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Sex-Specific Early Growth Hormone Response Genes in Rat Liver

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Pituitary GH-secretory profiles are sex dependent and regulate the sexually dimorphic expression of a large number of genes in the liver. The slow response of many sex-specific liver genes to changes in plasma GH status suggests that GH acts in the liver via both direct and indirect mechanisms organized in a hierarchical regulatory network. Presently, genome-wide liver transcription profiling was conducted to elucidate the global impact of pituitary hormone ablation on the sex specificity of rat liver gene expression and to identify sex-specific genes that respond rapidly to GH as candidates for direct targets of GH action. Hypophysectomy abolished the sex specificity of approximately 90% of 1032 sex-dependent genes, consistent with the dominant role of pituitary GH in regulating liver sexual dimorphism. Two major classes of sex-specific genes were identified: genes that were down-regulated after hypophysectomy and may be subject to positive GH regulation (461 class I genes), and genes that were up-regulated after hypophysectomy and may be subject to negative GH regulation (224 class II genes). Fifty class I sex-specific genes were induced, and 38 class II sex-specific genes were suppressed within 90 min of a physiological GH pulse, suggesting they are primary GH response genes. A further 71 sex-specific genes responded after a second GH treatment and may correspond to secondary response genes. Twenty four DNA-binding proteins were identified as early GH response genes, of which 15 were induced and nine were suppressed by GH. Five of these 24 genes displayed sex-specific expression, consistent with a hierarchical transcriptional network controlling sex-specific liver gene expression. Class II male-specific genes, such as *Cyp2a2* and *Cyp2c13*, were down-regulated within 30 min of GH pulse treatment, as determined by heterogeneous nuclear RNA analysis, suggesting that transcription of these genes is restricted to the GH-free interpulse period in adult male rat liver. We conclude that GH acts via both positive and negative regulatory mechanisms to establish and maintain the sex specificity of liver gene expression. (*Molecular Endocrinology* 22: 1962–1974, 2008)

GH REGULATES THE sexually dimorphic patterns of a large number of liver-expressed genes including various plasma and urinary proteins, cytochromes P450 (CYPs), and other enzymes of steroid and foreign compound metabolism, and various receptors and signaling molecules (1, 2). Sex differences in liver gene expression are dictated by the temporal patterns of circulating GH, which are sex dependent and under gonadal control. In the adult male rat, GH is secreted in a highly regular, pulsatile manner, with high peaks of hormone (200–300 ng GH/ml plasma) occurring approximately every 3.5 h, separated by periods during which GH levels are undetectable (≤2 ng/ml). By contrast, pituitary GH release in the adult female rat is more frequent and results in a near continuous presence of GH in circulation (3, 4). A key difference between male and female GH profiles is the length of the interpulse interval that is characteristic of adult males. This GH-free recovery period is required for the expression of male-specific genes such as *Cyp2c11* (5), whereas a more continuous plasma GH profile, characteristic of female rats, activates the transcription of female-specific genes such as *Cyp2c12* (6, 7). Continuous infusion of GH in male rats and mice overrides the pulsatile, male plasma GH pattern and feminizes the expression of many liver genes (8, 9).

GH binds to its plasma membrane receptor, which leads to the activation/tyrosine phosphorylation of signal transducer and activator of transcription (STAT) transcription factors. Upon activation, STAT proteins form homo- and heterodimers by reciprocal and complementary phosphotyrosine-SH2 domain interactions and, within minutes, translocate into the nucleus where they bind target DNA response elements and stimulate gene transcription (10). STAT5b responds directly to the male pulsatile GH pattern and is proposed to be a key regulator of the GH-responsive

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Abbreviations: CYP, Cytochrome P450; F-Hypox, hypophysectomized female; F-UT, untreated female; GO, gene ontology; HNF, hepatocyte-enriched nuclear factor; hnRNA, heterogeneous nuclear RNA (primary RNA transcript); Hypox, hypophysectomized; ID, identification; M-Hypox, hypophysectomized male; M-UT, untreated male; qPCR, quantitative real-time PCR; STAT, signal transducer and activator of transcription; TFS, total flagging sum.

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male-specific and female-specific liver *Cyp* genes (11). In adult male rats, high levels of active, tyrosine-phosphorylated STAT5b are induced by each successive plasma GH pulse, with little or no tyrosine-phosphorylated STAT5b detected during the GH-free interpulse period (12, 13). By contrast, in livers of female rats, which are stimulated by GH in a more continuous manner, nuclear STAT5b levels are generally much lower than the peak levels seen in male liver (14). The importance of STAT5b for sex-specific *Cyp* expression is highlighted by the loss of sexually dimorphic *Cyp* gene expression in STAT5b-deficient male mice (15, 16) and by the resistance of STAT5b-deficient hypophysectomized (Hypox) mice to GH pulse induction of sex-dependent liver *Cyps* normally expressed in intact mouse liver (8, 17). However, STAT5b alone is not sufficient to induce the adult male pattern of liver gene expression when activated precociously in prepubertal rats given periodic (pulsatile) injections of GH (12) or when activated by very low GH pulses given to Hypox rats (18), suggesting a requirement for additional factors. Furthermore, although STAT5b binding sites have been identified in the promoters of several male-specific genes, including rat *Cyp2c11*, *Cyp2a2*, and *Cyp4a2* and hamster *Cyp3a10*, these STAT5b-binding sites confer weak transcriptional responses in GH-stimulated cells (19, 20). Moreover, the down-regulation of male-specific genes after continuous GH treatment of male mice requires several days to take effect, suggesting an indirect regulatory mechanism (8).

Taken together, the above studies suggest that GH-activated STAT5b regulates sex-specific genes directly, in cooperation with other transcription factors, and also indirectly, via early response genes that may encode transcriptional activators and repressors or other factors that mediate the effects of STAT5b. Hepatocyte-enriched nuclear factor (HNF)4α is an example of a liver-enriched transcription factor that can cooperate with STAT5b to positively regulate certain male-specific genes while inhibiting the expression of a subset of female-specific genes in male liver (8, 21). HNF6, encoded by the *Onecut* gene, is a female-predominant liver transcription factor that is itself subject to GH regulation and may also contribute to the female-specific expression of genes such as *Cyp2c12* (22, 23). Two other continuous GH-dependent, female-specific nuclear factors, Cutl2/Cux2 and Trim24, reportedly act as transcriptional repressors and have been proposed to contribute to the loss of GH-regulated, male-specific liver genes expression seen in male mice deficient in either STAT5b or HNF4α (24).

The global regulatory role of GH with respect to sex-specific liver gene expression has been established by DNA microarray analysis (25). DNA microarrays have also been used to investigate the impact of hypophysectomy on liver gene expression in male rats, with changes in gene expression of up to approximately 2-fold reported for 29% of 720 detectable liver transcripts after hypophysectomy (26). However, the impact of pituitary hormone ablation in females, and any sex-differences in the effects of hypophysectomy, in particular on genes expressed in a sex-dependent manner, was not investigated. Microarray technology has been also applied to identify direct targets of GH/STAT5b-dependent gene induction (27) and gene suppression (28); however, whether sex-specific genes respond directly to GH was not investigated in these studies.

