

Characterization of Three Growth Hormone-Responsive Transcription Factors Preferentially Expressed in Adult Female Liver

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Plasma GH profiles regulate the sexually dimorphic expression of cytochromes P450 and many other genes in rat and mouse liver; however, the proximal transcriptional regulators of these genes are unknown. Presently, we characterize three liver transcription factors that are expressed in adult female rat and mouse liver at levels up to 16-fold [thymus high-mobility group box protein (Tox)], 73-fold [tripartite motif-containing 24 (Trim24)/transcription initiation factor-1 α (TIF1 α)], and 125-fold [cut-like 2 (Cutl2)/cut homeobox 2 (Cux2)] higher than in adult males, depending on the strain and species, with Tox expression only detected in mice. In rats, these sex differences first emerged at puberty, when the high prepubertal expression of Cutl2 and Trim24 was extinguished in males but was further increased in females. Rat hepatic expression of Cutl2 and Trim24 was abolished by hypophysectomy and, in the case of Cutl2, was restored to near-female levels by continuous GH replacement. Cutl2 and Trim24 were increased to female-like levels in livers of intact male rats and mice treated with GH continuously (female GH pattern), whereas Tox expression reached only about 40% of adult female levels. Expression of all three

genes was also elevated to normal female levels or higher in male mice whose plasma GH profile was feminized secondary to somatostatin gene disruption. Cutl2 and Trim24 both responded to GH infusion in mice within 10–24 h and Tox within 4 d, as compared with at least 4–7 d required for the induced expression of several continuous GH-regulated cytochromes P450 and other female-specific hepatic genes. Cutl2, Trim24, and Tox were substantially up-regulated in livers of male mice deficient in either of two transcription factors implicated in GH regulation of liver sex specificity, namely, signal transducer and activator of transcription 5b (STAT5b) and hepatocyte nuclear factor 4 α (HNF4 α), with sex-specific expression being substantially reduced or lost in mice deficient in either nuclear factor. Cutl2 and Trim24 both display transcriptional repressor activity and could thus contribute to the loss of GH-regulated, male-specific liver gene expression seen in male mice deficient in STAT5b or HNF4 α . Binding sites for Cutl1, whose DNA-binding specificity is close to that of Cutl2, were statistically overrepresented in STAT5b-dependent male-specific mouse genes, lending support to this hypothesis. (*Endocrinology* 148: 3327–3337, 2007)

GH REGULATES THE SEXUALLY dimorphic patterns of a large number of liver-expressed genes, including many receptors, signaling molecules, and enzymes of steroid and drug metabolism, especially cytochrome P450s (CYPs) (for reviews, see Refs. 1 and 2). These sex differences are dictated by the sexual dimorphism of plasma GH profiles, which is especially prominent in rats and mice and is a major determinant of the sex specificity of a large number of hepatic RNAs (3) and proteins (4). Plasma GH profiles are highly pulsatile in adult male rats, where high GH peaks occur every 3.5–4 h and are interrupted by periods of no measurable hormone, whereas adult female rats are characterized by more frequent and overlapping plasma GH peaks, resulting in a nearly constant presence of GH in circulation (5, 6). The length of the GH-free interpulse interval is a key determinant of the sex-specific effect that GH has on liver gene expression (7, 8). Continuous infusion of GH in male rats and mice

abolishes the male, pulsatile plasma GH profile and feminizes the expression of many liver genes (3, 9, 10).

GH signaling is initiated by GH binding to its membrane-bound receptor leading to activation/tyrosine phosphorylation of the GH receptor-associated protein tyrosine kinase Janus kinase 2 (JAK2) (11). JAK2, in turn, phosphorylates itself and the cytoplasmic domain of GH receptor, creating binding sites for signaling molecules implicated in downstream signaling, including signal transducer and activator of transcription (STAT) transcription factors. After tyrosine phosphorylation, STAT proteins dimerize and translocate to the nucleus, where they bind specific DNA elements and activate gene transcription (12). One STAT family member, STAT5b, is repeatedly activated by each incoming male plasma GH pulse and is considered to be a key mediator of the sexually dimorphic response of liver CYPs to GH in rats and mice (13, 14). In contrast to males, which are characterized by high episodic bursts of liver STAT5b activity, the level of tyrosine-phosphorylated, nuclear STAT5b is generally low in female liver (15–17). Additional transcription factors are likely to be required for establishing sexually dimorphic patterns of liver gene expression; however, as suggested by the absence of strong STAT5b response elements in the upstream sequences of several GH-regulated, male-specific liver CYP gene promoters (18, 19) and by the inability of STAT5b alone, when activated precociously in prepubertal rat liver, to induce male-specific liver gene ex-

First Published Online April 5, 2007

Abbreviations: Cutl2, Cut-like 2; CYP, cytochrome P450; HNF, hepatocyte nuclear factor; JAK2, Janus kinase 2; qPCR, quantitative PCR; STAT, signal transducer and activator of transcription; TIF1 α , transcription initiation factor-1 α ; Tox, thymus high-mobility group box protein; Trim24, tripartite motif-containing 24.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

pression (16). One such factor may be hepatocyte nuclear factor (HNF) 4 α , which can act in a synergistic manner with STAT5b to activate certain male-specific CYP promoters (20) and, like STAT5b, is required for the sex-dependent transcriptional control of several classes of GH-dependent liver genes (10, 21). Another liver-enriched transcription factor, HNF6, is expressed in a female-predominant, GH-regulated manner and may contribute to the female-specific expression of hepatic CYP gene 2C12 (22–24).

In the present study, we characterize three transcription factors, cut-like 2 (Cutl2), tripartite motif-containing 24 (Trim24), and thymus high-mobility group box protein (Tox), as novel, highly sexually dimorphic, liver-expressed factors that are positively regulated by the female-characteristic plasma GH pattern. Cutl2, also known as Cux2, belongs to a family of CCAAT displacement protein (CDP)/Cut homeodomain transcription factors involved in the control of proliferation and differentiation (25) and was reported to be expressed primarily in nervous tissue (26). Cutl2 binds DNA in a sequence-specific manner and may act as a transcriptional repressor (26, 27). Trim24, also known as transcription initiation factor-1 α (TIF1 α), is a transcriptional intermediary factor that interacts with ligand-bound nuclear receptors and is proposed to regulate transcriptional activity through chromatin remodeling (28, 29). The third female-specific factor, Tox, is a DNA sequence-independent high-mobility group box-containing protein previously implicated in the regulation of T cell development (30).

Materials and Methods

Continuous GH treatment of ICR mice

Adult ICR mice, 7–8 wk of age, were purchased from Taconic, Inc. (Hudson, NY) and maintained under standardized conditions of light and temperature. Male ICR mice were given GH as a continuous infusion via Alzet osmotic mini-pumps (Durect Corp., Cupertino, CA) for up to 14 d as described earlier (10). Briefly, pumps were filled with recombinant rat GH (purchased from Dr. A. F. Parlow, Harbor-UCLA Medical Center, Torrance, CA) dissolved in 70 mM sodium bicarbonate (pH 9.5) containing 137 mM NaCl and 100 μ g/ml rat albumin or, in the case of vehicle-treated mice, with protein-free buffer. GH was delivered at a rate of 20 ng/g body weight per h for time periods ranging from 10 h to 14 d. At each time point, six to seven GH-treated mice and two to three vehicle-treated mice were killed, and their livers were removed, frozen in liquid nitrogen, and stored at -80 C until use. Livers were also collected from untreated male ($n = 5$) and female ($n = 5$) ICR mice.

