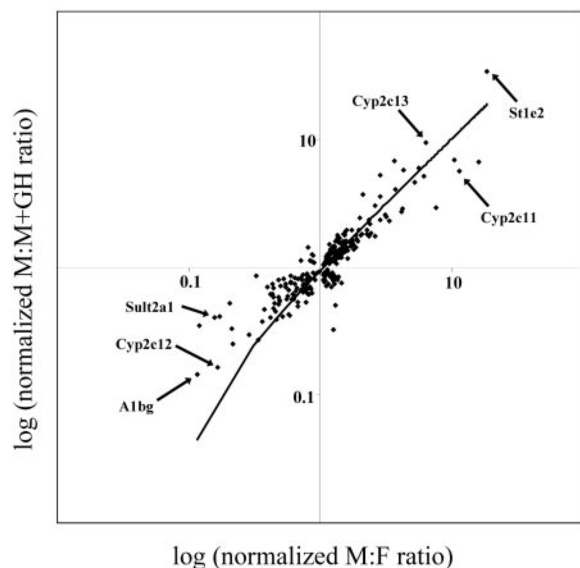


Fig. 1. Scatterplot of the Male:Female *vs.* Male:Male + GH Expression Ratios for Subset of Microarray Genes

Shown are male:female (x-axis) and the corresponding male:male + GH expression ratios (y-axis) plotted on a logarithmic scale with the origin at (1, 1) for 278 genes that were selected based on their highly reproducible expression ratios ($P < 0.1$) in either n = 4 or n = 8 arrays (genes represented on one, or both, microarray platforms, respectively). Genes mapping to the *upper right* quadrant (*e.g.* Cyp2c11, Cyp2c13, st1e2) are male specific and GH suppressed, and genes mapping to the *lower left* quadrant (*e.g.* A1bg, Cyp2c12, Sult2a1) are female specific and GH induced. The best-fit line ($y = 1.017x - 0.074$; $r = 0.814$) shows a slope close to 1 and an intercept close to 0, with a high correlation between the degree of sex specificity and GH responsiveness.

droxysteroid sulfotransferases (20). Male-predominant genes listed in Table 1 and shown by traditional methods to be subject to GH regulation include CYP2C11 (16, 17), CYP2C13 (36), CYP3A2 (37), CYP3A18 (38), CYP4A2 (30), corticosteroid 11β-dehydrogenase (39), carbonic anhydrase 3 (40), and sulfotransferases ST1E2 and 1C1 (20, 41). One of the male-predominant genes, CYP2C22 (42), was confirmed to be male predominant, but unexpectedly, was not found to be suppressed by continuous GH treatment (Table 3A). Thus, there may be multiple mechanisms for regulation of male-predominant liver gene expression. Earlier studies identified two distinct classes of GH-regulated, male-specific genes (15). Members of one class, represented by CYP2C11, are obligatorily dependent on pulsatile GH for expression, whereas members of the second class, which includes CYP2A2, CYP3A2, and CYP4A2, do not exhibit this GH dependence, as indicated by their full expression in hypophysectomized male rat liver and by their up-regulation to near male levels after hypophysectomy in female rat liver (16). However, both classes of male-specific genes are down-regulated by continuous GH treatment, a response that is distinct from that exhibited by CYP2C22 in the present study. Additional studies are required to further characterize the male-predominant, GH-regulated genes presently identified to determine which of these classes they belong to, and to elucidate the mechanisms that underlie their distinct responses to GH ablation by hypophysectomy.