The present study uses microarray technology to probe approximately 41,000 feature microarrays to characterize the effect of hypophysectomy on the sex specificity of rat liver gene expression on a global scale, and to identify sex-specific genes that respond to GH rapidly, and thus may be direct targets of GH action. Hypophysectomy is shown to result in a near global loss of liver sex specificity, and short-term GH pulse replacement studies enabled us to identify genes likely to be primary targets of GH action. These include several male-specific *Cyps* the expression of which was unexpectedly found to be rapidly down-regulated by a GH pulse, as well as several sex-specific transcription factors with the potential to mediate the effects of GH on downstream sex-specific targets.

RESULTS

Experimental Design

A large-scale expression study was conducted to characterize the effect of Hypox on the sex-specificity of liver gene expression, and to identify sex-specific genes that respond to GH rapidly and are thus likely to be direct targets of GH action. RNA was isolated from livers of male (M) and female (F) rats that were: 1) intact and untreated (UT); 2) hypophysectomized at adulthood (Hypox); 3) Hypox adult male rats that were treated with a single GH pulse and killed either 30, 60, or 90 min later (M-Hypox + GH); or 4) were treated with two GH injections, spaced 3–4 h apart, and killed 60 min after the second GH injection (M-Hypox + 2GH). RNA samples representing each of these four conditions were analyzed in six sets of competitive hybridizations to 41,012-feature oligonucleotide microarrays: 1) M-UT *vs.* F-UT; 2) M-Hypox *vs.* M-UT; 3) F-Hypox *vs.* F-UT; 4) M-Hypox *vs.* F-Hypox; 5) M-Hypox + GH *vs.* M-Hypox; 6) M-Hypox + 2GH *vs.* M-Hypox. Normalized ratios and *P* values were calculated for all six data sets using Rosetta Resolver software. Of the 41,012 probes, 4,150 met the threshold criteria [average expression ratio >2 (or <0.5) and a significance of $P < 0.005$] for at least one of the six data sets after elimination of redundant probes. A complete listing of these genes, along with their expression ratios, measured signal intensities in all six microarray experiments, and gene annotations is provided in Table S2 (published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).

Table 1. Sex Specificity of 4150 Genes of Interest in Intact and in Hypox Rat Liver

Sex Specificity in Intact, Untreated Rats	Sex Specificity in Hypox Rats	Gene Count	%
Male-specific	M > F	71	12
	F > M	1	0
	Non-sex-specific	517	88
	Total	589	
Female-specific	M > F	3	1
	F > M	22	5
	Non-sex-specific	418	94
	Total	443	
Sex-independent	M > F	66	2
	F > M	48	2
	Non-sex-specific	3004	96
	Total	3118	

Liver-expressed genes (4150) meeting the criteria of male-female ratio greater than 2.0 (or < 0.5) and $P < 0.005$ (genes of interest) are grouped according to the sex specificity of their expression in intact (UT) rats and secondarily according to the sex specificity of their expression in Hypox rats.

Sex Specificity in Intact and Hypox Rat Liver

Comparison of M-UT and F-UT liver gene expression profiles revealed sex specificity for 1032 of the 4150 genes of interest, similar to the number of sex-specific genes seen in mouse liver (15, 29, 30). Of the 1032 genes, 589 showed male-specific expression (M-UT:F-UT > 2.0), and 443 genes showed female specificity (M-UT:F-UT < 0.5). Pituitary hormone ablation led to a loss of sex specificity for approximately 90% of the sex-specific genes. Thus, 517 of the 589 male-specific genes (88%) and 418 of the 443 female-specific genes (94%) lost sex specificity after hypophysectomy (Table 1). Furthermore, many of the 71 male-specific genes and 22 female-specific genes that retained sex specificity after hypophysectomy showed a decrease in the sex specificity ratio. Of the sex-specific genes, less than 1% showed a reversal of sex specificity after hypophysectomy, and only 4% of the 3118 sex-independent genes of interest acquired sex specificity after hypophysectomy (Table 1).

Sex-Specific Response to Hypophysectomy

Many of the sex-specific genes responded to hypophysectomy in a highly sex-dependent manner (Table 2). Thus, of those sex-specific genes that responded to hypophysectomy in male liver only, 164 of 166 male-specific genes were decreased in expression and all 58 female-specific genes were increased in expression. In contrast, of those sex-specific genes that responded to hypophysectomy in female liver only, 120 of 121 male-specific genes were increased in expression whereas 137 of 138 female-specific genes were decreased. These patterns differ markedly from those of the sex-independent genes, where the distribution between genes increasing and genes decreasing in expression after hypophysectomy was independent of whether the change in expression occurred in males only, females only, or both sexes (Table 2). Moreover, for all 697 sex-independent genes that responded to hypophysectomy in both males and females, the response to hypophysectomy was in the same direction, *i.e.* either an increase or a decrease, in both sexes.

Clustering by Significance and Differential Expression

The general trends in gene expression, summarized above, and the impact of short-term GH treatment were further investigated by classification of the 4150 genes of interest using a binary flagging system (15), whereby each gene is assigned to a specific category, termed TFS (total flagging sum), based upon its expression ratio and *P* value in each of the six microarray experiments. Genes were thus classified into groups and subgroups based on their sex specificity in intact rats, their response to hypophysectomy, their sex specificity in Hypox rat liver, and their response to either one or two GH injections. Major gene groups are shown in supplemental Table S3, and a complete listing of gene groups is presented in supplemental Table S4. Analysis of the major sex-specific gene groupings (supplemental Table S3A) revealed two classes of sex-specific genes: 1) class I sex-specific genes, which are down-regulated in one, or both, sexes after hypophysectomy and thus require pituitary hormones for full expression; and 2) class II genes, which are up-regulated in either one or both sexes after hypophysectomy and are thus suppressed by pituitary hormones (Tables 2 and 3). An example of a well-characterized class I male-specific gene is *Cyp2c11*, the expression of which is dependent on the repeated stimulation by male plasma GH pulses and is down-regulated in male liver after hypophysectomy (5). *Cyp2a2* is a prototypic class II male gene; its expression does not require stimulation by plasma GH pulses but, rather, is repressed by the female plasma GH pattern (31–33). *Cyp2c12, Onecut1*/*Hnf6*, and *A1bg* are continuous GH-stimulated genes that serve as examples of class I female genes (6, 7, 23, 34). Class II female-specific genes have not been previously described in the rat; however, mouse *Cyp2b9* is representative of an analogous class of genes in mouse liver: it is highly female specific and is strongly up-regulated in Hypox male liver (8, 35). Class II female genes presently identified in rat liver include *Adh3*, *Adh4*, and *Igfbp1*.

Class I and class II sex-specific genes were further subdivided as shown in Table 3. Class IA male-specific genes are down-regulated in Hypox males but not females (164 genes). Thus, these genes are positively regulated by the male but not by the female pituitary hormone profile. In contrast, class IB male genes are down-regulated by hypophysectomy in both males and females, indicating that they require pituitary hormone for full expression in both sexes (70 genes).