Knockout mouse models

Livers were obtained from male and female STAT5b-deficient mice (8–10 wk; 129 \times BALB/c) (31), liver-specific HNF4 α -deficient mice (7 wk; 129/SV \times C57B6 \times FVB) (32) and corresponding control mice (10, 21). Livers from adult male and female somatostatin-deficient mice (11–12 wk) (33) were kindly provided by Drs. R. M. Luque and R. D. Kineman (University of Illinois at Chicago, Chicago, IL) (34).

Rats

Livers were collected from untreated, sham-operated, and hypophysectomized adult male and female adult Fischer 344 rats (9–13 wk of age) (35, 36). Where indicated, rats were subjected to GH treatment by continuous infusion via an Alzet osmotic mini-pump (20 ng of recombinant rat GH/g body weight per h for 7 d). Hypophysectomy was performed by the supplier of the rats (Taconic) at 8 wk of age. Livers from untreated Fischer 344 rats ranging in age from 4 d to 12 wk (16) were used for the postnatal developmental expression studies.

Western blot analysis of Cutl2

Liver nuclear extracts were prepared from freshly isolated livers from individual untreated adult male and female Fischer 344 rats, individual male rats given a continuous infusion of GH for 7 d, and pooled adult male and female ICR mice ($n = 8$ livers/group) using the method of Gorski et al. (37) as described previously (35). Proteins were resolved on 6% SDS-polyacrylamide gels (40 μ g nuclear extract protein per lane) and subjected to Western blot analysis with anti-Cutl2 Ab 356, kindly provided by Dr. A. Nepveu, McGill University, as described previously (27), except that the Tris-buffered saline was 20 mM Tris-HCl (pH 7.6) and 137 mM NaCl, and blocking was performed for 2 h at room temperature. For immunoprecipitation of Cutl2, male and female mouse liver nuclear extracts (1 mg protein per sample) were incubated overnight at 4 C with 1.2 μ l anti-Cutl2 Ab 356 in 0.6 ml buffer A [100 mM sodium phosphate (pH 7.4), 0.9% NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.5% IGEPAL CA-630 detergent, containing 1 mg/ml BSA and complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)]. Antibody-antigen complexes were collected using protein A-Sepharose CL-4B beads (GE Healthcare, Bio-Sciences Corp., Piscataway, NJ), washed three times with buffer A, once with 0.5 M NaCl, and once with PBS and analyzed by Western blotting. Whole-cell extracts prepared from 293T cells transfected with mouse Cutl2 cDNA (plasmid pMX139/Myc/Cux2/HA, a gift from Dr. A. Nepveu) (27) served as a positive control.

Total RNA preparation and quantitative PCR (qPCR)

Total RNA was purified from frozen liver using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA samples were treated with RQ1 RNase-free DNase (Promega, Madison, WI) for 1 h at 37 C followed by heating for 5 min at 75 C. RT of 1 μ g RNA per sample was then performed as described earlier (10) or using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). SYBR Green I-based qPCR assays were performed as described (10) using either SYBR Green PCR Master Mix or Power SYBR Green PCR Master Mix (Applied Biosystems). The following primer pairs were designed using Primer Express software (Applied Biosystems) and used for qPCR amplifications (GenBank accession numbers as indicated): mouse Cutl2 (NM_007804), 5'-CTCAAGACGAA-CACCGTCAT-3' (forward primer; exon 22) and 5'-GCGCATCTG-GACCTGTAGT-3' (reverse primer; exon 23-exon 22 junction; Fig. 1A); rat Cutl2 (XM_222184), 5'-ATTGGCCAGCGTGTGTTT-3' and 5'-CATCCGACAGGAAGTCTCA-3'; mouse Trim24 (NM_145076), 5'-GAGGCCCTCCGTCAAACAGAAC-3' and 5'-GAGCCAGAGCTC-CTCGACTT-3'; rat Trim24 (XM_575435), 5'-GAAGTATCTCCAGA-GGCAGTTGGT-3' and 5'-CAGCTTATCACACGCTTCCAGTAGAG-3'; mouse Tox (NM_145711), 5'-GTGAAGTGTGCGGCTAGT-3' and 5'-GGACCGTTTACCCAGACATC-3'; rat CYP2C11 (NM_019184), 5'-AGAGGAGGCTCAGTGCCTGT-3' and 5'-CCCAGGATAAAGGT-GGGATCA-3'; and rat CYP2C12 (NM_031572), 5'-GCTCACCCCTGT-GATCCCAA-3' and 5'-TGACATTGCAGGGAGCACAT-3'. Rat Tox (XM_342800) was assayed using the following two primer sets: 5'-GCACACTGCTCTCCAATTCCA-3' and 5'-CATGCTTGCTGCTGTCTGA-3' (primer set 1) and 5'-GAACATGGGAGGAACCAACGT-3' and 5'-ACTTGCTCCCAGGTGGAGAAG-3' (primer set 2). No amplification in rat samples was observed with either primer set. However, primer set 1, whose forward primer shows one mismatch to mouse Tox, successfully amplified Tox RNA from mouse liver. These findings suggest that Tox is not expressed in rat liver. The following qPCR primer pairs were used to determine the expression of different Cutl2 RNA variants (Fig. 1) in the livers of individual male and female ICR mice: for the exon 1A-containing variant (CJ065459), 5'-CGGCTCA-GAAGGAGCTTAG-3' (forward primer; exon 1A-exon 3 junction) and 5'-TTAAATTCGCGGAGTT-3' (reverse primer; exon 3); for the exon 1B-containing variant (U45665), 5'-CAGCCAGGACGCTCGGT-3' (exon 1B) and 5'-GCTCCGAGGCGACAGAACT-3' (exon 2); for the exon 1C-containing variant (NM_007804), 5'-CGTGCAAAGTC-CAGGGTCTT-3' (exon 1C) and 5'-GAAAGGGTCCCTGGCAAAG-3' (exon 1C); and for exon 1B- and exon 1C-containing variants together, 5'-ACGGAGTACGGCGGTGTTT-3' (exon 2) and 5'-TTAAATTCGCGGAGTT-3' (exon 3). Primers used to assay 18S rRNA assay were detailed previously (38).

