In vivo transfection of rat liver discloses binding sites conveying GH-dependent and female-specific gene expression

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Abstract
The sexually dimorphic mode of GH secretion leads to a sex-differentiated expression of many hepatic target genes. Expression of the a1bg gene in rat liver is specifically induced by the female pattern of GH secretion. In this study, we have used the a1bg promoter in in vivo transfection experiments to investigate molecular mechanisms of GH-mediated female-specific hepatic gene regulation. Rat liver transfection was achieved by rapid tail vein injection of large volumes of plasmid solution. Expression of reporter constructs showed that the 160 bp proximal part of the a1bg promoter contained elements directing sex-specific expression. In vitro footprinting analysis and electromobility shift assays identified binding of hepatic nuclear factor 6 (HNF6), signal transducer and activator of transcriptions (Stat5) and nuclear factor 1 (NF1) in liver nuclear extracts to the 160 bp proximal promoter. Transfection of mutated and/or deletion constructs showed that HNF6 and NF1 binding markedly enhanced expression in female livers, whereas Stat5 reduces the sex difference by enhancing expression more strongly in male than in female rat liver. Based on our present results, we propose that adjacent binding sites for NF1 and HNF6 constitute a gene regulatory unit of importance for transducing the female-specific effect of GH in rat liver.

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Introduction
Growth hormone (GH) has diverse physiological actions and gene-regulatory effects underlie many of the cellular effects of the hormone. GH secretion is sexually dimorphic in most species and this sex difference is particularly pronounced in rats (Eden 1979, Jaffe et al. 1998, 2002). The sexual difference in GH secretion starts to develop during the prepubertal period (25–30 days of age) and continues to mature during puberty. In male rats, GH is secreted in regular pulses every 3–4 h with undetectable levels in between peaks (Tannenbaum & Martin 1976). This differs from the more frequent pulses of low amplitude that occurs in females, which lead to a continuous presence of the hormone in plasma (Eden 1979). The sex-characteristic mode of GH secretion leads to a sex-differentiated expression of many hepatic target genes (Flores-Morales et al. 2001, Tolley-Egnell et al. 2001). CYP2C11 is the prototypical male-specific and GH-dependent gene expressed in rat liver and the JAK2–Stat5 signalling pathway has been identified as a transducer of the male-characteristic GH pattern (Waxman et al. 1995). Studies on the prototypical female-specific and GH-dependent rat liver gene, CYP2C12, have shown that Stat5, HNF6 and HNF4 are involved in the GH regulation, but the sexually dimorphic expression has not been possible to ascribe to these factors (Sasaki et al. 1999). It is evident that a multitude of signalling pathways, transcription factors and/or transcriptional coregulators are involved in GH-dependent sexual regulation of hepatic gene expression (Wiwi & Waxman 2004), but the sex-differentiating mechanisms remain an enigma. By utilizing suppressive subtractive hybridization (SSH), we have previously identified a number of sex-dependent rat liver genes induced by the female pattern of GH secretion (Gardmo et al. 2002). We found a1bg gene sequences to be more represented in the SSH assay than CYP2C12 sequences, which indicates a strong female expression of a1bg. In a subsequent study, we confirmed a female-specific and GH-dependent expression of the a1bg gene (Gardmo et al. 2001). The gene encodes a plasma protein, a1B-glycoprotein (A1BG), for which a function has remained elusive for a long time. However, A1BG was recently shown to bind cystein-rich secretory protein-3 (CRISP-3) with high affinity, and was suggested to protect against potentially toxic effects of CRISP-3 in the circulation (Udby et al. 2004). In this study, we have used the a1bg promoter to investigate molecular mechanisms of GH-mediated female-specific gene regulation.

Materials and methods

Animals and hormone therapy
Normal Sprague-Dawley rats, about 7 weeks of age, were obtained from BK Scanbur (Sollentuna, Sweden).
The rats had free access to standard laboratory chow and water and were maintained under standardized conditions of temperature and light. Normal male rats were ‘feminized’ with respect to the pattern of GH exposure by continuous infusion of bovine GH (bGH) for 6 days. The animals received 0.5 mg bGH/kg per day by means of Alzet 2001 minipumps (Alza Corp., Palo Alto, CA, USA) implanted subcutaneously. Recombinant bGH was a generous gift from American Cyanamid Co. (Princeton, NJ, USA). The animal experiments were conducted in accordance with humane care and approved by the Stockholm South Ethical Committee of the Swedish National Board of Animal Experiments.