cDNA-based microarrays were used in several earlier studies to identify target genes for GH action in the paradigm of liver gene expression (24–27). Although cDNA and oligonucleotide-based microarrays exhibit similar sensitivities for detecting changes in gene expression (31, 43), cDNA microarrays can be limited by their ability to discriminate between closely related DNA sequences because of the cross-hybridization that invariably occurs when a single arbitrary set of hybridization conditions is applied uniformly across the thousands of genes represented on the cDNA array (*c.f.* Refs. 44 and 45). The present study was carried out using microarrays constructed from 50-nucleotide-long oligonucleotides chosen to maximize gene specificity and matched with respect to length, GC content, melting temperature and designed to be free from secondary structure and self-annealing tendencies. These oligonucleotides were generally effective in distinguishing between closely related cDNAs within complex gene families and subfamilies, as exemplified by CYPs belonging to subfamily CYP2C. This gene subfamily includes seven closely related rat genes (75–86% nucleotide identity), all of which are expressed in liver, and six of which (CYPs 2C7, 2C11, 2C12, 2C13, 2C22, and 2C23) were represented on the arrays used in the present study. The percent identity between any given 50-nucleotide-long CYP2C oligomer on the array and nontarget CYP2C transcripts was generally between 40% and 70%, and in all cases was devoid of complementary sequences greater than 15 contiguous bases (Fig. 2 and data not shown). Cross-hybridization is unlikely under these conditions (43). As noted above, several rat CYP2C genes are GH regulated and sexually dimorphic in their expression, with CYPs 2C11, 2C13, and 2C22 being male dominant and CYPs 2C7 and 2C12 female dominant. These patterns of expression were verified in the present study, supporting the specificity of the oligonucleotide probes spotted on the arrays.

The two commercial microarrays used in this study encompassed a total of 5889 rat genes, enabling us to identify both known genes and novel genes that display sexual dimorphism and/or regulation by GH, including six sequences not represented in GenBank. Unlike a previous study of GH-regulated liver genes (24), the present study specifically sought to identify liver-expressed genes that are both sex dependent and GH regulated. Many of the sex-dependent, GH-regulated genes that we identified are involved either in steroid synthesis or in the metabolism of steroids and foreign compounds. Others encode serum proteins, structural proteins, receptors, membrane trafficking proteins, and transporters. Several of the genes identified code for signaling molecules such as nuclear

Table 6. QPCR Analysis of Select Regulated Genes Identified by Microarray Analysis (mean ± SE, n = 4 or 8 Individual Liver Comparisons)

	Accession No.	Common Name	QPCR		Microarray	
			Male:Female Ratio	Male:Male + GH Ratio	Male:Female Ratio	Male:Male + GH Ratio
6A.	AB030829	Carbonic anhydrase III	4.78 ± 1.13	16.58 ± 8.81	15.48 ± 4.78	7.58 ± 1.49
	J02657	Cytochrome P450 2C11	1680 ± 500	125 ± 54	21.12 ± 8.34	7.64 ± 2.64
	X79991	Cytochrome P450 3A18	11.98 ± 2.94	5.42 ± 3.03	3.07 ± 0.37	3.71 ± 0.36
	M33936	Cytochrome P450 4A2[a]	7.28 ± 2.99	5.80 ± 2.24	2.43 ± 0.34	1.81 ± 0.08
	D28560	Phosphodiesterase I/nucleotide pyrophosphatase 2	1.55 ± 0.37	2.00 ± 0.72	2.01 ± 0.14	2.44 ± 0.20
	AF054810	Transcobalamin II precursor (TCII)	1.50 ± 0.63	1.91 ± 1.11	2.16 ± 0.17	2.12 ± 0.23
6B.	AB012933	Acyl-CoA synthetase 5	0.38 ± 0.12	0.32 ± 0.04	0.57 ± 0.03	0.58 ± 0.06
	J03786	Cytochrome P450 2C12; steroid sulfate 15β hydroxylase	0.0013 ± 0.0002	0.0046 ± 0.0017	0.21 ± 0.04	0.21 ± 0.06
	U04733/S67064	Cytochrome P450 2C23; arachidonic acid epoxygenase	0.46 ± 0.12	0.42 ± 0.08	0.51 ± 0.04	0.52 ± 0.05
	X76453	H-ras-like tumor suppressor H-rev 107	0.21 ± 0.10	0.13 ± 0.06	0.51 ± 0.10	0.53 ± 0.06
6C.	AB008538	Activated leukocyte cell adhesion molecule; Alcam	0.068 ± 0.01	0.26 ± 0.05	0.65 ± 0.03	0.72 ± 0.07
	AF146044	Aminopeptidase A	0.48 ± 0.11	0.66 ± 0.13	0.74 ± 0.02	0.78 ± 0.07
	Y11283	Inter-α-trypsin inhibitor, heavy chain 4 (H4P)	0.52 ± 0.08	0.11 ± 0.01	0.69 ± 0.04	0.55 ± 0.05
	AJ250374	Phosphodiesterase I/nucleotide pyrophosphatase 1	0.53 ± 0.14	0.34 ± 0.12	0.75 ± 0.03	0.67 ± 0.07
	AF017185	Protein tyrosine phosphatase PTEN	0.59 ± 0.12	0.72 ± 0.13	0.74 ± 0.05	0.74 ± 0.07
6D.	L13619	Growth response protein CL-6, insulin-inducible	1.03 ± 0.34	0.22 ± 0.05	0.77 ± 0.11	0.40 ± 0.06
	X91234	Hydroxysteroid-17β, dehydrogenase type 2	0.73 ± 0.22	1.32 ± 0.32	0.52 ± 0.09	1.97 ± 0.41