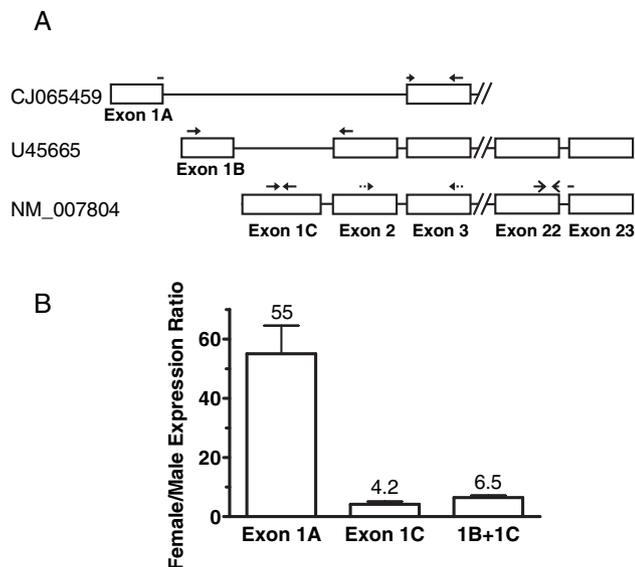


FIG. 1. Expression of Cutl2 transcript variants in mouse liver. **A**, Schematic representation of three different mouse Cutl2 RNAs and qPCR primer pairs used to determine their expression. Cutl2 transcript variants, based on the February 2006 build of the mouse genome, are identified by their GenBank accession numbers. Exon/intron structure shown is not drawn to scale; exons 1A and 1B are separated by 165 nucleotides and exons 1B and 1C by 1951 nucleotides. Exons 1–3 are noncoding. Exon 2 is absent from the exon 1A-containing RNA. The forward primer for this transcript (CJ065459) traverses the exon 1A/exon 3 junction, as indicated. The primer pair amplifying portions of exons 22 and 23 does not discriminate between the three Cutl2 RNA transcripts and was used to assay mouse Cutl2 in a majority of the experiments presented in this study. **B**, Female/male expression ratios of individual Cutl2 transcripts in ICR mouse liver. The ratios were determined for transcripts containing exon 1A, exon 1C, and the exon 1B plus exon 1C transcripts together (1B+1C), as indicated. Relative RNA levels were determined by qPCR analysis of liver RNA from wild-type male and female mice ($n = 5$ per group). The expression levels of each RNA transcript were normalized to the 18S rRNA content of each liver and then calculated relative to the average expression of the corresponding RNA transcript in males. Data shown are mean \pm SE. The calculated mean ratios are indicated above each bar. Levels of each Cutl2 transcript showed statistically significant differences between females and males (Student's t test, $P < 0.01$).

Analysis of sex-specific promoters for Cutl1/Cutl2 binding sites

Computational analysis of Cutl1/Cutl2 binding sites was carried out on a set of 21 group 1A male-specific genes, 18 group 2A male-specific genes, and 20 group 1B female-specific genes (39), selected from the top 35 sex-specific genes in each group, as determined by microarray analysis (39). The 207 liver-expressed genes that were identified by microarray analysis as non-sex-specific were selected as controls. The analysis was performed on an 8-kb region flanking the transcription start site of each gene (5 kb upstream plus 3 kb downstream). The motif discovery program CLOVER (40) was used to search for significant Cutl1/Cutl2 motifs from the TRANSFAC 7.0 public database using a P value threshold of 0.005 and a motif score threshold of 6.5. The matrix comparison algorithm POSSUM (41) was used to scan for Cutl1/Cutl2 binding sites using a score threshold of 7.8.

Results

Female-specific expression of Cutl2, Trim24, and Tox RNAs

Microarray analysis has identified more than 750 liver-expressed genes, including several encoding transcription

factors, that are more highly expressed in female compared with male mouse liver (39). qPCR was used to quantify the expression of three of these transcription factors, Cutl2, Trim24, and Tox, in livers of adult male and female mice and rats (Table 1). All three factors were found to be expressed at higher levels in female compared with male liver in each of the four mouse strains examined. The female specificity was highest for Cutl2 (up to 100-fold higher expression than in males) in both mouse and rat liver. The female specificity of Trim24 RNA was substantially higher in rats (73-fold) than in mice (2.5- to 6.5-fold; Table 1). Tox RNA could not be detected in male or female rat liver using two different qPCR primer pairs, one of which amplified mouse Tox RNA efficiently, suggesting the absence of Tox RNA in rat liver.

Mouse genomic and cDNA databases indicate the existence of at least three Cutl2 RNAs, which differ in the sequence of the first exon and in the presence or absence of exon 2 (Fig. 1A), with exons 1 and 2 both being noncoding. qPCR primers that distinguish these three transcripts were designed and then used to determine relative expression of each Cutl2 transcript in male vs. female ICR mouse liver (Fig. 1B). Strong female-specific expression was observed for the exon 1A-containing cDNA (female/male ratio = 55 ± 10), which was previously characterized as a spliced expressed sequence tag sequence (accession no. CJ065459). Reduced female specificity was apparent for the exon 1C-containing cDNA (NM_007804; female/male ratio = 4.2 ± 0.9). Amplification of the exon 1B-containing sequence produced very low signals, in both male and female samples (data not shown). When exon 1B- and exon 1C-containing transcripts were assayed together using a primer set targeting exons 2 and 3, a female/male expression ratio of 6.5 ± 0.7 was determined. Liver Cutl2 RNA is thus represented by at least two transcripts preferentially expressed in females, with the transcripts containing exons 1A and 1C, respectively, being the major and minor sex-specific transcripts.

TABLE 1. Female-specific expression of Cutl2, Trim24, and Tox RNAs in mouse and rat liver

Strain and species	Female/male expression ratio		
	Cutl2	Trim24	Tox
ICR mice	96 ± 18^b	6.5 ± 0.7^b	16 ± 2^b
Black Swiss mice	64 ± 6^b	3.9 ± 0.2^b	5.6 ± 0.3^b
129 \times BALB/c mice	107 ± 39^a	2.5 ± 0.6^a	9.5 ± 2.7^b
129/SV \times C57B6 \times FVB mice	33 ± 13^a	2.8 ± 0.4^b	3.3 ± 0.2^b
Fischer 344 rats	126 ± 10^b	73 ± 11^b	ND

Relative expression of each RNA was determined by qPCR analysis of wild-type male and female rat livers and mouse livers from the indicated strains. The expression levels in individual livers normalized to the 18S rRNA contents were calculated relative to the average expression in males. Data shown are mean \pm SE based on the following numbers of animals per group (males/females): $n = 5/n = 5$ for ICR mice, $n = 3/n = 5$ for Black Swiss mice, $n = 6/n = 5$ for the mixed background strain of the STAT5-deficient mice (129 \times BALB/c), $n = 8/n = 8$ for the mixed background strain of the HNF4 α -deficient mice (129/SV \times C57B6 \times FVB), $n = 9/n = 7$ for Fischer 344 rats. The differences in expression between females and males were subjected to statistical analysis using Student's t test. ND, Not detected.

^a $P < 0.05$.

^b $P < 0.01$.

Female specificity of liver nuclear *Cutl2* protein

Next, we investigated whether the strong female specificity seen for *Cutl2* RNA was reflected at the protein level. Liver nuclear extracts prepared from adult male and female mice were analyzed on Western blots probed with antibody to mouse *Cutl2* (27). Figure 2A shows that a protein with relative molecular mass of approximately 200 kDa, corresponding in size to *Cutl2* protein from 293T cells transfected with mouse *Cutl2* plasmid, is highly enriched in female compared with male mouse liver. This female specificity is seen both in unfractionated liver nuclear extracts (lane 6 vs. lane 5) and after immunoprecipitation with anti-*Cutl2* antibody (lane 4 vs. lane 3). Female-specific expression of *Cutl2* protein was also seen in liver nuclear extracts from individual rats (Fig. 2B), consistent with the sex specificity of rat *Cutl2* RNA.

Developmental profiles of *Cutl2* and *Trim24*

The sex specificity of many liver RNAs and proteins emerges at the onset of puberty (~4 wk of age in the rat), coincident with a strong increase in pituitary GH release (1, 42). Investigation of the expression of *Cutl2* and *Trim24* during postnatal rat development revealed female-predominant expression of both RNAs beginning at 4 wk of age (Fig. 3, A and B). Both RNAs were expressed in a sex-independent manner in prepubertal (4 d and 2 wk old) rat liver at levels

corresponding to approximately 15% (*Trim24*) and 70–80% (*Cutl2*) of 8-wk-old adult female liver. In males, *Cutl2* and *Trim24* both declined precipitously at 4 wk and were extinguished by 5 wk, whereas the expression in females increased after 4 wk and remained elevated through 12 wk of age. These developmental profiles contrast with those of the prototypical male-specific gene *CYP2C11* (Fig. 3C) and the female-specific gene *CYP2C12* (Fig. 3D). The GH pulse-regulated *CYP2C11* and the continuous GH-regulated *CYP2C12* were not expressed in neonatal and prepubertal rats; their expression was first detected at 4 wk of age in male and female liver, respectively.