Table 2. Impact of Hypophysectomy on Gene Expression

Impact of Hypox	Change in Expression in		
	M-Hypox Only[a]	F-Hypox Only[a]	M-Hypox and F-Hypox[a]
Male-specific genes			
Decrease (class I)	164	1	70
Increase (class II)	2	120	26
Other	0	0	17[b]
Female-specific genes			
Decrease (class I)	0	137	59
Increase (class II)	58	1	20
Other	0	0	14[c]
Sex-independent genes			
Increase	346	240	302
Decrease	289	233	395

Liver-expressed genes of interest (Table 1) were grouped based on their response to hypophysectomy in both males and females, and secondarily, based on whether gene expression was increased or decreased. The designation of class I and class II genes is as described in the text and in Table 3. Not tabulated are data for 1763 other genes of interest, namely, 189 male-specific genes, 154 female-specific genes, and 1313 sex-independent genes, the expression of which was not significantly changed after hypophysectomy (also see Table 3). A majority of these unchanged sex-specific genes actually lost sex specificity after hypophysectomy (*i.e.* male-female difference <2-fold), but the changes in gene expression did not reach the specified criteria of more than 2.0-fold and $P < 0.005$.

[a] All numbers shown are gene counts.

[b] Male-specific genes down-regulated in Hypox males and up-regulated in Hypox females.

[c] Female-specific genes down-regulated in Hypox females and up-regulated in Hypox males.

Class IC male genes are down-regulated by hypophysectomy in male liver, but are up-regulated by hypophysectomy in females (17 genes). Class IIA male-specific genes are selectively up-regulated in Hypox female liver, indicating strong negative regulation by the female pituitary hormone profile (120 genes),

Table 3. Classification of Sex-Specific Genes Based on Response to Hypophysectomy in Males and in Females

Class	Response to M-Hypox	Response to F-Hypox	Gene Count	%	Examples
Male-specific genes					
IA	Down	–	164	41	*Ncam2, Gluld1, Fgf21*
IB	Down	Down	70	18	*Mup4*
IC	Down	Up	17	4	*Cyp2c11*
IIA	–	Up	120	30	*Cyp2a2, Cyp2C13, Ca3*
IIB	Up	Up	26	6	*Cyp17a1*
Other			3[a]	1	
Total responsive to Hypox			400		
Unresponsive to Hypox			189		
Total male-specific			589		
Female-specific genes					
IA	–	Down	137	48	*Cutl2/Cux2*
IB	Down	Down	59	21	*Cyp2c12, Onecut1/Hnf6, A1bg*
IC	Up	Down	14	4	*Sult2a2*
IIA	Up	–	58	19	*Adh3*
IIB	Up	Up	20	7	*Adh4, Igfbp1*
Other			1[b]	0	
Total responsive to Hypox			289		
Unresponsive to Hypox			154		
Total female-specific			443		

Male-specific (400 genes) and 289 female-specific genes showing pituitary dependence are grouped into each of two classes (I, II) and subclasses (A, B, C) based on their response to hypophysectomy [up-regulation, down-regulation, or no significant change (–)] in males and females, as indicated. Class I sex-specific genes are positively regulated by pituitary factors, as shown by their down-regulation after hypophysectomy, whereas class II sex-specific genes are negatively regulated (*i.e.* up- regulated after hypophysectomy). The subclass designates whether the impact of hypophysectomy is seen in only one sex (A) or in both sexes (B). Genes that respond to hypophysectomy in both sexes but in the opposite direction in each sex are placed in subclass C.

[a] One gene down-regulated in F-Hypox only and two genes up-regulated in M-Hypox only.

[b] Gene is up-regulated in F-Hypox only.

Table 4. Impact of a Single or Two GH Injections Given to Hypox Male Rats on GH-Responsive Class I (panel A) and Class II (panel B) Sex-Specific Genes

A. Class I Genes	Up-Regulation by GH	All Class I Genes		Class IA		Class IB		Class IC	
		Gene Count	%	Gene Count	%	Gene Count	%	Gene Count	%
Class I Male genes (251)	After first injection	26	*53*	13	*41*	12	*92*	1	*25*
	After second injection only	23	*47*	19	*59*	1	*8*	3	*75*
	Total:	49		32		13		4	
Class I Female genes (210)	After first injection	24	*65*	8	*44*	14	*88*	2	*67*
	After second injection only	13	*35*	10	*56*	2	*13*	1	*33*
	Total:	37		18		16		3	

B. Class II Genes	Down-Regulation by GH	All Class II Genes		Class IIA		Class IIB	
		Gene Count	%	Gene Count	%	Gene Count	%
Class II male genes (146)	After fist injection	33	*61*	24	*65*	9	*53*
	After second injection only	21	*39*	13	*35*	8	*47*
	Total:	54		37		17	
Class II female genes (78)	After first injection	5	*26*	2	*15*	3	*50*
	After second injection only	14	*74*	11	*85*	3	*50*
	Total:	19		13		6	

Classes and subclasses of sex-specific genes are as defined in Table 3. Shown are class I genes induced by either one or two GH injections and class II genes suppressed by either one or two GH injections. "After first injection" designates genes that are up-regulated or down-regulated after both the first and the second GH injections. "After second injection only" designates genes that responded to the second GH injection but not to the first GH injection. Total number of genes in each class is shown in *parentheses at left.*

whereas class IIB male genes are up-regulated by hypophysectomy in both males and females, indicating negative regulation by pituitary hormones in both sexes (26 genes) (Table 3).

Class IA female-specific genes are down-regulated in Hypox females but not males, indicating positive regulation by the female, but not by the male, pituitary hormone profile (137 genes), whereas class IB female genes are down-regulated by hypophysectomy in both sexes (59 genes). Class IC female genes are down-regulated by hypophysectomy in female liver, but are up-regulated by hypophysectomy in males (14 genes). Class IIA female genes are up-regulated in Hypox male liver only, indicating a requirement for the male but not female pituitary hormone pattern for full expression (58 genes). Finally, class IIB female genes are up-regulated by hypophysectomy in both male and female liver, indicating negative regulation by pituitary hormones in both sexes (20 genes) (Table 3).

Response of Sex-Specific Genes to Short-Term GH Treatment

Of the sex-specific genes that showed a change in expression after hypophysectomy, 25–31% responded to short-term GH treatment in Hypox male liver, similar to the percentage of sex-independent genes of interest that responded rapidly to GH (24%) (supplemental Table S5). Moreover, 18–19% of the sex-specific genes the expression of which decreased after hypophysectomy were up-regulated after either one or two GH injections (49 of 251 class I male genes and 37 of 210 class I female genes; Table 4A and supplemental Table S6A). The induction seen in the case of the class I female genes may be a manifestation of their responsiveness to continuous GH stimulation.

Similarly, GH suppressed 24–36% of the sex-specific genes that were up-regulated after hypophysectomy (54 of 146 of class II male genes and 19 of 78 class II female genes; Table 4B and supplemental Table S6B). Class I and class II sex-specific genes that did not respond to short-term GH treatment may be secondary GH response genes or perhaps primary genes characterized by a delayed response; alternatively, these genes may require pituitary hormones other than GH for expression. In addition, class II genes not showing a GH response may include direct targets of GH, the mRNA half-life of which is too long to allow for a significant decrease within the 4–5 h time frame of the GH treatment.

