Sexual Dimorphism of Rat Liver Nuclear Proteins

REGULATORY ROLE OF GROWTH HORMONE*[S]

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Many genes are expressed in mammalian liver in a sexually dimorphic manner. DNA microarray analysis has shown that growth hormone (GH) and its sex-dependent pattern of pituitary secretion play a major role in establishing the sexually dimorphic patterns of liver gene expression. However, GH may exert effects on protein post-translational modification and nuclear localization that are not reflected at the mRNA level. To investigate these potential effects of GH, we used two-dimensional gel electrophoresis followed by LC-MS/MS to: 1) identify rat liver nuclear proteins whose abundance or state of post-translational modification displays sex-dependent differences; and 2) determine the role of the plasma GH profile in establishing these differences. Nuclear extracts prepared from livers of individual male ($n = 9$) and female ($n = 5$) adult rats, and from males given GH by continuous infusion for 7 days to feminize liver gene expression ($n = 5$ rats), were resolved by two-dimensional electrophoresis. Image analysis of SYPRO Ruby-stained gels revealed 165 sexually dimorphic protein spots that differ in normalized volume between male and female groups by >1.5-fold at $p < 0.05$. Sixty of these proteins exhibited female-like changes in spot abundance following continuous GH treatment. Comparison of male and GH-treated male groups revealed 130 proteins that displayed >1.5-fold differences in abundance, with 60 of these GH-responsive spots being sexually dimorphic. Thus, GH plays an important role in establishing the sex-dependent differences in liver nuclear protein content. Twenty-eight of the sexually dimorphic and/or GH-regulated protein spots were identified by LC-MS/MS. Proteins identified include regucalcin, nuclear factor 45, and heterogeneous nuclear ribonucleoproteins A3, D-like, and K, in addition to proteins such as GST, normally associated with cytosolic extracts but also reported to be localized in the nucleus. ***Molecular & Cellular Proteomics 3:1170–1180, 2004.***

Growth hormone (GH),[1] a polypeptide hormone secreted by the anterior lobe of the pituitary gland, plays a major role in the regulation of metabolism and longitudinal growth. Primary targets of GH action include the liver, skeletal muscle, and adipose tissue. In many species, including humans, the temporal pattern of pituitary GH secretion differs between males and females, resulting in sexually dimorphic plasma GH profiles (1–3). Sex differences in plasma GH profiles are especially prominent in rodents. In adult male rats, GH is released into circulation every 3–4 h, which gives rise to high plasma GH peaks separated by periods of very low to undetectable plasma GH (4). In contrast, adult female rats are characterized by lower yet more frequent, overlapping pulses of GH secretion, resulting in the presence of GH in plasma at nearly all times (5, 6). GH acts via these sex-dependent plasma profiles to establish sex differences in body growth rates (7) and in the sexually dimorphic expression of a large number of genes in the liver. These genes encode various receptors, signal transduction molecules, and enzymes of steroid and foreign compound metabolism, in particular cytochrome P450 (8–12). DNA microarray analysis has shown that GH represents a major determinant of sexually dimorphic liver gene expression in the rat model, with GH regulating the expression of 72 of 86 genes found to exhibit sex differences in expression (12). However, changes in gene expression at the mRNA level do not always lead to changes at the protein level. Moreover, GH may effect post-translational changes that modulate enzyme activities and intracellular protein patterns via signal transduction events that are not manifest at the level of gene expression and would not be detected by microarray analysis.

Two-dimensional (2D) gel electrophoresis coupled with MS is a powerful method to assess the state of protein expression in subcellular compartments, cells, and tissues and its response to regulatory factors, including hormone stimulation. Proteomic approaches have been used to identify changes in protein expression in diseased states, including cancer, diabetes, and inflammatory disease (13–16), in response to exposure to environmental pollutants (17) and certain nutrients (18), and during development (19–21).

In the present study, nuclear extracts prepared from rat liver were analyzed by 2D gel electrophoresis followed by LC-MS/MS in order to identify proteins that display sex-dependent differences in abundance and to determine the role of GH in establishing these differences. A total of 165 spots differentially expressed between untreated males and females were detected on the 2D gels, and 60 of these spots exhibited

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[1] The abbreviations used are: GH, growth hormone; 2D, two-dimensional; STAT5, signal transducer and activator of transcription 5; hnRNP, heterogeneous nuclear ribonucleoprotein; NF45, nuclear factor 45.

female-like changes in their abundance in response to continuous GH treatment. Twenty-eight sex-dependent and/or GH-regulated spots were identified, including several proteins that may contribute to GH signaling and regulatory events in the nucleus.

EXPERIMENTAL PROCEDURES

Animals and Preparation of Liver Nuclear Extracts—Adult male and female Fischer 344 rats (10–12 wk old) were purchased from Taconic, Inc. (Germantown, NY). Male rats were treated with GH by continuous infusion using an Alzet osmotic minipump as described earlier (22). Mini-pumps (Alzet model 2001; Alza Corp., Palo Alto, CA) filled with GH were implanted subcutaneously on the backs of anesthetized male rats to deliver GH at 2 μg per 100 g body per hour for 7 days. This treatment mimics the near-continuous plasma GH profile of adult female rats and feminizes the overall liver mRNA profile (12). Partially purified nuclear extracts were prepared from freshly excised individual rat livers according to the method by Gorski *et al.* (23) and frozen in aliquots, as described earlier (22). Nuclear extracts prepared in this manner are highly enriched in transcription factors such as signal transducer and activator of transcription 5 (STAT5) and hepatic nuclear factor 4, as evidenced by EMSA. STAT5 activity was assayed for individual liver nuclear extract samples by EMSA using a STAT5 DNA probe (22). Male nuclear extracts that exhibited low or no STAT5 DNA-binding activity compared with the most active sample in the group were designated as nuclear extracts with low STAT5 activity (24).