Genes shown in 6A and 6B were selected from those presented in Tables 1 and 2, respectively. Genes in 6C did not meet the threshold ratio for inclusion in Table 2. Genes in 6D showed unique patterns of expression by microarray analysis; these patterns were verified by QPCR.

[a] The oligonucleotide on the microarray did not distinguish CYP4A2 from CYP4A3; the latter gene is not sex dependent or GH regulated. These two genes were distinguished by the primers used for QPCR.

factors and phosphatases, some of which may potentially help mediate the effects of GH on sex-dependent liver gene expression. GH-regulated signaling molecules that are more highly expressed in female than male liver include the H-ras-like tumor suppressor H-rev 107, fibroblast growth factor 5 and several hormone, cytokine, and G protein-coupled receptors (Table 2). Potential signaling molecules found to be GH-regulated and more highly expressed in males include phosphodiesterase I/nucleotide pyrophosphatase 2, the protein kinase phosphorylase kinase γ-2, muscarinic acetylcholine receptor M2, and the cell growth regulator and gap junction component connexin 30 (Table 1). Further investigation will be required to ascertain whether these changes in mRNA expression are accompanied by corresponding changes in protein levels, and to elucidate the precise role that these proteins play in liver physiology and in the sexual dimorphism of liver function.

Many of the genes presently identified displayed moderate regulation by GH (*i.e.* 1.5- to 3-fold differences in expression in response to changes in GH status), as compared with the more than 100-fold differences in gene expression seen with the classic sexually dimorphic, GH pattern-regulated genes CYP2C11 and CYP2C12. These striking quantitative differences in the extent of sexual dimorphism and GH regulation were verified by QPCR (Table 6). This finding suggests that intrinsic signals distinct from GH, both inductive and repressive, contribute to the observed sexually dimorphic pattern of liver gene expression. QPCR also verified the expression patterns for several genes identified in the microarray