A substantial fraction (41–44%) of the GH-responsive class IA genes were induced after the first GH injection; the other 56–59% were induced after the second GH injection. In contrast, 88–92% of the class IB sex-specific genes induced by short-term GH treatment were already increased after the first GH injection, indicating they are likely to be direct GH targets (Table 4A). Supplemental Tables S2E and S2F list the top 100 early GH response genes (both sex specific and sex independent), with a complete listing pre-

sented in supplemental Tables S7–S9. In the case of the class IIA sex-specific genes suppressed by short-term GH treatment, 65% of the male-specific genes were down-regulated after the first GH injection, as compared with only 15% of the female-specific genes ($P < 0.005$; Fisher's exact test) (Table 4B). The high level of expression of these class IIA male genes in Hypox liver, and their rapid suppression by a physiological GH pulse, raises the possibility that transcription of these genes is restricted to the GH-free interpulse interval. Of note, six of the 12 early GH-responsive class IB male-specific genes belong to the *α2u-globulin/Mup* family; the expression of these genes is decreased by hypophysectomy in both sexes but retains partial intrinsic male specificity in Hypox rat liver. Moreover, 24 genes coding for DNA-binding proteins (a majority of them transcription factors) were early GH response genes, of which 15 were rapidly induced by GH treatment and nine were down-regulated (supplemental Table S10). Three of these early GH response genes were expressed in a female-specific manner (*Cux2/Cutl2, Onecut1/HNF6*, and *Ncl*), and three were expressed in a male-specific manner (Zfp37, *Bcl6, Pparg*). Seventy other genes coding for DNA-binding proteins were either induced or suppressed at the second GH time point only; of these, only two showed sex-specific expression (*Bhlhb8*, male-specific; and *Rfxdc1*, female-specific).

Gene Ontology (GO) Analysis

The sex-specific genes were found to be enriched for a variety of biological processes and metabolic functions, with class I male genes enriched for genes related to cell division/cell cycle, axonal fasciculation, and pheromone binding (*e.g. α-2u globulin/Mup* genes, in male class IB), whereas the class II female genes showed enrichment for alcohol metabolism (supplemental Table S11). Interestingly, the class I female genes, which are down regulated in Hypox liver, are enriched in several of the same processes and functions related to steroid and lipid metabolism as the class II male genes, which are up-regulated in Hypox liver. This suggests that the up-regulation of the latter genes may be, in part, a compensatory response to the loss of the class I female genes. Sex-specific genes were also enriched in several important hepatic KEGG metabolic pathways, including xenobiotic metabolism by cytochromes P450, fatty acid metabolism, bile acid metabolism, and androgen and estrogen metabolism. Early GH response genes identified in this study were enriched in several of the above-mentioned biological processes and metabolic functions, but in addition, were enriched for genes related to acute phase response, aldo-keto reductase activity, and Janus family of tyrosine kinases-STAT signaling pathway (genes rapidly up-regulated by GH) and genes involved in digestion and exopeptidase activity (genes rapidly down-regulated by GH) (supplemental Table S11).

Validation of Sex-Specific Early GH Response Genes

Microarray analysis identified 26 class I male genes and 24 class I female genes induced within 90 min of a single GH injection to Hypox rats (Table 4A), suggesting these are direct GH response genes. To confirm these findings, we performed quantitative real-time PCR (qPCR) analysis to measure mRNA levels, as well as heterogeneous nuclear RNA (hnRNA) levels, which correlate closely with results obtained in run-on transcription assays (36). In addition, the time course of induction was investigated by qPCR analysis carried out at individual time points, *i.e.* 30, 60, and 90 min after a single GH injection and 1 h after a second GH injection given 3–4 h after the first injection. Analysis of mRNA and hnRNA levels for two class IA genes showing male specificity verified their sex specificity and responsiveness to hypophysectomy and revealed GH-induced expression by 30 min (*Gluld1* hnRNA) and by 60 min (*Fgf21* mRNA and hnRNA) (Fig. 1A). Substantial increases in mRNA and hnRNA levels of *Onecut1/Hnf6*, a class IB female gene, were also seen 30 min after a single GH injection (Fig. 1B), in agreement with an earlier study of this gene (37) and validating the microarray data identifying it as an early GH response gene.

The suppressive action of GH was investigated by qPCR analysis of several class II sex-specific mRNAs and their cognate hnRNAs. Previous studies have shown that *Igfbp1* expression rapidly decreases in response to supraphysiological GH treatment (28, 38). Based on our microarray data, *Igfbp1* is a class IIB female gene that is up-regulated in both male and female liver after hypophysectomy, by 8.2-fold and 2.9-fold, respectively. *Igfbp1* did not meet the 2-fold microarray threshold for a response to short-term GH treatment, although a trend of a decrease was observed (M-Hypox + GH/M-Hypox ratio = 0.6; M-Hypox + 2GH/M-Hypox ratio = 0.7). Consistent with these array results, *Igfbp1* mRNA was not decreased in Hypox rat liver until 60 min after GH treatment; however, *Igfbp1* hnRNA was strongly down-regulated within 30 min of a single, physiological GH injection (Fig. 2A). Similar observations were made for *Adh4*, another class IIB female gene. Based on the microarray data, *Adh4* was up-regulated after hypophysectomy in both males and females and was down-regulated after two GH injections. A modest and progressive decrease of *Adh4* mRNA was seen 60 min after a single GH injection in Hypox males (Fig. 2A). However, hnRNA measurements showed that *Adh4* expression was substantially suppressed 30 min after a single GH injection (Fig. 2A), indicating that this gene is likely to be a direct target of GH suppression.

Three class IIA male-specific genes were also analyzed: *Ca3, Cyp2a2*, and *Cyp2c13*. Based on the microarray data, these three genes are male specific,