2D Gel Electrophoresis—Protein was precipitated from individual nuclear extract samples using a 2D Clean-Up Kit (Amersham Biosciences Corp., Piscataway, NJ) and redissolved in rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.62% DTT, 0.5% IPG buffer pH 3–10, and a trace of bromphenol blue. Samples (115 μg of protein) were separated by IEF using 24-cm Immobiline DryStrips pH 3–10 and an IPGphor apparatus (both from Amersham Biosciences Corp.). After a 12-h rehydration period at 20 °C at low voltage (30 V for 6 h followed by 60 V for 6 h), the running conditions were set to 200 V for 1 h, followed by 500 V for 1 h, 1,000 V for 1 h, a linear gradient to 8,000 V for 0.5 h, and then 8,000 V for 60,000 Vh (all steps at 20 °C). The strips were stored at −80 °C until subjected to SDS-PAGE in the second dimension. SDS-PAGE was performed using pre-cast homogeneous 12.5% polyacrylamide gels and an Ettan DALT*twelve* separation unit (both from Amersham Biosciences Corp). Each strip was equilibrated in 10 ml of equilibration buffer (50 mM Tris-HCl buffer, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and a trace of bromphenol blue) containing 1% (w/v) DTT, followed by equilibration in 10 ml of the same buffer containing 2.5% (w/v) iodoacetamide. Both equilibration steps were performed at room temperature. The strip was then sealed on top of the SDS gel using a 1% agarose solution. SDS-PAGE was performed at 25 °C at 2.5 W per gel for the first 30 min, followed by 19 W per gel for ~4.5 h. Gels were fixed for 1 h in 10% acetic acid containing 40% ethanol and stained in the dark at room temperature for at least 16 h with 250 ml per gel of SYPRO Ruby stain (Molecular Probes, Eugene, OR). Gels were destained in 7% acetic acid containing 10% methanol for 1 h and scanned (see below). After scanning and prior to spot excision, the gels were stored in 10–20% ethanol at 4 °C.

Image Analysis—SYPRO Ruby-stained gels were scanned with a FluorImager 595 instrument (Amersham Biosciences Corp.) using an excitation wavelength of 488 nm and an emission band-pass filter of 610 nm. Gel images were analyzed using ImageMaster 2D software, version 2002.01 (Amersham Bioscience Corp.). Protein spots were detected by automatic detection combined with extensive manual editing. Spots from each gel were matched to the spots on an artificial reference gel, first by planting seed matches, followed by automatic matching with visual inspection and evaluation of the matches produced. The reference gel was originally based on the image of one of the experimental gels, chosen arbitrarily. Additional protein spots not present on that gel were subsequently added to the reference gel from the other gels. After background subtraction, the spot volumes (spot areas multiplied by stain intensity) on each gel were normalized as a percentage of the total volume of those spots that were present on all gels. After this step, data were exported from ImageMaster 2D program to Excel and then imported to GeneSpring 6.1 software (Silicon Genetics, Redwood City, CA) for further analysis. Because GeneSpring is designed for analysis of gene expression data, the reference spot numbers for 2D gel spots were entered into the program as "gene identifiers," and individual normalized spot volumes were assigned as "signal" parameters. Normalized volumes were adjusted to 0.010 for all spots that were apparently absent or whose measured normalized volume was <0.010. Mean normalized volumes were calculated for spots within each of the three groups (untreated males (n = 9 gels), females (n = 5 gels), and GH-treated males (n = 5 gels)) and then compared between groups in a pair-wise fashion. Mean normalized spot volumes were also calculated for the two subgroups of untreated male nuclear extracts: males with high liver STAT5 activity (5 gels) and males with low liver STAT5 activity (4 gels). A threshold for the differences in mean normalized volumes of spots between groups was set at 1.5-fold. Gel groups were compared with respect to the statistical significance of spot volume differences using a Student's t test. Changes with $p < 0.05$ were considered to be statistically significant.

Determination of Protein pI Values and Molecular Masses—Protein pI values were calculated based on the pH gradient distribution within the Immobiline DryStrips published by the manufacturer. Molecular masses were calculated based on the migration of low molecular mass range protein markers (Bio-Rad, Hercules, CA) run on select second-dimension gels.

Identification of 2D Gel-separated Proteins—Protein spots to be identified were excised from the 2D gels using an Ettan DALT Spot Picker (Amersham Biosciences Corp.). Gel plugs containing the same protein spot were pooled from up to four gels and submitted for LC-MS/MS analysis to the UVIC-Genome BC Proteomics Centre (Victoria, BC, Canada). Samples were subjected to in-gel digestion for 4 h at 37 °C with 400 ng of trypsin per sample (Promega, Ann Arbor, MI) followed by reverse-phase chromatography with on-line MS using an Applied Biosystems/MDS Sciex Qstar LC-MS/MS Q-TOF mass spectrometer. Chromatography was performed on a PepMap100 nanocolumn (75 μm inner diameter × 15 cm, C18, 3 μm, 100 Å pore size; LC Packings/Dionex, Amsterdam, The Netherlands) with a gradient of 0–80% buffer B (80% ACN/0.1% formic acid in water) in buffer A (0.1% formic acid in water) over a period of 35 min. A mass spectrum in full scan mode was followed by four MS/MS spectra of the most abundant ions. MS data were analyzed using either MASCOT v. 1.9 (Matrix Science, London, United Kingdom) or ProID 1.0 ePack 2 software (Applied Biosystems, Foster City, CA). In the case of one protein (identified as heterogeneous nuclear ribonucleoprotein (hnRNP) D-like), data were analyzed with the SEQUEST software (Thermo Finnigan, San Jose, CA). ProID software searches against NCBI nonredundant database (March 15, 2003), with either no species specified or *Rattus norvegicus* specified, used the following parameters: one missed cleavage; a tolerance of 0.15 Da for both peptide and fragment ions; and carbamidomethyl cysteine and oxidized methionine as variable modifications. The results were written to a Microsoft Access database that was then queried with a confidence cut off of 50 and a score cut off of 15. For searches with MASCOT software, MASCOT script for Analyst was used to generate the peak list, and the charge state was determined from the survey scan. MS and MS/MS spectra were centroided at a 50% peak height