Responsiveness of rat liver *Cutl2* and *Trim24* to hypophysectomy and continuous GH treatment

Given the dependence of many sex-specific hepatic RNAs and proteins on pituitary GH, we investigated the impact of hypophysectomy and GH replacement on the expression of *Cutl2* and *Trim24* (Fig. 4). Hypophysectomy of female rats ablated the expression of both liver transcription factors, indicating a strong dependence on pituitary or pituitary-derived hormone(s). Continuous GH treatment of hypophysectomized male and female rats using an osmotic mini-pump (20 ng GH/g body weight per h for 7 d) (7) restored normal female liver levels of *Cutl2* (Fig. 4A). GH induced *Trim24* approximately 3.7-fold

FIG. 2. Western blot analysis of liver *Cutl2* protein. Nuclear extracts prepared from pooled mouse livers (A) or from individual rat livers (B) were resolved on a 6% SDS-polyacrylamide gel and subjected to Western blot analysis with anti-*Cutl2* antibody 356 in comparison with cDNA-expressed mouse *Cutl2*. A, Whole-cell extracts (40 μ g) from untransfected 293T cells (lane 1) or from 293T cells transfected with *Cutl2* cDNA (lane 2); *Cutl2* protein immunoprecipitated with anti-*Cutl2* antibody 356 from liver nuclear extract (1 mg) prepared from pools of adult male (lane 3) and female (lane 4) mouse liver (n = 8 livers per group); and adult male (lane 5) and female (lane 6) mouse liver nuclear extract (40 μ g/lane). B, Portion of Western blot showing, in lanes 1–2, cell extracts (40 μ g) from untransfected or *Cutl2* cDNA-transfected 293T cells, respectively; lanes 3–14, liver nuclear extracts prepared from individual adult male (lanes 3–6) and female (lanes 7–10) rats and from adult male rats given a continuous GH infusion for 7 d (lanes 11–14). Authentic, cDNA-expressed *Cutl2* protein (lane 2, A and B; arrow) runs close to the 204-kDa protein marker shown on the right.

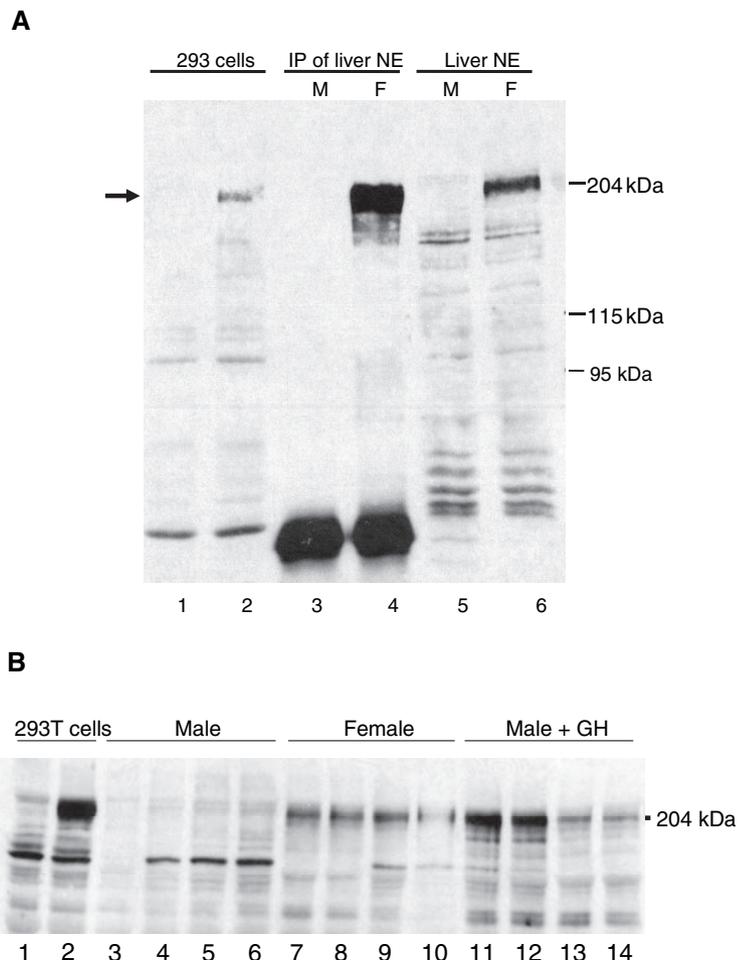
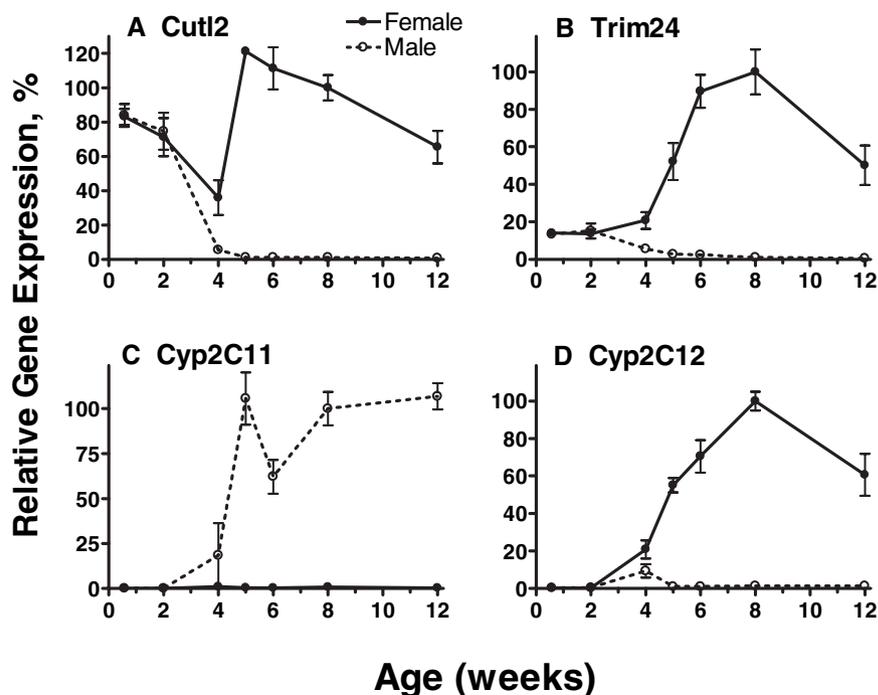


FIG. 3. Postnatal developmental profiles of rat liver *Cutl2* and *Trim24* RNAs in comparison with *CYP2C11* and *CYP2C12* RNAs. Expression of the four indicated RNAs was determined by qPCR analysis of liver RNA prepared from individual male and female rats ranging from 4 d to 12 wk of age. RNA levels were normalized to the 18S rRNA content of each liver and expressed as a percentage of the average level in 8-wk-old females (A, B, and D) or 8-wk-old males (C), which were set to 100%. The data shown are mean \pm SE for each group (n = 3–6 livers).



in hypophysectomized males and females; however, this increase did not reach statistical significance (Fig. 4B).