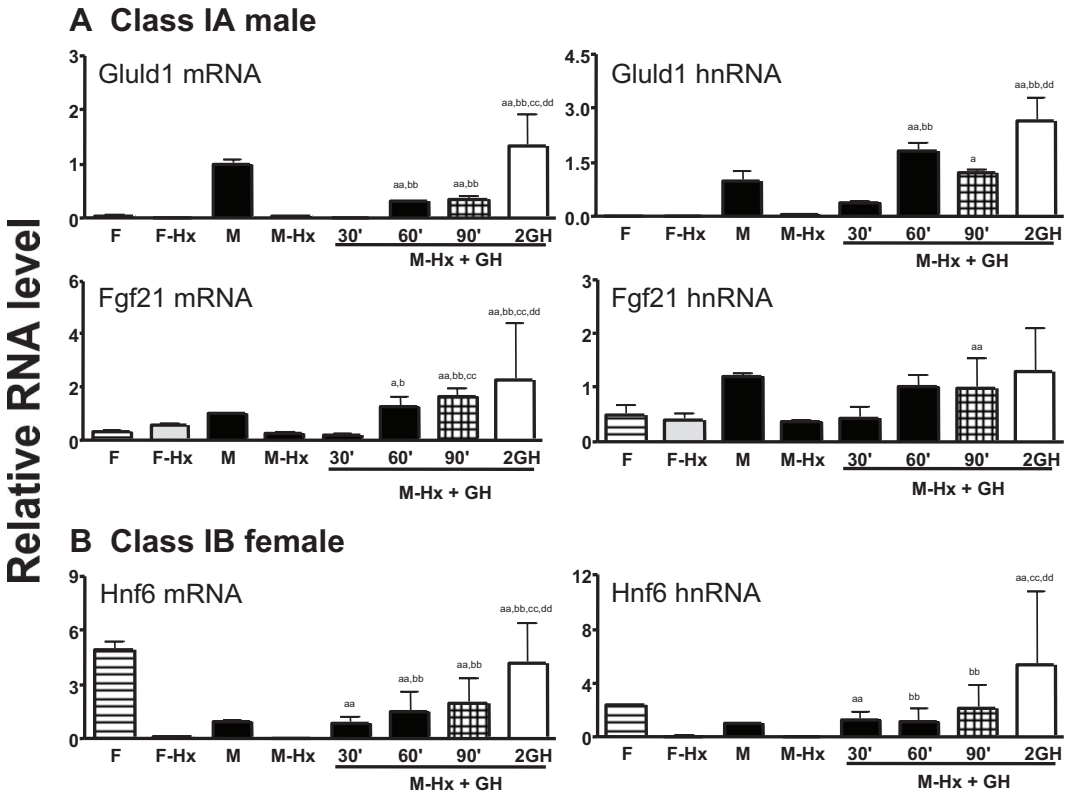

Fig. 1. qPCR Analysis of Class I Sex-Specific Genes Induced by Short-Term GH Treatment

Liver RNA samples were prepared from the liver of the following groups of rats: untreated males (M, n = 8); Hypox males (M-Hx, n = 6); Hypox males treated with a single GH injection and killed after 30 min (M-Hx+GH, 30′, n = 2), 60 min (M-Hx+GH, 60′, n = 3), and 90 min (M-Hx+GH, 90′, n = 3); Hypox males killed 1 h after a second GH injection given 3–4 h after the first injection (M-Hx+2GH, n = 3), untreated females (F, n = 7), and Hypox females (F-Hx, n = 7). RNAs were reverse transcribed to cDNA and assayed for the indicated mRNAs and hnRNAs by qPCR using primers shown in supplemental Table S1. Data shown are mean ± SD values (analysis of pooled RNA from UT male and female and Hypox male and female liver RNAs) or mean ± SE values (analysis of individual liver RNAs prepared from Hypox rats treated with GH). Data were normalized to the 18S RNA content of each pooled cDNA sample, with the UT male group set at a value of 1. For M-Hx + GH (30′, 60′, 90′, and 2GH) *vs.* M-Hx: ^{a}, $P < 0.05$; and ^{aa}, $P < 0.01$; for M-Hx + GH (60′, 90′ and 2GH) *vs.* M-Hx + GH (30′): ^{b}, $P < 0.05$; and ^{bb}, $P < 0.01$; for M-Hx + GH (90′ and 2GH) *vs.* M-Hx + GH (60′): ^{c}, $P < 0.05$; and ^{cc}, $P < 0.01$; for M-Hx + 2GH *vs.* M-Hx + GH (90′): ^{d}, $P < 0.05$; and ^{dd}, $P < 0.01$.

up-regulated (derepressed) in Hypox females, unchanged in Hypox males, and either suppressed by a single GH pulse (*Ca3*), suppressed after two GH pulses (*Cyp2a2*), or largely unresponsive to short-term GH treatment (*Cyp2c13*). These overall trends were validated by mRNA qPCR (Fig. 2B, *left*). Moreover, hnRNA analyses show that all three male-specific genes were rapidly suppressed, within 30 min of a single GH pulse (Fig. 2B, *right*). Thus, these genes are likely to be direct targets of the negative regulatory action of GH. Results were validated using a second set of hnRNA primers for the three genes showing GH suppression of hnRNA, but not mRNA, at the 30-min time point (*Igfbp1*, *Adh4*, and *Cyp2a2*; supplemental Fig. S1 published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Sex specificity and responsiveness to hypophysectomy and GH treatment were also verified for six other class I male genes, three class II male genes, and two class I female genes (supplemental Figs. S2–S4).

DISCUSSION

Microarray technology was used to characterize the impact of pituitary hormone ablation on sex-specific gene expression in rat liver and to identify early GH response genes (primary targets of GH) that are sex specific and may potentially mediate the regulatory effects of GH on liver sex specificity. Approximately 90% of more than 1000 sex-specific liver genes were shown to be under pituitary control, demonstrating a near-global dependence of sex-specific gene expression on the pituitary gland, and consistent with the widespread regulatory role of GH with respect to the sex specificity of liver-expressed genes reported earlier (25). Two distinct classes of sex-specific liver