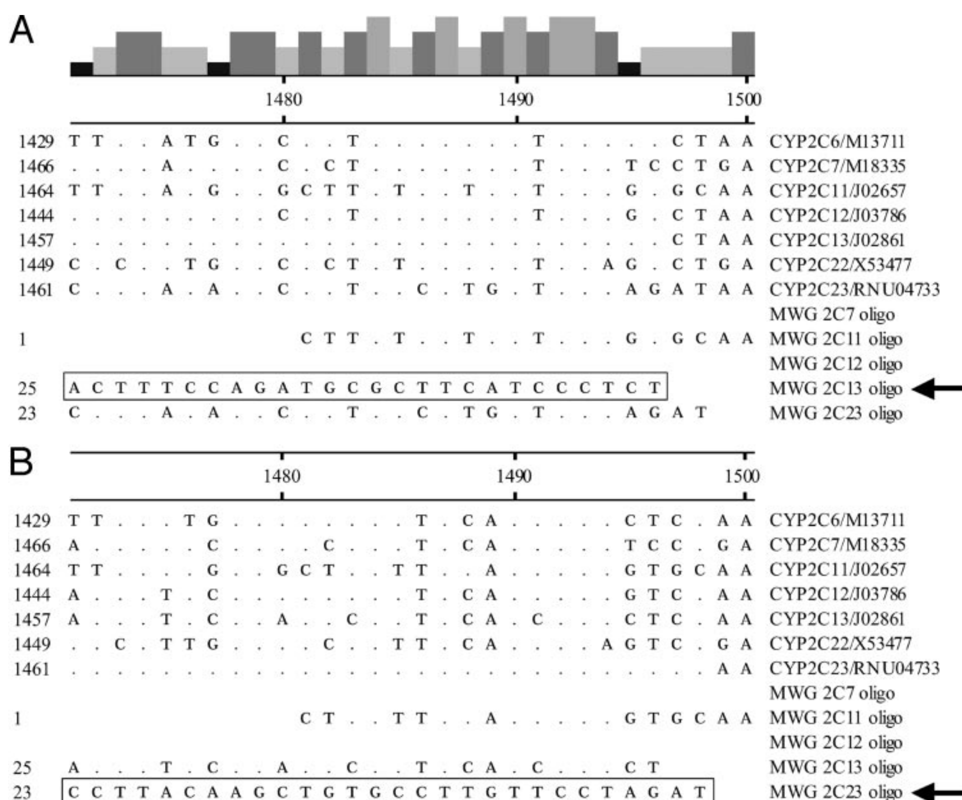


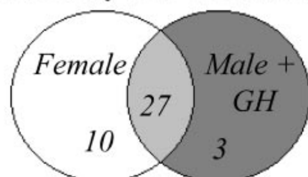
Fig. 2. Sequence Specificity of the Arrayed 50-Nucleotide-Long Oligomers Used to Detect Rat CYP2C Family Members

Shown are portions of each rat CYP2C mRNA sequence (cDNA nucleotide positions as shown on the *left*, and GenBank accession numbers shown on the *right*, as indicated). Also shown are portions of the 50-nucleotide sequences spotted on the array for CYP2C11, 2C13, and 2C23, with nucleotides that are identical shown as *dots* and mismatches to the CYP2C oligonucleotide sequence as indicated. A, Comparisons to the 3′-most 26 nucleotide segment of the CYP2C13 oligonucleotide spotted on the array (*boxed*); B, comparisons to the 3′-most 28 nucleotide segment of the CYP2C23 oligonucleotide. Sequences of each full-length CYP2C cDNA and of the indicated CYP2C oligonucleotides were aligned using DNAStar Megalign software (Madison, WI). Each of the oligonucleotides is seen to display multiple sequence differences with each of the six other CYP2C family members. *Bars at top* represent relative degree of sequences identity between the cDNAs for each of the positions shown. Percent sequence identities to the nontarget CYP2C genes over the entire 50-nucleotide length of each CYP2C oligonucleotide were as follows: 46–68% (CYP2C7 oligo), 38–46% (CYP2C11 oligo), 40–62% (CYP2C12 oligo), 40–76% (CYP2C13 oligo), and 36–56% (CYP2C23 oligo).

studies that were not previously known to be sexually dimorphic and GH-regulated (transcobalamin I precursor, phosphodiesterase I/nucleotide pyrophosphatase 2, acyl-coenzyme A (CoA) synthase 5, and H-ras-like tumor suppressor H-rev 107). Several other genes that were found by microarray analysis to be more highly expressed in female than in male liver, but did not meet our threshold ratio criteria for inclusion in Table 2, were also analyzed by QPCR and shown to be female predominant and induced by continuous GH treatment (Table 6C). The absence of these five genes from Table 2 serves as an example of false negatives, which occur frequently in microarray analysis. False positives are also an unavoidable feature of microarray analysis. However, this possibility may be minimized by the requirement that the genes listed in Tables 1–4 show a consistent pattern of regulation in at least three of four independent pairs of male:female livers and a corresponding number of male:GH-treated male livers, and by the fact that a majority of the genes listed in Tables 1–4 gave expression ratios that were tightly clustered in the four pairs of livers analyzed, and thus highly significant (table entries shown in bold). Nevertheless, it will be important to validate the patterns of gene expression profiling obtained in the present microarray studies through the use of alternative, more traditional methods, such as the QPCR analyses presented in Table 6.