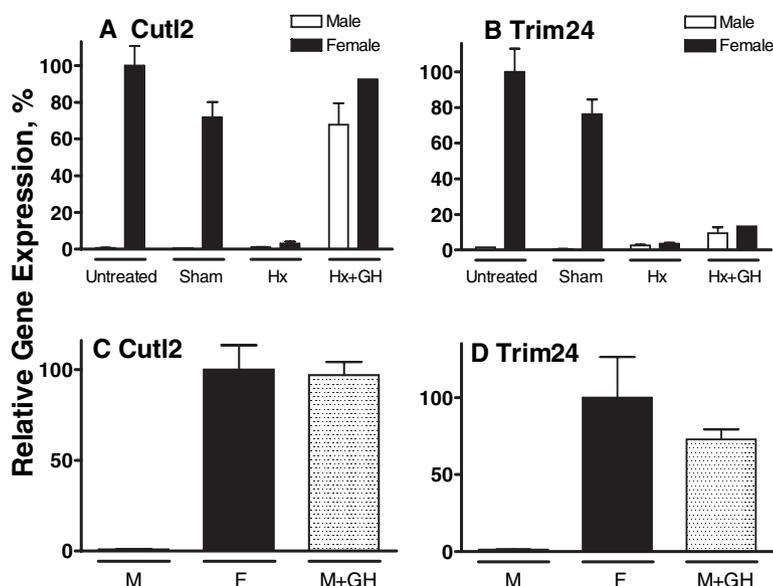
The GH responsiveness of *Cutl2* and *Trim24* was further established by the strong induction of both genes in livers of intact male rats given a 7-d continuous GH infusion (Fig. 4, C and D). This treatment also increased *Cutl2* protein in male liver nuclear extracts to normal female levels (Fig. 2B, lanes 11–14 vs. lanes 7–10). The substantial induction of *Trim24* after continuous GH treatment of intact males but not hypophysectomized males (Fig. 4, D vs. B) suggests a requirement for pituitary-dependent factor(s) other than GH, such as thyroid hormone and corticosteroids, for full female expression of *Trim24*. By contrast, *Cutl2* RNA was fully feminized by continuous GH treatment of intact and hypophy-

sectomized rats, *i.e.* even in the absence of other pituitary hormones. Neither gene was induced after hypophysectomy of male rats (Fig. 4, A and B), indicating that *Cutl2* and *Trim24* are not subject to the strong negative regulation by the male GH profile seen with female-specific liver genes such as *Cyp2b9* (10, 43).

Response to continuous GH in mouse liver

The impact of continuous GH treatment was investigated over a time course to determine the temporal relationship between changes in circulating GH profiles, induction of *Cutl2* and *Trim24*, and the feminization of hepatic gene expression. This study was carried out in mice, where the

FIG. 4. Effect of hypophysectomy and continuous GH infusion on *Cutl2* and *Trim24* RNA in rat liver. *Cutl2* and *Trim24* RNA levels were determined by qPCR analysis of livers isolated from individual untreated (n = 3 livers per group), sham-operated (n = 2 per group), and hypophysectomized (Hx; n = 4 per group) male and female rats and from hypophysectomized rats given a continuous GH infusion via an osmotic pump (+GH) for 7 d (n = 3 males and n = 1 female) (A and B). RNA levels were also determined in individual livers from untreated male (M) and female (F) rats (n = 4–5 per group) and control male rats treated with continuous GH via an osmotic mini-pump for 7 d (n = 5) (C and D). Data were analyzed as in Fig. 3, except that the RNA levels (mean \pm SE for individual livers) are shown relative to the average level in untreated females, which was set to 100%.



time-dependent effects of continuous GH treatment on sex-specific *Cyp* genes and other liver-expressed genes are well established (10) and where the effects of continuous GH on the expression of *Tox* could be determined. Adult male mice were infused with GH continuously for time periods ranging from 10 h up to 14 d using osmotic mini-pumps. This treatment eliminates the GH-free interpulse period that is required for male liver gene expression and feminizes hepatic enzyme profiles (10). qPCR analysis revealed a time-dependent increase in all three RNAs. In the case of *Cutl2* and *Trim24*, the induction of RNA levels was evident within the first day of GH infusion. *Cutl2* RNA was up-regulated approximately 10-fold on d 1, 50-fold on d 2, and 80-fold on d 7, corresponding to approximately 85% of its adult female level (Fig. 5A). *Trim24* increased about 4-fold, to 60% of its adult female level, within 1 d, and additional increases were seen at later times (Fig. 5B). The early and substantial increases in *Cutl2* and *Trim24* RNAs contrast with the response of *Tox* RNA, whose induction was noticeably slower, reaching only about 40% of female levels after 7–14 d of continuous GH treatment (Fig. 5C).

The responsiveness of *Cutl2*, *Trim24*, and *Tox* to changes in plasma GH profiles was further investigated in mice deficient in somatostatin, a hypothalamic suppressor of pituitary GH release whose absence leads to substantial increases in basal levels of GH between plasma pulses and increased expression of several female-specific hepatic genes (33). As shown in Fig. 6, all three RNAs were increased to normal female levels or higher in somatostatin-deficient male mouse liver, resulting in a loss of sex specificity in the somatostatin knockout strain. This increase reached approximately 150-fold in the case of *Cutl2* RNA. In contrast, in somatostatin-deficient female liver, *Cutl2* RNA but not *Trim24* or *Tox* RNA was up-regulated by about 2-fold, consistent with the modest effect that somatostatin deficiency has on the overall plasma GH profile in females (33).

Dependence on *STAT5b*

STAT5b is a GH pulse-responsive transcription factor whose expression is required for hepatic expression of many sex-dependent genes (39). The impact of *STAT5b* deficiency on *Cutl2*, *Trim24*, and *Tox* RNA levels was therefore investigated. In male mice deficient in *STAT5b*, *Cutl2* RNA was up-regulated approximately 40-fold, to a level equal to 40% of wild-type female liver (Fig. 7A). *Trim24* and *Tox* were increased by about 2- and 10-fold, respectively, and reached levels similar to wild-type females (Fig. 7, B and C). In contrast, the loss of *STAT5b* in females had no effect on gene expression. Thus, sex specificity is markedly decreased (*Cutl2*) or abolished (*Trim24* and *Tox*) in the absence of *STAT5b*.