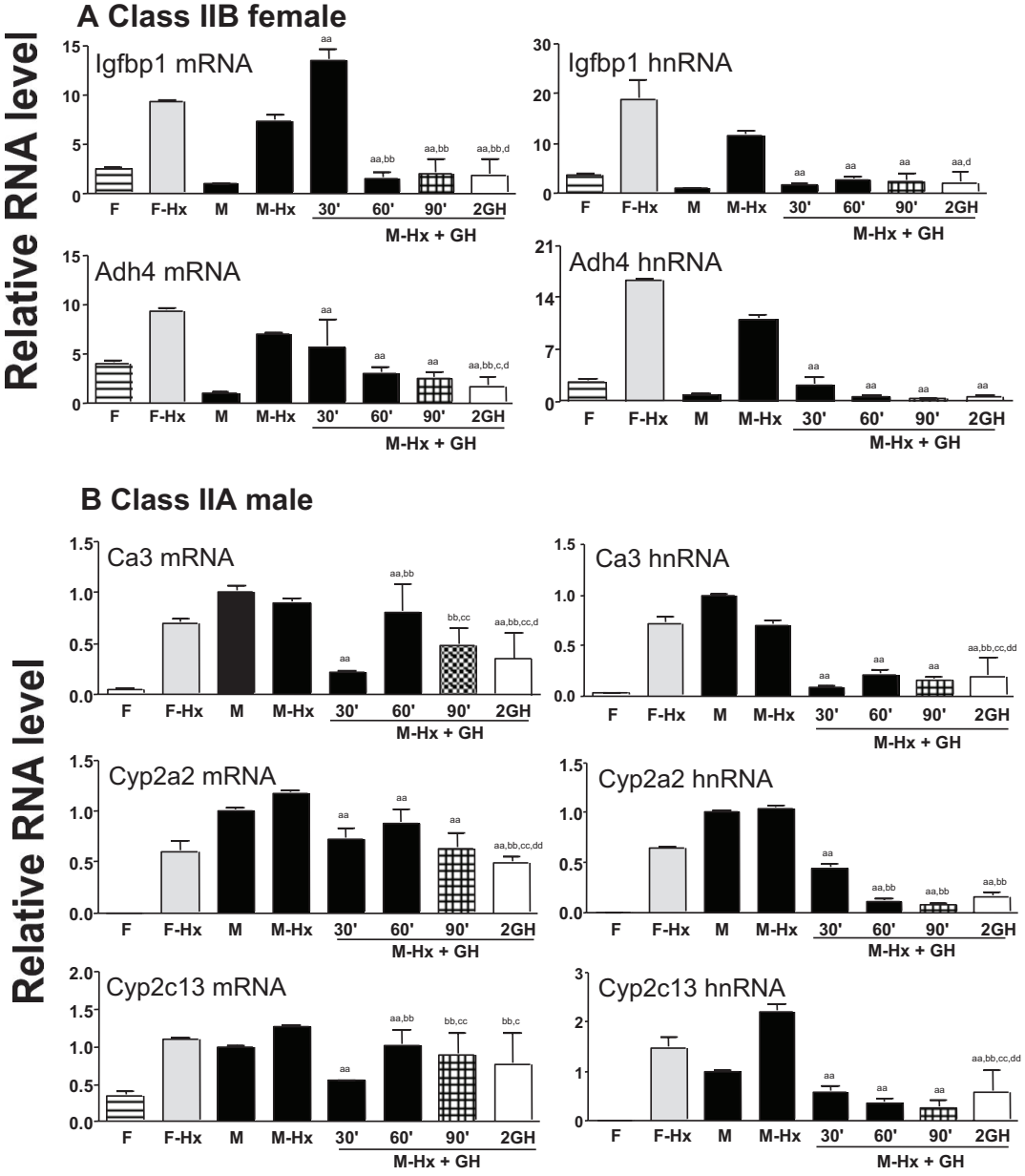

Fig. 2. qPCR Analysis of Class II Sex-Specific Genes Repressed by Short-Term GH Treatment

Liver RNA samples described in Fig. 1 were assayed for the indicated mRNAs and hnRNAs by qPCR. Data presentation and statistical analyses are as described in Fig. 1.

genes were identified: genes positively regulated by pituitary hormones, as evidenced by their down-regulation after hypophysectomy (class I genes), and genes negatively regulated by pituitary hormones, as shown by their up-regulation after hypophysectomy (class II genes). Individual class I and class II sex-specific genes described previously at both the RNA and protein level include *Cyp2c11*, a class I male gene that requires pulsatile plasma GH stimulation for expression, and *Cyp2c12*, a class I female gene that is induced by the more continuous female plasma GH pattern (1, 2). Class II male genes, such as *Cyp2a2*, *Cyp3a2*, and *Cyp4a2*, do not require male plasma GH pulse stimulation, as evidenced by their full expression in Hypox male liver. Rather, these genes are strongly repressed by the female plasma GH profile, which explains their up-regulation to near male levels in Hypox female liver (31–33, 39). The present studies also identified a second, novel class of female-specific genes in rat liver, termed class II female genes, which are repressed by the male pituitary hormone profile, as indicated by their up-regulation in Hypox male liver. This response is analogous to that of a corresponding set of female-specific genes described earlier in mouse liver and exemplified by mouse *Cyp2b9* (8, 35). These class I and class II sex-specific genes play distinct but overlap-

ping roles in liver metabolism and physiology, as determined by GO analysis. The greatest functional similarity was seen between class I female genes and class II male genes, suggesting the latter group of male-specific genes, the expression of which is up-regulated in Hypox female liver, compensates, in part, for the associated loss of the class I female genes.

A significant number of the class II sex-specific genes were down-regulated by short-term GH treatment (supplemental Table S8). GH is thus the key pituitary factor the ablation of which leads to the up-regulation of these genes after hypophysectomy. Moreover, the rapid down-regulation of these genes by GH indicates they are direct targets of the suppressive action of GH and that their mRNAs have a short half-life. Presumably, there are other class II sex-specific genes that are rapidly down-regulated by GH but could not be detected over the 4–5 h time frame of the present study owing to their long mRNA half-lives. This conclusion is supported by our studies of *Cyp2a2*, *Cyp2c13, Igfbp1*, and *Adh4* because the suppression of these genes by GH at 30 min was only apparent when examined at the level of hnRNA. Other sex-specific genes that did not respond to short-term GH treatment could be secondary response genes or perhaps may be primary response genes characterized by delays in transcription and/or subsequent stages of RNA elongation and processing (40). Alternatively, these genes may be dependent on other pituitary hormones for expression. Our finding that class II male-specific genes, such as *Cyp2a2* and *Cyp2c13*, are directly repressed by a single physiological pulse of GH leads us to propose that gene expression is limited to the approximately 2-h GH-free internal between plasma GH pulses. Furthermore, the marked suppression of these and other male-specific genes in male rats given GH as a continuous infusion (9, 31, 41) supports the hypothesis that these genes are repressed at all times in female liver due to the near-continuous presence of GH in circulation. Further study is required to test this hypothesis, which predicts that transcription of these class II male-specific genes is intermittent in males and is restricted to the plasma GH-free interpulse interval.

Several of the primary GH target genes identified in the present study are induced by GH via either STAT5b or STAT5a. These genes include *Igf1* (42), *Cish* (43), and *Onecut1*/*Hnf6* (37). Rapid induction by GH (within 90 min) was presently observed for 50 class I sex-specific genes. These genes are candidates for direct regulation by STAT5b, which is rapidly activated by GH treatment *in vivo* (within 10–15 min) (44, 45) and is required for sex-specific liver gene expression (15, 46). Genes rapidly induced by GH (supplemental Table S7) include the male-specific genes *Gluld1, Fgf21*, and several α*2u globulin*/*Mup* genes, as well as female-specific genes such as *Cux2*/*Cutl2* and *Onecut1*/*Hnf6*. The rapid suppressive effect of GH may also be regulated by STAT5b, as recently described for *Igfbp1*, where GH-activated STAT5b impairs the action of FoxO1, a transcription factor that positively regulates *Igfbp1* (28). A STAT5b-dependent repression mechanism could also characterize the suppression of class II male genes, such as *Cyp2a2*, at the time of each plasma GH pulse, as discussed above. Indeed, in studies using adenoviral vectors delivering dominant-negative and constitutively active forms of STAT5b, STAT5b was shown to be a major mediator of the rapid suppressive effects of GH; however, STAT5b-independent mechanisms appeared to dominate the inductive effects of GH (27, 28). STAT5-dependent repression may readily be identified through these types of studies, where a dominant-negative STAT5 only needs to partially inhibit endogenous STAT5 signaling to detectably reverse STAT5-dependent gene repression. However, to conclusively establish the STAT5 dependence of gene induction using this experimental approach, the dominant-negative STAT5 construct needs to be potent and fully effective in blocking endogenous STAT5 activity, which is difficult to achieve. Accordingly, STAT5b may mediate a larger fraction of the rapid gene induction responses that the 20% value reported earlier (27).