Whereas the microarray analysis reported here is by no means comprehensive, it has led to the identification of several distinct groups (classes) of liver-expressed genes, each of which is unique with respect to its responsiveness to sex-dependent hormonal factors, of which GH is seen to be the major, but apparently not the sole determinant (Table 5). Conceivably, genes within each of the classes represented by Tables 1 and 2 are likely to share common regulatory

A. Female-dominant Genes compared to Genes induced by GH Treatment in Males



B. Male-dominant Genes compared to Genes suppressed by GH Treatment

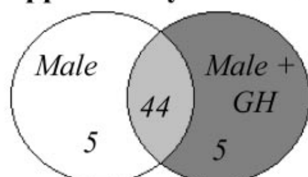


Fig. 3. Venn Diagram Representations of Liver Gene Expression Profiles of Adult Male and Female Rats, and of Male Rats Treated with GH by Continuous Infusion

A, The intersection highlights 27 genes that are up-regulated in common in female and continuous GH-treated male liver as compared with untreated male rat liver (Table 2). Ten genes were female dominant but not induced by GH treatment (Table 3, B and C), and three genes were induced by GH treatment but were not sex specific (Table 4A). B, The intersection highlights 44 genes that are down-regulated in common in female and continuous GH-treated male liver as compared with untreated male rat liver (Table 1). Five genes (Table 3A) were male dominant in their expression but were not responsive to GH treatment of males, whereas four other genes were suppressed by GH treatment but were not sex specific, and one gene was suppressed by GH treatment but was female dominant (Tables 4B and 3C).

mechanisms that enable them to respond to continuous GH treatment by gene up-regulation (Table 2) or down-regulation (Table 1). As GH-regulated, sexual dimorphic liver gene expression is primarily, if not exclusively, controlled at the level of transcription initiation (18, 19), future studies designed to elucidate common promoter region sequences and upstream regulatory elements may provide further insight into the mechanisms that underlie the sexual dimorphism of hepatic gene expression.

MATERIALS AND METHODS

Animals

Adult male and female Fischer 344 rats (8–10 wk of age) (n = 4/group) (Taconic, Inc., Germantown, NY) were untreated or were treated with rat GH (rGH-B-14-SIAFP, National Institute of Diabetes and Digestive and Kidney Diseases), given as a continuous infusion using an Alzet osmotic minipump delivering 2 μg GH/100 g body weight·h for 7 d. Intact, untreated male and female rats were killed and livers were collected, frozen in liquid N_2 and stored at −80 C.

RNA Isolation

Total liver RNA was isolated from frozen liver samples using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). Poly(A) RNA was then isolated using a commercial kit (Ambion, Austin, TX). RNA samples having an A_{260}/A_{280} ratio of at least 1.8 were deemed suitable for use in subsequent analyses. Total liver RNA and Poly(A) RNA samples were treated with deoxyribonuclease I (Ambion, Austin, TX) as described previously (46) and their integrity was checked on Northern blots probed for CYP2C6 mRNA (47), which is expressed at high levels in both male and female rat liver.