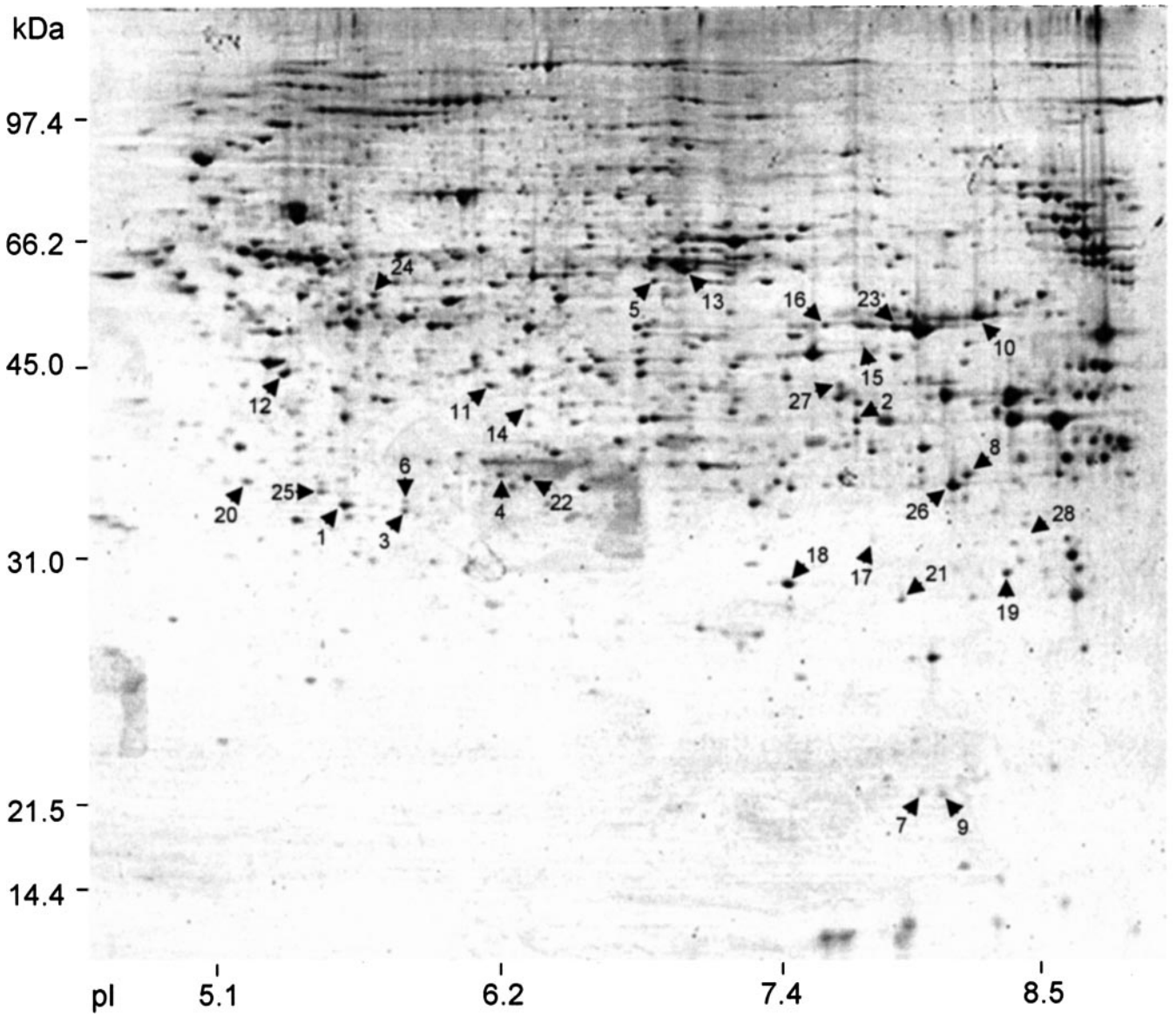

FIG. 1. **Resolution of rat liver nuclear extract proteins on a 2D gel stained with SYPRO Ruby.** Male liver nuclear extract proteins (115 μg) were focused on a pH 3–10 IPG strip and then separated on a 12.5% SDS-PAGE gel, stained, and visualized as described under "Experimental Procedures." Protein spots identified by LC-MS/MS (*arrowheads*) are marked by their spot numbers (c.f., Table III).

and a merge distance of 0.1 or 2 amu, respectively. The parameters for MASCOT searches against the MSDB database (with either no species specified or *R. norvegicus* specified) were as follows: one missed cleavage; carbamidomethyl cysteine and oxidized methionine as variable modifications; and 0.5- and 0.3-Da mass tolerance allowed for peptide and fragment ions, respectively.

RESULTS

Liver nuclear extracts prepared from individual male and female rats were analyzed by 2D gel electrophoresis to identify nuclear proteins whose abundance or state of post-translational modification displays sex-dependent differences. To determine the role of GH in establishing these sex differences, we also analyzed liver nuclear extracts prepared from male rats given GH as a 7-day continuous infusion. This treatment mimics the near-continuous plasma GH levels that are characteristic of adult female rats and feminizes the expression of GH-dependent liver proteins in males. A threshold of 1.5-fold was set to evaluate changes in spot abundance between liver groups.

A representative SYPRO Ruby-stained 2D gel of male rat liver nuclear extract is shown in Fig. 1. Between 900 and 1,200 spots were resolved on each gel. Comparison of male and female liver nuclear samples revealed 165 spots that exhibited $>$1.5-fold differences in abundance at $p < 0.05$. One hundred eight of the 165 sexually dimorphic spots were elevated in males compared with females (male-dominant spots), and 57 spots were female-dominant. The female-to-male normalized volume ratio (fold-difference in expression) ranged from 0.02

TABLE I

Summary of 165 nuclear protein spots showing sex-dependent patterns of expression in male rat liver

Protein spots exhibiting >1.5-fold difference in abundance between untreated males and females at $p < 0.05$ were grouped according to their male or female dominance and their response to continuous GH treatment of males. Protein spots that were induced or suppressed ≥1.5-fold by GH at $p < 0.05$ are listed as a subset of the total number of spots in each GH response group. Values listed are numbers of spots in each category.

	Response to continuous GH treatment				
	Induction (no. of spots)		Suppression (no. of spots)		No change (no. of spots)
	Total no. of spots	$p < 0.05$	Total no. of spots	$p < 0.05$	
Male-dominant (108 spots)	4	0	45	20	59
Female-dominant (57 spots)	15	8	2	0	40

TABLE II

Summary of 130 nuclear protein spots showing responsiveness to continuous GH treatment in male rat liver

Protein spots exhibiting >1.5-fold difference in abundance between untreated and GH-treated males at $p < 0.05$ were grouped according to their response to continuous GH treatment of males and according to their sex dependence. Protein spots that were male- or female-dominant at $p < 0.05$ are listed as a subset of the total number of spots in each group.