Bioinformatics analysis

The database search for putative binding sites for transcription factors was made using MatInspector (www.genomatix.de) and Patch (www.gene-regulation.com).

Reporter constructs

Genome walking (Rat GenomeWalker Kit, Clontech) was used to clone 2.3 kb of the 5’ flanking region of the rat a1bg gene into a T/A vector (Advantage PCR Cloning Kit, Clontech). The following gene-specific primers, GSP1-ACCAGTGTGAGGTGGCCAGGTGTCGA and GSP2-GAGGCTCTGGCCGGTAGTTCAAGGTCCACT, were used. Luciferase constructs were generated by PCR’s followed by subcloning into the BglII and MluI sites of the pGL3-Basic vector (Promega, Madison, WI, USA). To obtain the 2.3 kb-Luc, 1 kb-Luc and 160 bp-Luc constructs the 5’ primer tggtgacgctAT-TAGGATTTTTTAGTTCTG (BglII) and the 5’ 0 0 primer gcctttcgtagatctGGTGGGTCTTCAGTCCAGCA, ttggtgacgctCTGCAATTTTGATTATTCTTAT and ttgc-gccagcgtGACAGGATATAATTACACAT respectively, were used. Nucleotides included for cloning purposes are indicated by lowercase letters. The Transformer Site-Directed Mutagenesis Kit (Clontech), was used to mutate the distal Stat5 site in the 2.3 kb-Luc construct, mut5Stat5-Luc. The 5’ phosphorylated oligonucleotides were GCCCCTACCTTaCAGtAAGGGCAAACAAA for mutation of the 5’Stat5 site and TAAAATCGATA- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACACA; (KAACTGTTCATTACTTTCTTTCAGGACAGGATATAAT- TACAC.
gel. The following oligonucleotides were used: HNF6, CACATTATTAGTATGACATTAT; HNF6mut, CACATTAttatTTGACATTAT; Stat5, ACCTTCCAGGAAG; Stat5mut, ACCTTACGATTACGCGTCCAGTGGTGT; –116/–89, TGCTGATCTCTCGTGAGGCGCTCCCGAGCTGTTCGAGCTGGT; –116/–107, TGCTGATCTCTAGCTGTCCGATGCCAAGTAGTTGT; –103/–89, CTGTCCGCACTGCCCAGTATGTTGT. Mutated bases are indicated by lowercase letters. In the presence of 1 μg poly(dI-dC) in binding buffer (20 mM Heps pH 7.9, 4% Ficoll, 0.2 mM EDTA, 0.5mM TTTT and 150 mM (HNF6), 90 mM (Stat5) or 200 mM (–116/–89) KCl), 2 (HNF6, –116/–89) or 15 μg (Stat5) nuclear protein extract was incubated with the probe for 30 min. For supershift analysis, the antibody (Stat5) nuclear protein extract was incubated with the unlabelled probe for 10 min before the labelled probe, while for competition the unlabelled probe for 30 min. For supershift analysis, the antibody (Stat5) nuclear protein extract was incubated with the unlabelled probe for 10 min before the labelled probe, while for competition the unlabelled probe for 30 min. The labelled probe was visualized on a 6% sequencing gel alongside with sequencing reactions. The gels were dried on Whatmann paper and exposed to X-ray film. The sequencing reactions were made with Sequenase version 2.0 DNA polymerase and T7 Sequencing mixes, both from USB Corp (Cleveland, OH, USA), together with the end-labelled primer used for the PCR amplification of the footprint template.

### Statistical analysis

Data are the mean ± S.E.M. Comparisons between groups were made by Student’s t-test or, when appropriate, one-way ANOVA followed by Newman–Keuls’ test. *P* < 0.05 was considered significant.

### Results

To render studies on GH-dependent and sex-specific mechanisms possible, we set out to establish an in vivo system with a promoter directing female-specific expression in rat hepatocytes. A 2.3-kb fragment of the a1bg promoter was cloned by the use of `genome walking` and fused to a luciferase reporter, 2.3 kb-Luc. To examine whether this part of the promoter conveyed the sexually dimorphic expression in rat liver, female and male rats were transfected in vivo by rapid tail vein injection. As shown in Fig. 1, generated luciferase activity was 3.5 times higher in female than in male rat livers. That sexually differentiated expression of 2.3-kb-Luc was due to the female pattern of GH secretion was corroborated by the female level of expression of the construct in ‘feminized’ male rats, i.e. male rats continuously treated with GH to mimic the female pattern of GH exposure. Thus, the a1bg promoter and rapid tail vein injection for transfection

![Figure 1](https://example.com/figure1.png)
of hepatocytes in vivo can be used to investigate molecular mechanisms whereby GH induces female-specific expression of a gene.