Oligonucleotide Microarray Experiments

The expression of sex-dependent and GH-regulated liver mRNAs was investigated using two commercial DNA microarrays, Pan Rat Liver Oligonucleotide array and 5K Rat Oligonucleotide array (MWG Biotech, Inc., High Point, NC). These arrays are respectively comprised of oligonucleotide sequences 50 nucleotides in length selected to represent unique (gene specific) sequences, generally derived from the coding sequence or 3′-untranslated region of each mRNA. The Pan Rat Liver and 5K Rat arrays used respectively represent 1353 unique liver-expressed genes (1408 features, including replicates; Pan Rat Liver array) and 5535 unique rat genes (5760 features, including replicates; 5K Rat array). Together, these microarrays represent 5889 unique rat genes, of which 999 genes are represented on both arrays. Poly(A) RNA purified from individual livers was labeled with Cy3-deoxyuridine triphosphate (dUTP) or Cy5-dUTP (PerkinElmer Life Sciences Inc., Boston, MA) in a reverse transcription reaction using 1 μg Poly(A) RNA and Superscript II (ribonuclease H mutant) enzyme (Invitrogen Life Technologies, Carlsbad, CA) at 39 C for 2 h. Unincorporated deoxynucleotide triphosphates were removed using the Qiaquick PCR-purification kit (QIAGEN, Valencia, CA). Cy5-labeled male liver cDNA was mixed with Cy3-labeled female or Cy3-labeled GH-treated male liver cDNA followed by cohybridization of the mixed cDNA population to the microarray for 18 h at 42 C. Arrays were washed according to the manufacturer's protocol. RNA from each of four individual sets of male, female and GH-treated male liver was converted to cDNA and labeled in duplicate. One series of eight hybridizations (four hybridizations with male + female liver cDNA, and four hybridizations with male + GH-treated male liver cDNA; n = 4 livers/treatment group) was carried out with the Pan Rat Liver arrays, and a second series containing the same eight hybridizations (n = 4 livers/group) was carried out with the 5K Rat arrays. Dye swapping experiments using a single pair of male and female RNA samples, respectively labeled with Cy3- and Cy5-dUTP, were performed to verify the independence of the microarray results from the nature of the cyanine dye label.

Data Acquisition and Analysis

Fluorescent cDNA bound to each microarray was detected with a GenePix 4000B array scanner (Axon Instruments, Foster City, CA) using a fixed laser power intensity and the minimum photomultiplier tube setting necessary to avoid bleaching while allowing for detection of features with faint fluorescent intensities. The manufacturer's GenePix 3.0 software package was used to locate individual spots, quantitate the Cy3- and Cy5-fluorescence intensity at each spot, and determine background signal intensities. Data from spots that were determined to result from hybridization anomalies or microarray errors (*e.g*. dust particles, fibers, or spotting anomalies including bleeding around the actual spotting area) were excluded from the analysis. The data were imported into GeneSpring 6.0 (Silicon Genetics,

Redwood City, CA). Fluorescence intensity values were calculated by subtracting the local median background value from the median foreground value for each spot. The use of median values instead of mean values reduced the effect of outliers generated by imperfections in GenePix's automatic spot detection protocol. Average background values were determined to be 40 ± 8 (Cy5 channel) and 51 ± 13 (Cy3 channel), mean ± SD. Lowess normalization, using 20% of the data for smoothing, was applied to the Cy3 channel for each microarray spot on each array using GeneSpring software. Lowess-normalized Cy3 values below 10 were set to a baseline value of 10. The ratio of the normalized channels (Cy5/Cy3) as reported by GeneSpring was used for expression analysis. All genes included in our analysis and listed in Tables 1–4 exhibited expression differences of 1.5-fold or higher *(i.e.* at least 50% greater expression in the Cy5 sample than in the Cy3 sample) or 0.66-fold or lower that were reproducible in microarray slides representing at least three of four male:female or male:male+ GH liver pairs, or at least two of three liver pairs for those genes where only three of the four microarrays gave data of suitable technical quality. The threshold values of ≥1.5 and ≤0.66 were chosen so as to not exclude genes whose expression is known to be sex dependent and/or GH regulated by other, independent studies, but whose microarray expression ratios did not satisfy a 2.0-threshold value. Genes with reproducible expression ratios between 0.8 and 1.2 were not considered to be sex specific (male:female comparisons) or GH regulated (male: male + GH comparisons). For those genes that are represented on both microarrays, the mean ratio and SE values were calculated based on n = 8 replicates. Expression ratios for all other genes were calculated based on n = 4 unless one of the replicates returned no data for that spot. Average fluorescence intensity values reported in Tables 1–4 are normalized values and were corrected for background fluorescent intensity. Expression ratios for the approximately 6000 genes examined on the 16 microarray slides used in this study are available as published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. The data are also available for query or download from the Gene Expression Omnibus (GEO) web site at NCBI (http://www.ncbi.nlm.nih.gov/geo).