	Expression in untreated rats				
	Male-dominant (no. of spots)		Female-dominant (no. of spots)		Nonsex specific (no. of spots)
	Total # spots	$p < 0.05$	Total # spots	$p < 0.05$	
Down-regulated by GH (67 spots)	34	20	1	0	32
Up-regulated by GH (63 spots)	4	0	21	8	38

to 21 for these 165 spots. Comparison with GH-treated male gels revealed that a substantial fraction of the sexually dimorphic spots—66 of 165 spots (40%)—were responsive to continuous GH treatment (greater than 1.5-fold change in abundance between untreated males and GH-treated males; Table I). Sixty of these 66 sexually dimorphic, GH-regulated spots (or 36% of the 165 sexually dimorphic spots) exhibited female-like changes in abundance, with 15 female-dominant spots induced and 45 male-dominant spots suppressed by continuous GH treatment. For 28 of the 60 spots (8 female-dominant and 20 male-dominant), the GH-induced changes were significant at $p < 0.05$ (Table I). The preponderance of female-like responses exhibited by these 60 sexually dimorphic protein spots (*i.e.* GH induction of female-dominant spots and GH suppression of male-dominant spots) demonstrates that the continuous GH treatment is a feminizing factor that regulates the sex-dependent expression of these proteins in rat liver. Four male-dominant spots were induced and two female-dominant spots were suppressed by continuous GH; however, none of these GH-induced changes was statistically significant. The sex-dependent pattern of expression of these six protein spots, and that of the 59 male-dominant and 40 female-dominant spots that did not respond to continuous GH treatment (Table I), may be governed by regulatory factors other than GH.

Comparison of untreated male and continuous GH-treated male nuclear extracts revealed 130 protein spots that were responsive to continuous GH-treatment (>1.5-fold change in abundance between the two groups) at $p < 0.05$. Sixty-seven of these spots were down-regulated and 63 were up-regulated in the GH-treated males (Table II). The fold-change in spot abundance (male + GH:male normalized volume ratio) ranged from 0.07 (protein suppressed by GH) to 6.4 (protein induced by GH). Sixty of the 130 GH-responsive spots (46%) displayed sexually dimorphic expression patterns. For 55 of the 60 spots, the response to continuous GH treatment in males matched the gender-dependent pattern of expression. Thus, 34 spots down-regulated by continuous GH treatment were also down-regulated in females as compared with males, and 21 spot up-regulated in the GH-treated males were more abundant in females than in males. GH thus plays a dominant role in establishing sex-dependent differences in the nuclear protein profile.

The identity of 28 nuclear protein spots was established by nanoLC-MS/MS (Table III). Mean normalized spot volumes, their ratios, and the *p* values for a Student's *t* test are shown in Table III and the 2D spot mobilities are shown in Fig. 1. Male-dominant spots chosen for identification were present on all nine untreated male gels, but could be absent from any number of the female gels. Similarly, the female-dominant spots identified were present on all five female gels. Of the 28 spots identified, 21 were down-regulated and 7 were up-regulated in females compared with untreated males. The sex-dependent differences in abundance surpassed the significance test for all but two protein spots (hnRNP K and glutamate dehydrogenase; Table III). Seventeen of the 28 spots were responsive to GH, as they exhibited female-like changes in abundance in the continuous GH-treated males (*i.e.* either induction or suppression; Table III). For 13 of these spots, the GH-induced changes were statistically significant.

TABLE III

Sexually dimorphic rat liver nuclear proteins identified by LC-MS/MS

Sex differences and GH responses that are statistically significant ($p < 0.05$) are shown in bold. Ratios listed are the ratios of the mean normalized spot volumes for the indicated groups. *M*, untreated males; *F*, females; *M+GH*, GH-treated males. Also listed are mean normalized spot volumes with standard deviation for each group and the *p* values for a Student's *t*-test comparing the mean normalized spot volumes of the indicated groups.