Next, we analysed truncated constructs in order to map sex-responsive regions. The 1 kb-Luc construct was much less expressed in females than 2.3 kb-Luc and the expression was not significantly different between the sexes. Transfection of 160 bp-Luc resulted in luciferase activity comparable with the activity of 2.3 kb-Luc in females and, surprisingly, a significant 2.7-fold sex difference (Fig. 1). This indicated the presence of a negative regulatory element in the −1 kb to −160 bp region, which was supported by the higher expression of (−1 kb/160 bp)del-Luc than of 1 kb-Luc. These results show that both the 2.3 kb and the 160 bp proximal parts of the a1bg promoter direct sex-specific expression of the reporter gene, and that a negative regulatory element resides in the −1 kb to −160 bp region.

Computer analysis of the 2.3 kb a1bg promoter fragment revealed two putative Stat5 sites and one HNF6/HNF3 binding site at −2077/−2069, −69/−61 and −137/−128 respectively (Fig. 2A). As mentioned earlier, corresponding sites are involved in GH-mediated expression of CYP2C12 (Sasaki et al. 1999). Mutation of the proximal Stat5 site (3’Stat5) and/or the HNF6/HNF3 site led to reduced expression in in vivo transfected females, whereas mutation of the distal Stat5 site (5’Stat5) had no effect (Fig. 2B). In male rats, expression of 2.3 kb-Luc was not affected by the HNF6/HNF3 mutation (mutHNF6-Luc), whereas mutation of 3’Stat element (mut3’Stat5-Luc) reduced expression. The expression of these two constructs was sex-differentiated, higher in females than in males. Expression of mut3’Stat5HNF6-Luc, with both sites mutated, appeared sex-differentiated, but the difference did not reach statistical significance, P = 0.077. To this end, we conclude that the GH-dependent sexually dimorphic expression conveyed by the 2.3 kb a1bg promoter is enhanced by the HNF6/HNF3 site and, if anything, reduced by the proximal Stat5 site in that the impact of the 3’Stat5 mutation was more pronounced in males.

Next, we verified the binding of Stat5 and HNF6 to the respective site by electromobility shift analysis (EMSA) using female-derived liver nuclear extracts. As shown in Fig. 3A, Stat5 bound to the a1bg proximal Stat5 site, 3’Stat5 and the mutated oligonucleotide was unable to compete for the binding. Similarly, HNF6 bound to the a1bg HNF6 oligonucleotide, but in this case, the mutated oligonucleotide was able to compete for binding when added in large excess (Fig. 3B, lanes 5 and 6). However, as shown in Fig. 3C, the HNF6 binding capacity of the mutated oligonucleotide was clearly reduced. A 20 molar excess of the mutated oligonucleotide had only a marginal effect on the binding of HNF6 (Fig. 3C, lane 6), whereas a 20 molar excess of unlabelled probe (Fig. 3C, lane 3) completely abolished binding. Supershift analysis with an HNF6 antibody revealed a complex with a slightly lower mobility than the HNF6 complex (Fig. 3B, lane 7). By extending the electrophoresis run and including nuclear extract from hypophysectomized rats, devoid of GH and thereby lacking HNF6 (Lahuna et al. 1997), the two different complexes were clearly visualized. The complex with the lower mobility is most probably due to the binding of HNF3, in analogy with what was shown by Lahuna et al. for the CYP2C12 HNF6 binding site; HNF3 can bind to the site in the absence of HNF6 (Lahuna et al. 1997). To summarize the EMSA results, Stat5 and HNF6 could bind to their respective site in the a1bg promoter in vitro, and the mutations introduced in respective site abolished binding of the corresponding factor.