Statistical Analysis

A one-sample *t* test (GeneSpring 6.0 software) was applied to the distribution of natural logs of the ratios for each gene. The *t* test implemented by the GeneSpring software package calculates the *P* value for the distribution of natural logs as compared with 0. A filter ($P < 0.05$) was applied to the *P* values to determine the statistical significance of each gene's differential expression for each DNA array type (Pan Rat Liver and 5K Rat array). Expression ratios that met the $P < 0.05$ criterion are shown in bold in Tables 1–4. Multiple testing correction methods, such as Bonferroni or Holm step-down, were not applied to the *P* values because these options, available in the GeneSpring 6.0 software package, depend heavily on the independence of each gene's expression and thus filter out many *bona fide* regulated genes to avoid all type I errors; they are thus too restrictive in their effort to avoid false positives (48).

A Pearson's χ^2 test was applied using MATLAB 6.1.0.450 (The MathWorks, Inc., Natick, MA) on a table of the gene counts for each observed relation between sex dominance and GH regulation (Table 5) to validate the correlation between the two experimental paradigms (sex specificity and GH regulation). The numbers for these relationships were set into a 3 × 3 table and the test was applied to determine whether the observed distribution differs significantly from the expected normal distribution. Any element in the table less than five was increased by pseudo-counts to five to fit the assumptions of the χ^2 test (*i.e.* no observation should be less than 5). The value of the non-GH-responsive, non-sex-specific genes was set at an underestimated value of 2475, to take into account potential experimental error. A χ^2 value of 2993, corresponding to $P \ll 0.001$, was calculated. The same high level of significance was found when a second calculation for the χ^2 test was made underestimating the value of the non-GH-responsive, non-sex-specific genes as 20, to reduce the bias on the expected distribution due to the large difference between 2475 and the other table entries. An equally high level of significance was found when a χ^2 test for a 2 × 2 table of only up-regulated and down-regulated genes for each condition was calculated.

Real-Time PCR Studies

The expression profiles of select genes represented on the microarrays were examined by a reverse transcription real-time QPCR method using an ABI Prism 7700HT instrument (Applied Biosystems, Foster City, CA) and SYBR green detection. cDNA was prepared from individual livers, and each cDNA was assayed in triplicate PCRs. Briefly, 1 μg of total liver RNA was treated with deoxyribonuclease I and reverse transcribed with oligo deoxythymidine$_{20}$-VN primers (V = any nucleotide but thymidine) using the GeneAmp RNA-PCR kit (Applied Biosystems) in a reaction volume of 20 μl as recommended by the manufacturer and described earlier (46). cDNA samples were used at a dilution of 1:100 in QPCR assays using the SYBR Green mix kit (Applied Biosystems, Foster City, CA). PCRs (4–5 μl total volume per well) were carried out in triplicate in individual wells of a 384-well plate. With each cycle of PCR, more SYBR Green dye molecules bind to the newly synthesized double-stranded DNA amplicon, resulting in an increase in fluorescence in real-time. Gene-specific primers were designed using Primer Express software (Applied Biosystems). Primer sequences used for the 17 genes shown in Table 6 are published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. Primer specificity and the extent of possible cross-hybridization with other genes in GenBank was evaluated by basic local alignment and search tool analysis (BLAST). Primer concentrations of 300–600 nM were generally found to be optimal. Assays were performed using liver cDNA prepared from n = 4–8 individual male, female and continuous GH-treated male livers, all separate from the livers used in the microarray analysis. QPCR amplification of 18S rRNA was carried out in parallel to normalize the input cDNA for each PCR. Products of mock reverse transcription reactions for each of the liver RNA samples were included as negative controls to verify the absence of amplification signal attributable to contamination by genomic DNA.

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