	Spot no.	Protein name	F/M ratio	M+GH/M ratio	Male spot volume (mean ± S.D.)	Female spot volume (mean ± S.D.)	Male + GH spot volume (mean ± S.D.)	F *vs* M *p* value	M+GH *vs* M *p* value
Sexually dimorphic and GH-regulated proteins	1	Estrogen sulfotransferase	**0.02**	**0.08**	0.468 ± 0.131	0.010 ± 0.000	0.036 ± 0.032	0.000	0.000
	2	Hydroxyacid oxidase	**0.05**	**0.07**	0.186 ± 0.078	0.010 ± 0.000	0.014 ± 0.005	0.000	0.000
	3	Estrogen sulfotransferase	**0.08**	**0.08**	0.131 ± 0.065	0.010 ± 0.000	0.010 ± 0.000	0.001	0.001
	4	*N*-hydroxyarylamine sulfotransferase	**0.13**	**0.42**	0.145 ± 0.065	0.019 ± 0.009	0.061 ± 0.048	0.001	0.028
	5	Cysteine sulfinic acid decarboxylase	**0.15**	**0.24**	0.100 ± 0.068	0.015 ± 0.007	0.024 ± 0.008	0.019	0.031
	6	Estrogen sulfotransferase	**0.17**	**0.17**	0.060 ± 0.029	0.010 ± 0.000	0.010 ± 0.000	0.003	0.003
	7	Peptidylprolyl isomerase A	**0.19**	0.30	0.111 ± 0.078	0.021 ± 0.016	0.034 ± 0.024	0.027	0.055
	8	Phytanoyl-CoA dioxygenase	**0.21**	**0.13**	0.424 ± 0.162	0.087 ± 0.086	0.053 ± 0.018	0.001	0.000
	9	Peptidylprolyl isomerase A	**0.21**	0.34	0.147 ± 0.105	0.031 ± 0.040	0.051 ± 0.014	0.037	0.067
	10	CMP-*N*-acetylneuraminic acid synthetase	**0.39**	**0.59**	1.388 ± 0.407	0.546 ± 0.554	0.815 ± 0.157	0.007	0.011
	11	hnRNP K	0.48	**0.30**	0.180 ± 0.080	0.086 ± 0.073	0.054 ± 0.031	0.052	0.006
	12	NF45	**0.49**	0.64	0.341 ± 0.135	0.166 ± 0.134	0.217 ± 0.112	0.038	0.107
	13	Glutamate dehydrogenase	1.93	**2.51**	0.428 ± 0.137	0.825 ± 0.741	1.073 ± 0.476	0.133	0.002
	14	hnRNP D-like	**3.08**	1.58	0.056 ± 0.021	0.173 ± 0.019	0.089 ± 0.056	0.000	0.133
	15	Glutaryl-CoA dehydrogenase	**3.33**	**2.94**	0.022 ± 0.006	0.073 ± 0.025	0.064 ± 0.027	0.000	0.001
	16	Betaine homocysteine methyltransferase	**4.65**	**2.88**	0.167 ± 0.088	0.776 ± 0.766	0.481 ± 0.340	0.031	0.020
	17	Alcohol sulfotransferase A	**20.96**	**6.36**	0.010 ± 0.000	0.210 ± 0.180	0.064 ± 0.058	0.005	0.014
Sexually dimorphic but not GH-regulated proteins	18	GST Yb2	**0.10**	0.80	0.390 ± 0.227	0.038 ± 0.058	0.311 ± 0.174	0.006	0.512
	19	GST Yb1	**0.12**	0.72	0.300 ± 0.142	0.035 ± 0.039	0.215 ± 0.140	0.002	0.303
	20	Regucalcin	**0.15**	0.71	0.222 ± 0.104	0.033 ± 0.018	0.159 ± 0.117	0.002	0.314
	21	GST Yrs-Yrs (GST θ)	**0.22**	0.71	0.093 ± 0.058	0.020 ± 0.014	0.067 ± 0.052	0.019	0.411
	22	Glycerol-3-phosphate dehydrogenase	**0.26**	0.84	0.175 ± 0.110	0.046 ± 0.032	0.147 ± 0.161	0.027	0.704
	23	Betaine homocysteine methyltransferase	**0.30**	0.77	0.725 ± 0.150	0.216 ± 0.207	0.558 ± 0.345	0.000	0.224
	24	Fibrinogen γ chain precursor	**0.31**	1.03	0.145 ± 0.045	0.044 ± 0.017	0.148 ± 0.067	0.001	0.904
	25	Hydroxyanthranilate dioxygenase	**0.32**	1.18	0.139 ± 0.054	0.045 ± 0.053	0.165 ± 0.134	0.008	0.615
	26	L-lactate dehydrogenase	**0.38**	0.94	0.990 ± 0.217	0.380 ± 0.249	0.933 ± 0.234	0.000	0.651
	27	hnRNP A3	**1.67**	1.34	0.139 ± 0.066	0.232 ± 0.056	0.186 ± 0.084	0.021	0.265
	28	Alcohol sulfotransferase	**11.70**	1.00	0.010 ± 0.000	0.117 ± 0.061	0.010 ± 0.000	0.000	N/A[a]

[a] N/A, not applicable; the *p* value cannot be calculated because all normalized spot volumes in each group (males and GH-treated males) had an identical value of 0.010.

Eleven of the 28 spots were not responsive to continuous GH treatment (spots 18–28; Table III). A summary of the proteins identified (accession numbers, number of peptides identified, sequence coverage, and theoretical and experimental pI and mass values) is presented in Table IV. Identified peptides and their characteristics are shown in a Supplemental Table. Fig. 2 presents portions of representative male, female, and GH-treated male 2D gels, with select sexually dimorphic protein spots identified by MS marked by arrows.

We also attempted to detect sexually dimorphic, GH-regulated protein spots whose abundance might correlate with the activity status of the transcription factor STAT5b. STAT5b (the major form of STAT5 in the liver) plays a key role in mediating sexually dimorphic effects of GH on liver gene transcription (25). Liver STAT5 activity directly correlates with the plasma GH levels in male rats, with nuclear STAT5 DNA-binding activity high at the time of each plasma GH pulse, and STAT5 activity low between GH pulses (24, 26). To detect sex-dependent, GH-regulated proteins whose nuclear abundance correlates with the occurrence of a male GH pulse, the nine untreated male gels were divided into two subgroups: males

TABLE IV

Summary of nuclear proteins identified by LC-MS/MS

Multiple entries for the same protein spot, including multiple values for sequence coverage and theoretical pI and M_r values, are listed when distinct protein database entries could not be distinguished based on the experimental MS data. Theoretical pI and M_r data were calculated based on the known protein sequence using the "calculate pI/MW" tool at www.expasy.ch. Additional information, including the sequences of identified peptides, observed ion masses, and the search scores, are available as supplemental data to the manuscript (http://www.mcponline.org).