Codependence on *HNF4 α*

Many, but not all, sexually dimorphic mouse liver genes are codependent on *HNF4 α* and *STAT5b* for sex-dependent expression (10). We therefore investigated the impact of *HNF4 α* deficiency on the expression of *Cutl2*, *Trim24*, and *Tox* using a liver-specific *HNF4 α* knockout mouse model (32). All three factors were strongly up-regulated in *HNF4 α* -

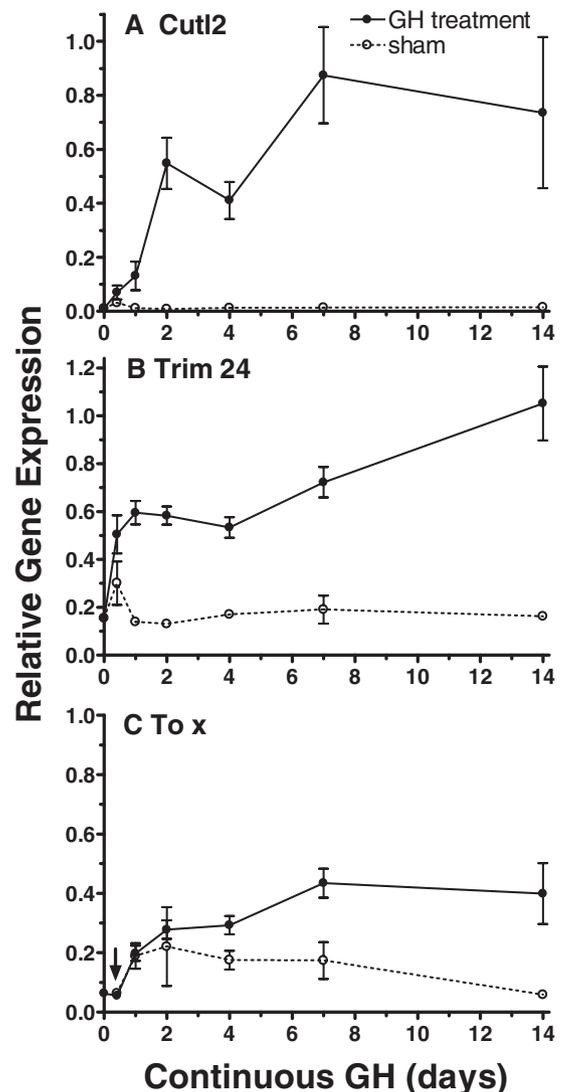


FIG. 5. Response of *Cutl2*, *Trim24*, and *Tox* RNAs to continuous GH infusion in mice. Male ICR mice were implanted with osmotic mini-pumps delivering a continuous infusion of GH or vehicle control for time periods ranging from 10 h to 14 d. Individual livers were isolated and analyzed for *Cutl2*, *Trim24*, and *Tox* RNAs by qPCR. Data shown are based on the following number of individual mice per group: $n = 6-7$ (each GH-treated time point) and $n = 2-3$ (each vehicle-treated control group; sham). RNA levels (mean \pm SE) were normalized to the 18S rRNA content of each liver and are presented relative to the average untreated female liver level, which was set to 1. All three RNAs showed stress-dependent responses to osmotic mini-pump implantation at the 10-h time point, as indicated by RNA increases in the vehicle-treated control group (dashed lines). The stress response of *Tox* RNA, evident at several of the early time points, was quite substantial and was indistinguishable for vehicle control and GH-treated livers at the 10-h point (increase up to 80–95% of the untreated female control for both groups). *Tox* RNA data for the 10-h time point (arrow) was adjusted for the stress response by multiplying the relative expression levels in GH- and vehicle-receiving groups by the untreated male/vehicle control expression ratio.

deficient male liver (Fig. 8). More modest increases in expression were seen for *Trim24* and *Tox*, but not *Cutl2*, in *HNF4 α* -deficient females. Overall, the loss of *HNF4 α* led to loss of female specificity for all three factors, as was also seen in the case of *STAT5b* deficiency (Fig. 7).

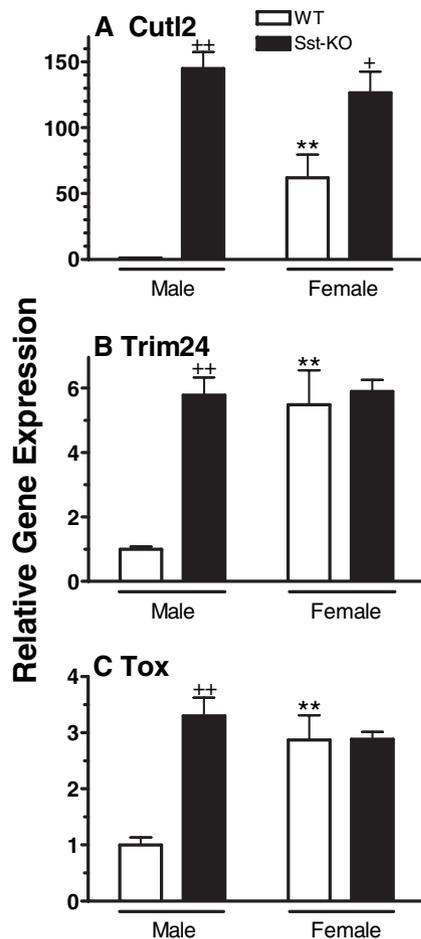


FIG. 6. Effect of *Sst* gene disruption on mouse liver expression of *Cutl2*, *Trim24*, and *Tox*. RNA levels were determined by qPCR analysis of individual livers from wild-type (WT) male ($n = 6$) and female ($n = 3$) mice and from somatostatin (*Sst*)-deficient (KO) male ($n = 5$) and female ($n = 7$) mice. RNA levels in individual livers were normalized to the 18S rRNA content and presented relative to the average RNA level in wild-type male mice, which was set at 1. Data shown are mean \pm SE for each group. Data were analyzed using Student's *t* test: + and ++, $P < 0.05$ and $P < 0.01$, respectively, for somatostatin-deficient male (or female) vs. wild-type male (or female); * and **, $P < 0.05$ and $P < 0.01$, for wild-type (or somatostatin-deficient) female vs. wild-type (or somatostatin-deficient) male.

Cutl2 binding sites in sex-specific hepatic genes

Sex-specific genes have been grouped into sets of coexpressed genes based on their dependence on STAT5b for expression in males and in females (39). The two largest sets of coexpressed sex-specific genes, male-specific group 1A and female-specific group 1B, are regulated by STAT5b in a positive manner (group 1A) and in a negative manner (group 1B), respectively. Both gene sets were analyzed to determine whether DNA sequence motifs likely to be associated with *Cutl2* binding are statistically overrepresented in comparison with a control (background) set comprised of liver-expressed genes that are not sex specific or subject to STAT5b-dependent regulation; the latter genes are expected to have a random distribution of *Cutl2* binding sequences. *Cutl2* has a DNA-binding specificity closely related to that of *Cutl1* (27), whose binding specificity is well studied and, unlike

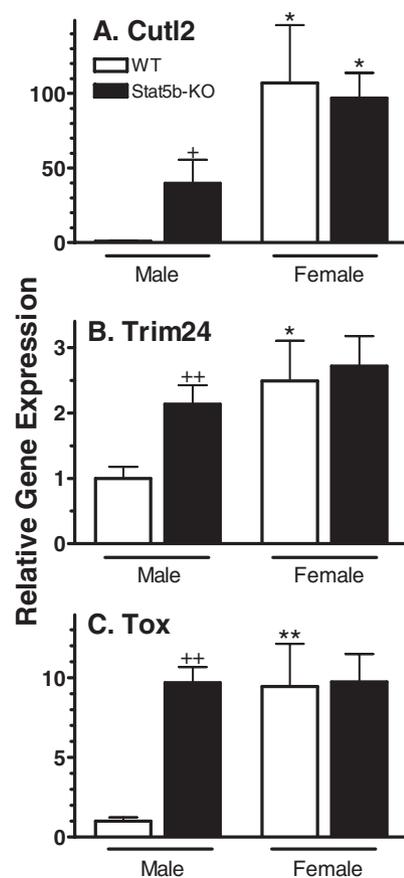


FIG. 7. Effect of *Stat5b* gene disruption on mouse liver expression of *Cutl2*, *Trim24*, and *Tox*. RNA levels were determined by qPCR analysis of individual livers from wild-type (WT) male ($n = 6$) and female ($n = 5$) mice and STAT5b-deficient (KO) male ($n = 6$) and female ($n = 7$) mice. Data were analyzed as described in Fig. 6, with the wild-type male RNA level set to 1.0 for each gene, and graphed as mean \pm SE for each group. Data were analyzed using Student's *t* test: + and ++, $P < 0.05$ and $P < 0.01$, respectively, for STAT5b-deficient male (or female) vs. wild-type male (or female); * and **, $P < 0.05$ and $P < 0.01$, respectively, for wild-type (or STAT5b-deficient) female vs. wild-type (or STAT5b-deficient) male.