The rapid induction of several class I female genes by GH is probably a manifestation of their responsiveness to continuous GH stimulation, as seen here in the context of the GH-free background of a Hypox rat, rather than a response to GH pulse treatment *per se*. This response could involve STAT5a, which has been shown to play a role in sex-specific gene expression, particularly in female liver (29). Rapid GH induction characterized a significantly greater fraction (88–92%) of those class I genes that were dependent on pituitary hormone for expression in both males and females (designated class IB genes; Table 3), as compared with genes that showed a pituitary dependence in only one sex (class IA genes; 41–44%), indicating direct GH regulation of the class IB genes via a mechanism that is common to both sexes. Direct GH induction also characterizes the sex-independent GH-responsive genes *Igf1* and *Socs2*, where STAT5b is a key transcriptional activator (27, 47, 48). Conceivably, class IB sex-specific genes may have strong binding sites for GH-activated STAT5b, which would enable them to be activated both in male liver, where the high but pulsatile plasma GH levels are associated with high nuclear concentrations of STAT5b, and in female liver, where the lower but more continuous plasma GH levels are associated with lower levels of active, nuclear STAT5b (12, 14). Similarly, sex-independent genes that are STAT5b dependent, such as *Igf1* and *Socs2*, may have stronger STAT5b binding sites than the STAT5b-regulated class IA male-specific genes, which could explain why the sex-independent genes are expressed in female liver at the same level as in male liver, despite the much lower peak STAT5b levels in the females. Further investigation is required to determine whether the sex-specific early GH response genes presently identified contain an overrepresentation of paired STAT5b-binding sites, which characterize many direct STAT5b

target genes (43, 49), or in the case of the class I female genes, if binding sites for STAT5a (50) are overrepresented.

At least some of the sex-specific genes that did not respond to short-term GH treatment may be regulated by GH via indirect mechanisms, as was previously suggested based on the rather long time (several days) required for certain sex-specific mouse liver mRNAs to respond to changes in plasma GH status (8). GH regulation of these genes may be mediated by sex-specific transcription factors that serve as direct targets of GH. Indeed, three class I female-specific DNA-binding proteins, Cutl2 (*Cux2* gene product), HNF6 (*Onecut1* gene product), and Nucleolin were found to be rapidly induced by GH, in agreement with an earlier study in the case of HNF6 (37). Cutl2 is a female-specific transcription factor that has the potential to repress male-specific genes in female liver (24), whereas HNF6 is a female-predominant factor that also has a Cut domain and may contribute to the female-specific expression of *Cyp2c12* (22, 23). Among the class IIA sex-specific genes suppressed by GH, two encode transcription factors showing male specificity (supplemental Table S10). Of these, the transcriptional repressor *Bcl6* showed the highest sex-specificity (M/F = 15.8). Further studies are required to determine whether these or other sex-specific signaling molecules are involved in the regulation of downstream sex-specific targets by GH/STAT5b. Eighteen other DNA-binding protein genes that were rapidly induced (11 genes) or suppressed (seven genes) by GH did not show sex specificity in their expression (supplemental Table S10). Presumably, a subset of these genes contributes to the secondary effects of GH that are common to male and female liver.

In conclusion, GH confers sex specificity in rat liver through both positive and negative regulation, which may involve both direct and indirect mechanisms. Future studies will be required to determine the role of the early GH-responsive transcriptional regulators identified in this study in the global pattern of sex-specific liver gene expression. Finally, the new insights into the complex regulatory networks involved in the sex-dependent patterns of *Cyps* and other liver-expressed genes provided by the present studies may further our understanding of the role of GH in regulating the sex specificity in human liver of genes such as *CYP3A4* (2), which plays a major role in human hepatic drug and steroid metabolism.

MATERIALS AND METHODS

Animal Treatments and Liver RNA Isolation

Male and female Fischer 344 rats, untreated or hypophysectomized at 8 wk of age (150–165 g), were purchased from Taconic Farms, Inc. (Germantown, NY). Rats were housed on Care Fresh bedding (catalog no. 2680; Scott's Distributing, Inc., Hudson, NH) on a 12-h light, 12-h dark cycle with free access to food (Pro Lab Rat/Mouse/Hamster 3000; catalog no. 6600, Scott's Distributing, Inc.) and drinking water. The completeness of hypophysectomy was confirmed by the absence of weight gain over a 4-wk period after surgery (weights monitored three times/wk, with final weights all ≤initial weight at time of surgery). Rats showing trends of increasing weight over the 4-wk monitoring period were excluded from the study. Hypox male rats were treated with a single ip injection of rat GH at 6 μg/100 g of body weight (rGH-B-14-SIAFP; National Hormone and Pituitary Program, NIDDK) and killed 30, 60, or 90 min later. A second group of Hypox male rats was given two ip injections of GH, spaced either 3 or 4 h apart, and then killed 60 min after the second GH injection. GH pulse treatments used in this study were given to Hypox male rats because a plasma GH pulse is the natural form of GH stimulation in males. GH pulse treatment of Hypox females, a nonphysiological form of GH replacement, was not tested in this study. Rats were euthanized by brief CO_2 treatment followed by cervical dislocation. Livers were excised, flash frozen in liquid N_2, and stored at −80 C. Total RNA was isolated from individual livers using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Livers from the following six groups were used for microarray analysis: intact, untreated male rats (M-UT; n = 7); intact, untreated female rats (F-UT; n = 6); Hypox male rats (M-Hypox; n = 6); Hypox female rats (F-Hypox; n = 7); Hypox male rats treated with a single GH injection and killed 30, 60, or 90 min later (M-Hypox + GH; n = 4), and Hypox male rats treated with two GH injections (M-Hypox + 2GH; n = 5). Liver RNA from M-Hypox rats given a single GH injection and killed 30, 60, and 90 min later were pooled for the microarray analysis because of the small numbers of individuals (n = 2 or 3) available in each group. These RNAs were assayed individually in the qPCR analysis, as described below.

Microarray Platform

The Agilent Whole Rat Genome Microarray platform (catalog no. G4131F, 4 x 44K slide format; Agilent Technology, Palo Alto, CA) was used for global gene expression analysis. This microarray contains 41,012 rat cDNA probes (features), each comprising a single 60-oligomer oligonucleotide sequence. In annotation assignments made by Aarathi Sugathan of this laboratory, accession numbers could be assigned to 39,308 of the 39,688 probes for which the manufacturer provided chromosomal location information: rat GenBank accession numbers were assigned for 36,383 of the probes; rat Ensembl transcript identifications (IDs) for 168 other probes; and non-rat accession numbers for 2,757 probes for which no rat annotations were available. Together, these probes encompass 23,642 unique rat accession numbers and 2,270 unique nonrat accession numbers and represent 16,947 rat Unigene IDs plus 5,941 nonrat Unigene IDs (Unigene build 166). Two or more probes mapping to a common accession number indicate redundancy in the microarray platform. Each of the probes assigned a unique accession number is herein referred to as representing a distinct gene (*i.e.* gene product). The true number of unique transcripts represented on the array is likely to be fewer than this number due to probes mapping to regions of the rat genome that are poorly (or incorrectly) annotated. Liver RNA samples having an RNA Integrity Analysis number greater than 8.0, determined using an Agilent Bioanalyzer 2100, were used for microarray analysis.