	Spot no.	Accession no.[a]	Protein name[b]	Sequence coverage (%)[c]	Peptides identified (no.)[d]	pI		M_r, (kDa)	
						Theoretical	Experimental	Theoretical	Experimental
Sex-dependent and GH-regulated	17	P22789	Alcohol sulfotransferase A (ST-40)	35	11	8.03	7.96	33.1	31.9
	16	O09171	Betaine homocysteine *S*-methyltransferase	29	8	8.02	7.69	45.0	53.3
	10	Q8K2G7	CMP-*N*-acetylneuraminic acid synthetase (M)	15	6	8.37	8.36	48.0	54.5
	5	Q64611	Cysteine sulfinic acid decarboxylase	6	3	6.84	6.94	55.2	60.3
	1	P52844, P52845, P49889	Estrogen sulfotransferase[e]	8	2	5.78, 5.57	5.63	35.5, 35.4	35.7
	3	Q99ND5, P49890	Estrogen sulfotransferase[f]	15	5	5.77, 5.65	5.87	35.4, 35.3	35.5
	6	Q99ND5, P52844, P49890	Estrogen sulfotransferase[g]	21	7	5.77 5.78, 5.65	5.87	35.3–35.5	36.3
	13	P10860	Glutamate dehydrogenase, mitochondrial	14	6	8.05	7.08	61.4	61.8
	15	Q60759, Q6P8N6	Glutaryl-CoA dehydrogenase (M)	11	4	8.82, 8.98	7.86	48.6	51.0
	14	Q9Z130	hnRNP D-like (M)	28	11	6.85	6.42	33.6	44.5
	11	P61980	hnRNP K	15	6	5.39	6.24	51.0	46.8
	2	Q07523	Hydroxyacid oxidase 3	32	13	7.90	7.82	39.0	42.7
	4	A49098	*N*-hydroxyarylamine sulfotransferase	37	10	6.09	6.30	35.7	37.9
	12	Q12905	NF45 (H)	18	6	8.27	5.43	44.7	48.0
	7	CSRTA	Peptidylprolyl isomerase A	28	5	8.34	8.09	17.9	19.3
	9	CSRTA	Peptidylprolyl isomerase A	29	4	8.34	8.19	17.9	19.2
	8	AAF15971	Phytanoyl-CoA dioxygenase, peroxisomal	43	13	8.75	8.31	38.6	36.8
Sex-dependent but not GH-regulated	28	P15709	Alcohol sulfotransferase (ST-20)	18	6	8.59	8.52	33.0	31.6
	23	O09171	Betaine homocysteine *S*-methyltransferase	21	8	8.02	8.00	45.0	53.5
	24	P02680, FGRTGA, FGRTGB	Fibrinogen γ chain precursor[h]	16 16	7 7	5.39, 5.78, 5.56	5.79	50.6, 49.5, 50.5	58.7
	18	P08010	GST Yb2 (chain 4)	75	18	7.30	7.52	25.6	29.5
	19	P04905	GST Yb1 (chain 3)	58	13	8.42	8.48	25.8	29.4
	21	P30713	GST Yrs-Yrs (GST θ)	26	5	7.90	8.05	27.3	28.1
	22	O35077	Glycerol-3-phosphate dehydrogenase [NAD+]	26	9	6.34	6.38	37.2	37.9
	27	Q8BG05	hnRNP A3 (M)	21	7	9.10	7.74	39.6	45.8
	25	P46953	3-Hydroxyanthranilate 3,4-dioxygenase	25	7	5.57	5.53	32.6	36.4
	26	A23083	L-lactate dehydrogenase chain M	40	16	8.45	8.25	36.4	36.0
	20	Q925W3, Q03336	Regucalcin, senescence marker protein SMP30	29	8	5.27, 5.40	5.26	33.4	36.9

[a] Swiss-Prot or NCBI accession numbers.

[b] Database comparison to rat proteins, unless indicated otherwise: *M*, mouse; *H*, human.

[c] Percentage of the entire protein sequence represented in the peptides identified.

[d] Multiple forms of the same peptide were counted as a single peptide.

[e] The following estrogen sulfotransferases correspond to the accession numbers listed: isoform 1 (P52844), isoform 2 (P52845), and isoform 3 (P49889).

[f] The following sulfotransferases correspond to the accession numbers listed: estrogen sulfotransferase (Q99ND5) and estrogen sulfotransferase, isoform 6 (P49890).

[g] The following sulfotransferases correspond to the accession numbers listed: estrogen sulfotransferase (Q99ND5), estrogen sulfotransferase, isoform 1 (P52844), and isoform 6 (P49890).

[h] The following fibrinogen γ chain precursors correspond to the accession numbers listed: fibrinogen γ (P02680), fibrinogen γ-A (FGRTGA), and γ-B (FGRTGB) chain precursor.