Cutl2, is represented by binding site matrices in the TRANSFAC database (44). These matrices were used by the motif analysis program CLOVER (40) to analyze 8 kb of DNA surrounding the transcription start sites of 21 group 1A male genes for the occurrence of *Cutl1*/*Cutl2* binding sites. A set of 207 liver-expressed genes that is not sex-specific and does not display STAT5b dependence served as a negative control. Binding sites represented by two *Cutl1* TRANSFAC matrices were found to be statistically overrepresented, at $P = 0.002$ and at $P = 0.004$, in the group 1A male genes (Table 2), suggesting that the repressor activity of *Cutl2* (27) may contribute to the silencing of male gene expression in female liver. No such overrepresentation of binding sites was observed in a set of 20 group 1B female genes or in a second, distinctly regulated group of 18 male genes (group 2A), whose members require STAT5b for expression in both male and female liver (39). The prevalence of *Cutl1*/*Cutl2* binding sites in group 1A male genes was confirmed using the matrix comparison algorithm POSSUM (Table 2 legend).



FIG. 8. Effect of *Hnf4 α* gene disruption on mouse liver *Cutl2*, *Trim24*, and *Tox* RNA. RNA levels were assayed in livers of wild-type (WT) and HNF4 α -deficient (KO) male and female mice by qPCR (n = 8 livers per group). Data were analyzed as in Fig. 6, with the wild-type male level set to 1.0, and graphed as mean \pm SE for each group. Data were analyzed using Student's *t* test: + and ++, $P < 0.05$ and $P < 0.01$, respectively, for HNF4 α -deficient male (or female) vs. wild-type male (or female); * and **, $P < 0.05$ and $P < 0.01$, respectively, for wild-type (or HNF4 α -deficient) female vs. wild-type (or HNF4 α -deficient) male.

Discussion

STAT5b is a plasma GH profile-responsive transcription factor that plays a major role in establishing or maintaining sex-specific liver gene expression, with approximately 90% of male-specific genes down-regulated to wild-type female levels and approximately 60% of female-specific genes up-regulated in STAT5b-deficient male mice, as revealed by microarray analysis (39). Several observations suggest that some, perhaps many, of these gene-regulatory effects of STAT5b are indirect, most notably the substantially delayed response of female-specific *Cyp* genes and other genes to changes in plasma GH profiles (10). These earlier findings prompted us to investigate novel signaling molecules and transcription factors that are expressed in liver in a sex-dependent manner and may potentially contribute to the STAT5b-dependent regulation of liver gene expression. Presently, three such liver transcription factors were characterized with respect to their sex specificity, postnatal develop-

mental expression, GH regulation, and dependence on STAT5b and HNF4 α for hepatic female specificity.

The three transcription factors investigated, *Cutl2*, *Trim24*, and *Tox*, were all expressed in liver in a female-specific manner, as determined by qPCR analysis of liver RNA. These results were confirmed at the protein level by analysis of liver nuclear extracts in the case of the CCAAT displacement protein/Cut transcription factor family member *Cutl2*, which exhibited the highest sex specificity (female:male ratio \sim 100) in both rats and mice. Female-predominant expression (female:male \sim 3) has previously been observed for another Cut domain protein, the liver-enriched transcription factor HNF6, which contributes to GH regulation of the female-specific rat gene *CYP2C12* (23, 24). In the case of *Trim24*, a transcriptional intermediary factor 1 family member, the female/male specificity ratio in rats (\sim 73) was substantially higher than in mice (2.5–6.5, depending on the strain). *Tox*, an HMG box-containing transcription factor, exhibited up to 16-fold higher expression in female compared with male mouse liver. The delayed response of *Tox* to continuous GH treatment, compared with *Cutl2* and *Trim24* (Fig. 5), rules out *Tox* as an early, upstream regulator of sex-dependent liver genes. Moreover, no expression of the predicted rat homolog of mouse *Tox* (95% nucleotide identity in their region of overlap) could be detected in either female or male rat liver. This absence indicates *Tox* does not play a fundamental, conserved regulatory role in the sex-dependent actions of GH in the liver.

High-level, female-like expression of *Cutl2* and *Trim24*, and to a lesser extent *Tox*, could be induced in males given a continuous infusion of GH using osmotic mini-pumps. This treatment overrides the endogenous male, pulsatile plasma GH pattern and mimics the more continuous female GH pattern, leading to global feminization of liver gene expression (3). The dependence of these transcription factors on the female GH pattern was also demonstrated, in the case of *Cutl2*, in hypophysectomy and continuous GH replacement experiments and, in the case of all three factors, by their increased expression in mice deficient in somatostatin, an inhibitor of pituitary GH secretion. Somatostatin-deficient mice have a significantly elevated plasma GH baseline associated with feminization of several sex-dependent hepatic RNAs (33, 45). The mechanisms responsible for the female-specific expression of *Cutl2*, *Trim24*, and *Tox* are uncertain. Regulation of these genes is not likely to be mediated by the low but sustained level of activated STAT5b found in intact adult female liver (15), insofar as STAT5b is dispensable for the high-level expression of these genes seen in female mice (Fig. 7). Moreover, *Cutl2* and, to a lesser extent, *Trim24* are expressed at a substantial level in both male and female rats before puberty (Fig. 3), at which time hepatic STAT5b activity is low or undetectable (16). The regulatory mechanisms for *Cutl2* and *Trim24* are also likely to be distinct from those of other female-specific, continuous GH-dependent genes, such as rat *CYP2C12*, which is not expressed before puberty (Fig. 3), and mouse *Cyp3a16* and *Cyp3a41*, which require at least 7 d to respond to continuous GH infusion in males (10). Conceivably, other continuous GH-regulated, female-enriched transcription factors, such as HNF3 β and HNF6 (21, 22, 24), could contribute to the female specificity of these