Sample Preparation, Hybridization, and Data Acquisition

Liver RNA pools were prepared for two independent sets of biological replicates for the M-UT, F-UT, M-Hypox, and F-Hypox groups (n = 3–4 livers per pool; two pools per group). A single pool of liver RNA (n = 4 livers from M-Hypox rats treated with a single GH injection and killed either 30, 60, or

90 min later) was used for the M-Hypox + GH group, and a single pool (n = 5 livers) for the M-Hypox + 2GH group. These RNA pools were used in six separate sets of competitive hybridization experiments in a loop design: 1) M-UT *vs.* F-UT; 2) M-Hypox *vs.* M-UT; 3) F-Hypox *vs.* F-UT; 4) M-Hypox *vs.* F-Hypox; 5) M-Hypox + GH *vs.* M-Hypox; 6) M-Hypox + 2GH *vs.* M-Hypox. Sample labeling, hybridization to microarrays, scanning, analysis of TIFF images with Agilent's feature extraction software, calculation of linear and LOWESS normalized expression ratios, and initial data analysis using Rosetta Resolver (version 5.1; Rosetta Biosoftware) were carried out at the Wayne State University Institute of Environmental Health Sciences microarray facility (Detroit, MI) as detailed elsewhere (21). Dye-swapping experiments were carried out for each of the six hybridization experiments, as follows. The Alexa 555-labeled cDNA from one of the two M-UT pools was mixed with the Alexa 647-labeled cDNA from one of the two F-UT pools. Similarly, Alexa 647-labeled cDNA from the second M-UT pool was mixed with Alexa 555-labeled cDNA from the second F-UT pool. Together, these two mixed cDNA samples are considered a fluorescent reverse pair (dye swap). Dye swaps were similarly carried out for each of the five other competitive hybridization experiments, except that for experiments 5 and 6, a single pool of M-Hypox + GH liver cDNA, or a single pool of M-Hypox + 2GH liver cDNA, was used in each half of the fluorescent reverse pair. Two microarrays, one for each mixed cDNA sample, were hybridized for each of the six fluorescent reverse pairs, giving a total of 12 microarrays. Expression ratios obtained in this study are available for query or download from the Gene Expression Omnibus web site (http://www.ncbi.nlm.nih.gov/geo) as GEO series GSE-11529.

Statistical Analysis

A filter ($P < 0.005$) was applied to the *P* values obtained from Rosetta Resolver to determine the statistical significance of each gene's differential expression for each of the six microarray experiments. A fold-change filter of 2.0-fold was combined with the above *P* value filter to reduce the false discovery rate to less than 1%. Thus, of the 41,012 features (probes) included on the array, 6,932 met the 2.0-fold expression filter for at least one of the six microarray comparisons. The number of probes expected to meet the combined threshold ($P < 0.005$ and >2.0-fold change in expression) by chance is $0.005 \times 6{,}932$, or 35 genes. The actual number of probes meeting the combined threshold was 4,621, corresponding to an apparent false discovery rate of 35 per 4,621 or 0.76%. No major change in the overall gene distribution patterns was observed when the threshold stringency was either relaxed (>1.5-fold change and $P < 0.05$) or increased (>2.25-fold change and $P < 0.001$).

A system of binary and decimal flags was used to cluster the microarray data based on the expression ratios and *P* values obtained for each gene in all six microarray experiments, as described previously (15). Average ratios meeting the threshold of 2.0-fold change and $P < 0.005$ contributed to the binary- and decimal-based flag. Thus, genes with a M-UT *vs.* F-UT microarray ratio meeting both criteria were assigned a binary flag value of 1, whereas genes meeting the criteria for the microarrays that analyzed M-Hypox *vs.* M-UT, F-Hypox *vs.* F-UT, M-Hypox *vs.* F-Hypox, M-Hypox + GH *vs.* M-Hypox, and M-Hypox + 2GH *vs.* M-Hypox were assigned, respectively, binary flag values of 2, 4, 8, 16, and 32. Genes not meeting these criteria were assigned flag values of 0. The sum of these binary-based flag values defines the whole number portion of the flag and was used as a simple method to identify which of the six microarrays met our criteria for inclusion for any given gene of interest, regardless of the direction (up or down) of the regulation. The flag value was then extended using decimal values of 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0.000001 or 0.2, 0.02, 0.002, 0.0002, 0.00002, and 0.000002 for each of the six microarrays, to indicate the direction of regulation between the two conditions on the microarray. Thus, average ratios for M-UT *vs.* F-UT microarray greater than 2.0 were assigned a decimal value of 0.1, to indicate up-regulation, whereas average ratios less than 0.5 were assigned a value of 0.2, to indicate down-regulation. The five other microarray ratios were similarly flagged, by advancing to a new decimal position for each microarray (*i.e.* the M-Hypox: M-UT flag is in the hundredths position, and so on). For each gene, the resulting binary sum describes which microarray ratios met the selection criteria, and the six-digit decimal value describes the direction of regulation (Total Flagging Sum, TFS; see supplemental Tables S3 and S4). Of the 41,012 probes, 4,621 met the threshold criteria (average expression ratio >2-fold and a significance of $P < 0.005$) for at least one of the six data sets and were included in our analysis. In each case where two or more probes for the same transcript (as defined by a common accession number assignment) gave the same pattern of regulation, as indicated by mapping to the same TFS group (*i.e.* redundant probes), only the probes with the best *P* values were retained. The number of genes meeting the threshold criteria was thus reduced to from 4621 to 4150. These 4150 genes of interest, as well as the 471 redundant probes excluded from further analysis, are presented in supplemental Table S2.

GO Enrichment Analysis

Gene lists were analyzed for enrichment of GO categories and biological pathways using DAVID (http://david.abcc.ncifcrf.gov), a web-accessible bioinformatics database (51, 52). Each gene was categorized per the GO Consortium designations using biological process and molecular function ontology levels (53). Categories enriched in each list as compared with the species background were identified using the DAVID *P* value, and those with a *P* value < 0.01 were selected for subsequent analysis. Enrichment in KEGG pathways was determined with DAVID, with results presented for pathways significant at $P < 0.05$.

qPCR Analysis

Liver RNA samples were prepared from the following eight groups of rats: untreated males (M, n = 8); Hypox males (n = 6); Hypox males treated with a single GH injection and killed after 30 min (n = 2), 60 min (n = 3) and 90 min (n = 3); Hypox males killed 1 h after a second GH injection given 3–4 h after the first injection (n = 3), untreated females (n = 7) and Hypox females (n = 7). Liver RNA samples were converted to cDNA and assayed for the expression of individual genes by qPCR (8). Amplification of a single, specific product during qPCR cycling was verified by examination of the dissociation curve of each amplicon. For untreated males, untreated females, Hypox males, and Hypox females, qPCR analysis was performed on pooled liver cDNA samples; for Hypox rats treated with GH, individual liver cDNA samples were used. Relative RNA levels were determined after normalization to the 18S RNA content of each sample. Statistical analysis was carried out by one-way ANOVA and Bonferoni posttest with the level of significance set at $P < 0.05$ using GraphPad Prism software version 4 (GraphPad Software, Inc., San Diego, CA). qPCR primer design was carried out using Primer Express software (Applied Biosystems, Foster City, CA), and all primers were verified with respect to their specificity for the target transcript by BLAST-like alignment Tool analysis (BLAT) of the rat genome (November 2004 assembly) at http://genome.ucsc.edu/ cgi-bin/hgBlat. Where indicated, primary nuclear transcripts (hnRNA) were assayed using qPCR primer sets that amplified across an exon-intron junction. Primer sequences are shown in supplemental Table S1.

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