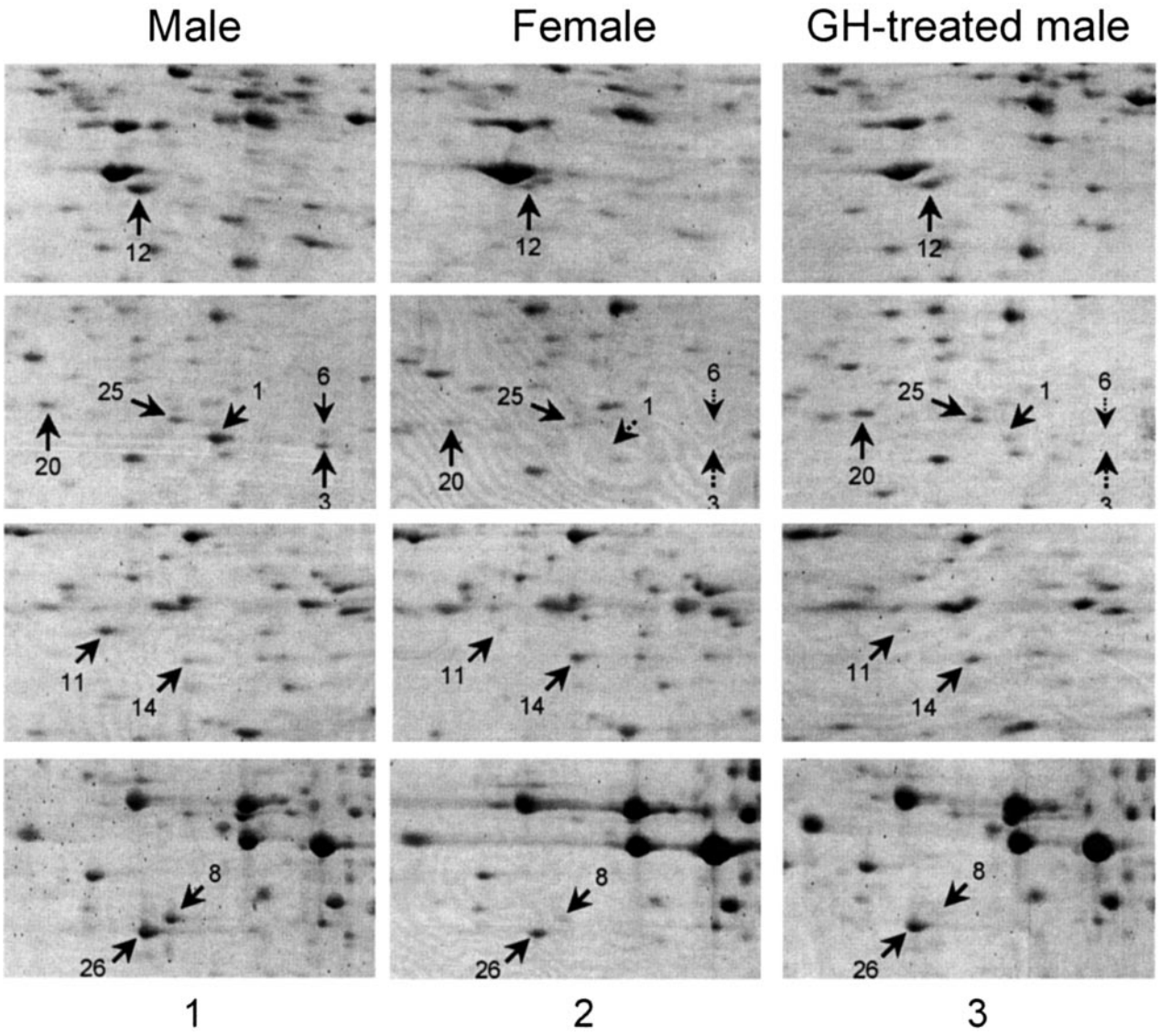

FIG. 2. **Portions of SYPRO Ruby-stained 2D gels highlighting select sex- and/or GH-regulated liver nuclear proteins.** Shown are four separate regions of individual 2D gels from each of three groups (male, female, GH-treated male liver nuclear extract samples in *columns 1–3*, respectively). Each row across corresponds to a separate 2D gel region (c.f., Fig. 1). The protein spots marked correspond to NF45 (spot 12), regucalcin (spot 20), 3-hydroxyanthranilate 3,4-dioxygenase (spot 25), various estrogen sulfotransferases (spots 1, 3, 6), hnRNP K (spot 11), hnRNP D-like (spot 14), L-lactate dehydrogenase (spot 26), and phytanoyl-CoA dioxygenase (spot 8). *Dashed arrows* indicate the positions of those spots that were not visible on the gels.

with high nuclear STAT5 activity (five gels) and males with low STAT5 activity (four gels). Mean normalized volumes were calculated for protein spots within each subgroup and compared with corresponding values in other groups. Comparison of the high and low STAT5 groups revealed 48 spots that exhibited >1.5 differences in abundance between the groups at $p < 0.05$ (low STAT5 to high STAT5 normalized spot volume ratio = 0.27–5.4). Thirteen of these spots were down-regulated and 35 were up-regulated in low STAT5 males compared with high STAT5 males.

Comparison of the 48 spots with the 28 spots differentially expressed at $p < 0.05$ between females and untreated males, and between GH-treated males and untreated males (all nine male samples) (Tables I and II), revealed no spots in common. Comparison of the 48 spots with 21 spots that differed in abundance (at $p < 0.05$) between females and high STAT5 males, and between GH-treated males and high STAT5 males, also revealed no spots in common. However, comparison of the 48 spots with 34 spots that were up- or down-regulated (at $p < 0.05$) in females and GH-treated males compared with low STAT5 males revealed seven spots in common. All seven spots were up-regulated in the low STAT5 males as compared with high STAT5 males, females, and GH-treated males. The identity of these spots was not established.

DISCUSSION

2D gel electrophoresis combined with LC-MS/MS was employed to identify rat liver nuclear proteins whose abundance or state of post-translational modification was sex-dependent and to determine the role of GH in establishing this dependence. This study complements recent DNA microarray analyses, which demonstrated that plasma GH profiles play a major role in establishing sex-dependent gene expression in rat liver (12).

Comparison of 2D gel images of nuclear extracts from untreated males and females revealed 165 protein spots that displayed a statistically significant sex-dependence. Thus, 14–18% of the 900–1200 nuclear protein spots resolved on these gels were sexually dimorphic in their abundance. Sixty of the 165 spots (36%) were GH-regulated and displayed female-like changes in expression following continuous GH treatment of male rats. This percentage is lower than the percentage of sex-dependent liver mRNAs that displayed GH regulation in microarray analysis in the same rat liver model (72 of 86 RNAs, or 84%) (12). When 2D gel images from GH-treated males and untreated male were compared, 130 spots exhibiting a statistically significant response to continuous GH treatment were detected (11–14% of the total nuclear spots). A substantial fraction of these spots (55 spots, or 42%) displayed sex-dependent patterns of expression that correlated with their response to the "feminizing" effects of continuous GH treatment. Taken together, these results indicate that GH plays an important role in establishing sex-dependent patterns of rat liver nuclear proteins. This role, however, is less prominent than that displayed by GH in establishing sexually dimorphic differences in liver mRNA expression (12). Because 60% (99 spots) of the sexually dimorphic protein spots were not responsive to GH treatment, there must be other factors, in addition to GH, that contribute to establishing and maintaining the sex-dependent patterns of liver nuclear proteins in the rat model. Moreover, 70 of the 130 GH-regulated nuclear proteins did not exhibit sex-specificity, indicating that a substantial subset of liver nuclear proteins can be regulated by GH in a manner that is independent of the sex-dependent effects that GH exerts on liver gene expression. This regulation may include GH signaling pathways that do not necessarily lead to changes in gene transcription, *e.g.* GH signaling leading to changes in protein phosphorylation mediated by mitogen-activated protein kinase or protein kinase C (27).

The identity of 28 protein spots was established by MS (Table III). Ten of these spots correspond to proteins with established nuclear localization. These include nuclear factor 45 (NF45) (28), cytidine monophospho-*N*-acetylneuraminic acid synthetase (29), and three hnRNPs, D-like (30), A3 (31), and K (32), all of which are primarily nuclear in their localization. hnRNPs are known to shuttle between the nucleus and the cytoplasm (33). Another identified protein, regucalcin, is found in both the nucleus and the cytoplasm (34). Nuclear localization has been also reported for several predominantly cytosolic proteins identified in the present study, L-lactate dehydrogenase (35, 36) and GSTs Yb1 and Yb2 (37), and for glutamate dehydrogenase, which is primarily found in the mitochondrial matrix (38, 39). Peptidylprolyl isomerase A (cyclophilin A), a cytosolic protein, has been found on 2D gel maps of nuclear extracts from human hepatoma HepG2 cells (18) and human lymphocytes (40). For several other proteins identified in the present study, nuclear localization was not reported previously. These proteins are associated with peroxisomes (hydroxyacid oxidase 3, phytanoyl-CoA dioxygenase), mitochondria (glutaryl-CoA dehydrogenase), the Golgi apparatus (fibrinogen), and the cytoplasm (betaine homocysteine *S*-methyltransferase, *N*-hydroxyarylamine sulfotransferase, cysteine sulfinic acid decarboxylase, 3-hydroxyanthranilate 3,4-dioxygenase, glycerol-3-phosphate dehydrogenase, alcohol sulfotranserases, and estrogen sulfotransferases). The presence of these proteins in liver nuclear extracts could perhaps be due to contamination of the sucrose gradient-purified nuclear preparations. It seems more likely, however, to represent *bona fide* nuclear localization, in view of the dynamic nature of the nucleus and its permeability to cytosolic proteins of lower molecular mass. Indeed, several of the latter proteins are monomeric and $<$40 kDa in molecular mass, a size small enough to gain entry to the nucleus even in the absence of a nuclear localization signal.