TABLE 2. Cutl1/Cutl2 binding sites identified in group 1A male-specific genes

Male-specific gene	TRANSFAC motif ID	Location	Strand	Sequence	Motif score
<i>Cyp4a12</i>	M00106	-3446	-	ggcatcaacc	7.04
<i>Moxd1</i>	M00106	-4903	-	ccgatcgacc	7.45
	M00104, M00106	-210	+	catcgattcc	6.94, 7.77
	M00104, M00106	1966	+	aatggatggg	6.63, 6.84
	M00106	2688	+	catggatgcc	7.24
<i>Gstpi</i>	M00104, M00106	1845	-	cccatcaatg	6.99, 8.26
<i>Aoh1</i>	M00104, M00106	-3859	+	catggatccc	6.57, 7.94
	M00106	-629	-	cacatcaatc	7.56
	M00106	-229	+	catagatctg	6.61
<i>Dkk4</i>	M00106	-4935	-	gggatccatt	7.29
<i>Ugt2b38</i>	M00104, M00106	-4444	+	gatcgatcca	7.11, 7.28
	M00106	-2683	-	cacatcaatg	7.46
	M00106	-778	-	gggatggatc	7.59
<i>Sftpb</i>	M00106	-4198	-	gggatccatg	7.66
	M00106	-2812	-	gacatcaatg	7.26
<i>Il1r2</i>	M00104	-4797	-	gcatggata	6.66
	M00106	-3468	-	catgatcaatc	8.01
	M00106	-2471	+	catggatctg	6.8
	M00104	-2169	+	tattgatgac	7.3
	M00106	-495	-	cccatccatt	6.66
<i>Lamc3</i>	M00106	-537	-	gggatcattt	6.62
	M00104, M00106	103	+	catcgatggc	8.16, 8.3
	M00106	405	-	cccatccatg	6.7
<i>Lck</i>	M00106	-4919	+	gattgatctc	6.6
	M00106	-3263	-	cggatggatg	6.94
<i>LOC382109</i>	M00104, M00106	-4882	-	gggatccatc	6.65, 7.98
	M00106	-3427	-	cacatcaatc	7.52
	M00104, M00106	-1513	-	gggatccatc	6.65, 7.98
<i>Hba-x</i>	M00106	-4973	+	catccatggc	6.54
	M00106	2687	-	cacatcaatt	6.95
<i>4921504102Rik</i>	M00104, M00106	-4281	-	gggatcaata	6.73, 6.92
	M00106	-2812	-	cacatcaatc	7.61
<i>Irx2</i>	M00106	-3684	+	gatagatctg	7.14
	M00106	-2884	-	gggatcattc	6.89
	M00104, M00106	-1681	+	aattgatccc	7.56, 8.9
<i>Ruvb12</i>	M00106	-3498	+	catggatctg	6.7
	M00104, M00106	-1057	+	gattgatggc	6.97, 7.97
<i>Sox15</i>	M00104, M00106	-4135	+	aatcgatcgc	9.79, 8.74

Shown are the Cutl1/Cutl2 binding sites, characterized by the two indicated TRANSFAC matrices, that were identified in 16 of 21 group 1A male-specific genes by CLOVER analysis. The location of each binding site is shown relative to the transcription start site of each gene, along with the genomic DNA strand, binding sequence, and associated motif score. Two motif scores are presented for binding sites that matched both TRANSFAC matrices. In several cases, binding sites that overlapped with those shown were found on the opposite strand with an offset of two nucleotides (not shown). The preponderance of Cutl1/Cutl2 sites in group 1A male-specific genes was confirmed using POSSUM to scan for binding sites, which were found in 11 group 1A genes but only three genes from female group 1B, five genes from male group 2A, and three to five genes in each of three randomly selected sets of 21 background genes (not shown).

genes. Indeed, computational analysis revealed the presence of binding sites for both HNF3 β and HNF6 within clusters of transcription factor binding sites associated with *Cutl2* and *Trim24* (data not shown). Additional study is required to determine the role, if any, that these HNF factors play in expression of *Cutl2* and *Trim24*.

Although STAT5b may not be required for hepatic expression of *Cutl2*, *Trim24*, or *Tox*, it may, nevertheless, regulate these transcription factors, as indicated by the substantial increases in their expression in livers of male mice deficient in STAT5b. This apparent de-repression of all three transcription factors in STAT5b-deficient males could indicate that the corresponding three genes are directly repressed by GH pulse-activated STAT5b, or perhaps by a STAT5b-dependent factor. This scenario seems unlikely for *Cutl2* and *Trim24*, however, because their expression in males was not elevated after hypophysectomy (Fig. 4), where liver STAT5b is inactive due to the absence of stimulation by circulating GH. An alternative possibility is that the increased expres-

sion seen for the three genes in STAT5b-deficient liver reflects a loss of STAT5b-dependent feedback inhibition in the hypothalamus (46), which may lead to changes in plasma GH profiles that mimic an adult female pattern and thereby induce *Cutl2*, *Trim24*, and *Tox* expression.

Trim24, also known as TIF-1 α , is a nonhistone chromosomal protein kinase that forms complexes with TIF-1 β /KAP-1 and certain KRAB motif-containing zinc finger repressors and exerts transcriptional repression activity, which is proposed to involve histone deacetylation and chromatin remodeling (28, 47, 48). *Trim24* binds tightly to euchromatin, in particular at the borders between euchromatin and heterochromatin, and may positively modulate the interaction of liganded nuclear receptors, and perhaps other sequence-specific DNA-binding proteins, with their cognate binding sites (29, 48, 49). The female-specific expression of *Trim24*, which reached 73-fold in the case of rat liver, could play an important role in GH-dependent chromatin remodeling associated with expression of sex-dependent genes (1), poten-

tially amplifying the sex-dependent effects of transcriptional regulators, such as HNF4 α and HNF6, which exhibit more modest female predominance (~3- to 6-fold) in expression and activity. Additional study will be required to investigate these and other possible consequences of the sex specificity of Trim24.

Cutl2, also known as Cux2, is a sequence-specific DNA-binding protein that was reported to be expressed exclusively in the central and peripheral nervous systems (26). The absence of Cutl2 RNA in adult mouse liver reported in earlier studies may readily be explained if the liver samples analyzed were from male mice. Presently, we found Cutl2 to be a female-specific, nuclear protein in both mouse and rat liver whose migration on SDS gels (relative molecular mass ~200 kDa) was somewhat slower than would be expected based on its predicted size of 1426 (mouse) or 1613 amino acids (rat) but is consistent with the apparent molecular weight of the similar length (1486 amino acids) human Cutl2 protein in a neuroblastoma cell line (27). Cutl2 contains three conserved Cut repeats and one COOH-terminal Cut homeodomain, which act in combination to dictate the sequence-specific DNA-binding activity of Cutl2. In contrast to Cutl1 (CCAAT-displacement protein), which exhibits both transcriptional repression and *trans*-activation functions, Cutl2 has, thus far, been exclusively associated with transcriptional repression of target genes (27). As in the case of Cutl1, repression by Cutl2 could involve displacement of the transcription factor CCAAT/enhancer-binding protein, which itself is subject to GH regulation (50, 51).

Conceivably, Cutl2 may contribute to sex-specific liver gene expression by repressing the expression of certain male-specific genes in female liver. This hypothesis is supported by our finding that binding sites for Cutl1, whose DNA-binding specificity is close to that of Cutl2 (27), were statistically overrepresented in a set of coexpressed male-specific mouse genes belonging to group 1A (39) but not in a corresponding set of female-specific mouse genes (group 1B) or in a distinctly regulated set of male-specific genes (group 2A). This hypothesis is also consistent with the dramatic induction of the male-specific rat gene *CYP2C11* at the onset of puberty, *i.e.* at the same time that *Cutl2* expression is silenced in male rat liver. Similarly, the substantial up-regulation of Cutl2 seen in adult male mice deficient in HNF4 α could contribute to the associated suppression of certain male-specific *Cyp* genes and other GH-regulated genes in this animal model. Additional studies will be required to test these hypotheses and to evaluate the potential role that Cutl2 and the other factors characterized in this study may play in the global expression patterns of sex-specific, GH-regulated genes in rat and mouse liver.

Acknowledgments

We thank Dr. Alain Nepveu, McGill University, for generously providing Cutl2 plasmid and antibody.

Received August 30, 2006. Accepted March 23, 2007.

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This work was supported in part by National Institutes of Health Grant DK33765 (to D.J.W.).

Disclosure Statement: The authors have nothing to disclose.

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