All 28 nuclear proteins identified in the present study were differentially expressed in untreated males as compared with females, the differences in abundance being statistically significant for all but two of the proteins (Table III). Sexually dimorphic hepatic expression at the mRNA or/and protein level has been demonstrated previously for several of these proteins. These include the male-predominant proteins *N*-hydroxyarylamine sulfotransferase (12, 41), hydroxyacid oxidase 3 (12), cysteine sulfinic acid decarboxylase (42), regucalcin (43), GSTs Yb1 and Yb2 (11, 44), and various estrogen sulfotransferases (12, 45), and the female-predominant alcohol sulfotransferases (12, 46–48). The sexually dimorphic pattern of expression of the other proteins identified in this study and listed in Table III is reported here for the first time.

Seventeen of the proteins identified were both GH-regulated and differentially expressed in untreated males *versus* females (Table III). GH regulation at the mRNA or/and protein level has been shown earlier for several of these proteins: hydroxyacid oxidase 3 (12), *N*-hydroxyarylamine sulfotransferase (12, 41), estrogen sulfotransferase (12, 45), and alcohol sulfotransferases (12, 48, 49). GH responsiveness is demonstrated here for the first time for phytanoyl-CoA dioxygenase, cysteine sulfinic acid decarboxylase, glutaryl-CoA dehydrogenase, peptidylprolyl isomerase, hnRNP K, hnRNP D-like, hnRNP A3, NF45, betaine homocysteine *S*-methyltransferase, and CMP-*N*-acetylneuraminic acid synthetase. Interestingly, GH has been shown to regulate the expression of alcohol

sulfotransferase at the level of gene expression (12, 48) and the expression of GSTs Yb1 and Yb2 at the gene and protein level (11, 44). In the present study, however, these proteins did not display changes in nuclear intensity in response to GH treatment. This finding raises the possibility that GH may differentially regulate protein *versus* mRNA levels (alcohol sulfotransferase) and/or nuclear *versus* cytoplasmic protein abundance (GSTs).

Interestingly, two 2D gel spots identified as the same protein (betaine homocysteine *S*-methyltransferase) exhibited different patterns of regulation: spot 16 was up-regulated both in females and GH-treated males as compared with untreated males, while spot 23 was more abundant in untreated males than in females and did not respond to continuous GH (Table III). This suggests that betaine homocysteine *S*-methyltransferase is post-translationally modified in a sex-dependent manner.

In contrast to proteins such as hydroxyacid oxidase 3, *N*-hydroxyarylamine sulfotransferase, and estrogen sulfotransferase, which were found to be sexually dimorphic and GH-regulated both in the present study and in our recent DNA microarray study (12), several of the proteins identified in the present study were not identified as sexually dimorphic or GH-responsive at the mRNA level in our earlier DNA microarray analysis (12). These proteins include phytanoyl-CoA dioxygenase, GST Yrs-Yrs (GST θ), and glycerol-3-phosphate dehydrogenase. Explanations for this apparent discrepancy include the high likelihood of false negatives in the microarray analysis and the possibility that GH may have effects on protein localization or post-translational modification, which would not be seen in an analysis of mRNA levels.

The majority of the proteins identified herein are enzymes. Nuclear function has been reported for L-lactate dehydrogenase, which functions as a single-stranded DNA binding protein in the nucleus (35). Another enzyme, glutamate dehydrogenase, displays an RNA-binding activity of unknown physiological relevance (50). The three hnRNPs identified (A3, D-like, and K) bind pre-mRNA or both pre-mRNA and DNA (hnRNP K) and regulate diverse cellular processes, including chromatin remodeling, transcription, RNA splicing, mRNA transport, translation, and turnover (51). NF45, a double-stranded RNA binding protein, has been reported to stimulate gene expression in mammalian cells (52). Regulcacin is a regulatory protein of Ca^{2+}-dependent signaling that can inhibit protein kinase and phosphatase activity (53–56). Of all the proteins identified, NF45 and the three hnRNPs are potentially of greatest interest, insofar as they are sexually dimorphic, GH-regulated nuclear proteins with the ability to regulate gene transcription and/or translation, and conceivably could contribute to the GH-dependent regulation of gene expression seen in liver. In particular, hnRNP K acts as a docking platform that integrates signaling cascades and responds to a variety of growth factors by induced phosphorylation on tyrosine and serine, which modulates its DNA- and RNA-binding and other activities (57–59).

None of the sexually dimorphic or GH-regulated proteins identified in this study are transcription factors. The fact that the transcription factor STAT5b was not identified as a regulated liver nuclear protein was especially surprising, given that tyrosine-phosphorylated STAT5b protein levels differ markedly between females and males (24), a finding that was verified for the individual liver nuclear extracts analyzed on our 2D gels by Western blotting and EMSA (data not shown). One possible explanation is that the concentration of STAT5b in the liver nuclear extracts was too low for detection by the SYPRO Ruby protein stain. Indeed, four well-resolved spots corresponding to the variously phosphorylated forms of STAT5b were readily detected by STAT5b Western blotting of a 2D gel of a male nuclear sample with high STAT5 activity. These spots were not, however, detectable by SYPRO Ruby staining, indicating that the abundance in nuclear extracts of STAT5b protein, and probably that of many other transcription factors (18), is too low for detection by this method. Further enrichment of the nuclear extract with respect to transcription factors and low-abundance DNA-binding proteins may be useful in this regard.

In conclusion, the present analysis of rat liver nuclear extracts has shown that GH plays an important role in establishing sex-dependent patterns of hepatic nuclear proteins. This role of GH in nuclear protein regulation is somewhat less prominent than the role that GH plays in the regulation of sexually dimorphic expression of rat liver mRNAs. This latter finding is not unexpected, given the large number of post-transcriptional factors, including protein stability, post-translational modifications, and intracellular localization, all of which may contribute to sex differences in the nuclear proteomic profile.

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