Figure 2 Liver expression of a1bg-luciferase constructs. (A) Stat5 and HNF6 consensus sequences and corresponding sites in the 2.3 kb a1bg promoter alongside with the used mutations. (B) Female (black bars) and male (open bars) rats were injected in the tail vein with 50 μg mutated and/or deleted 2.3 kb a1bg-Luc construct and 50 μg pRL-TK in saline. The animals were killed 20 h after injection and liver samples were assayed for dual luciferase activities. The data are expressed as normalized luciferase activities and represent the mean ± S.E.M. of the number of animals given within parentheses. The animals injected with the 2.3 kb-Luc construct are from Fig. 1. Significant differences, P < 0.05, are indicated by different superscripts within one sex and by an asterisk between females and males.
Since mutation of the HNF6 site reduced but did not abolished the sex-difference, in vitro footprinting analysis of the 160 bp promoter region was carried out to further analyse the binding of liver nuclear proteins to this part. Three different regions were clearly protected from DNase I digestion (Fig. 4A). The one located at −140/−124 corresponds to the binding site for HNF6 (−137/−128). The other two were located at −116/−107 and −103/−89, separated from one another by only three basepairs. A weak footprint in the area of the 3′Stat5 site was indicated and was more or less pronounced in different experiments using different nuclear extracts (data not shown). To examine the functional relevance of the −116/−89 region, a deletion of this region in 2.3 kb-Luc was made, (−116/−89)del-Luc, which was injected into female and male rats. As shown in Fig. 4B, the generated Luciferase activities were comparable with those generated from the mutHNF6-Luc construct. Likewise, in comparison with expression of 2.3 kb-Luc, (−116/−89)del-Luc expression levels were reduced in female rats but similar in males. These results indicate that in addition to HNF6, a factor(s) binding to the −116/−89 region contributes to the female-specific expression of aHbg. We then examined the expression of a −116/−89 deletion construct in which also the HNF6 site was mutated, (−116/−89)delmutHNF6-Luc, and to our surprise, the generated luciferase activities were reduced in both sexes (Fig. 4B). This is in contrast to that mutation/deletion of the sites separately only affected the expression in female livers. Deletion of the −116/−89 region in combination with mutation of both the HNF6 and the 3′Stat5 elements, (−116/−89)delmut3′-Stat5HNF6-Luc, generated luciferase activities that were similar to the expression of (−116/−89)delmutHNF6-Luc (Fig. 4B). Taken together, these results indicate that the −116/−89 region contains a site(s) of importance for the GH-dependent and female-specific expression of the aHbg gene, and that the impact of this region together with the HNF6 site is more complex than mere enhancement of the expression in females.

In an attempt to identify factors binding to the −116/−89 region, bioinformatics was used to search for potential binding sites. No similarity to known mammalian binding sites was revealed at the −116/−107 site, while the −103/−89 site harboured potential binding sites for several transcription factors, such as NFI, Oct-1 and Oct-2, C/EBP-α and C/EBP-β and activator protein-1 (AP-1). An oligonucleotide corresponding to both the footprints, −116/−89, was used in EMSA experiments with female-derived liver nuclear extracts and antibodies against the putative binding factors. As shown in Fig. 5, the NFI antibody attenuated formation of the specific complex(es). The Oct antibodies had marginal reducing effect on complex formation and none of the other antibodies used, directed against C/EBP-α C/EBP-β or c-Fos, affected the formed complex(es) (data not shown). We also sought to differentiate the binding of rat liver nuclear proteins to the −116/−89 probe by addition of 100 molar excess of unlabelled oligonucleotides corresponding to the −116/−107, −103/−89 or...
sequences. While an excess of unlabelled $-116/-89$ oligonucleotide completely abolished the protein binding, excess of the $-116/-107$ or $-103/-89$ oligonucleotide had little effect. By extension of the electrophoresis run at least three different bands could be discerned, suggesting formation of several complexes on the $-116/-89$ site, which involve NF1. We conclude that the $-116/-89$ region does not contain any known binding sites and NF1 can bind to the $-103/-89$ region, possibly in concert with Oct-1 and/or with other factors.

**Discussion**

*In vivo* transfection techniques are imperative for examining mechanisms whereby sex-specific GH secretion regulates gene transcription because sex-specific effects of the GH patterns on gene transcription are difficult to study *in vitro*, i.e. using primary hepatocytes in culture or hepatic-derived cell lines. Effects of the female-specific, continuous GH exposure can be studied *in vitro* in cultured primary hepatocytes, but the long-term effects of male-characteristic intermittent exposure to GH have, until recently (Thangavel et al. 2006), not been possible to obtain in that system.
Rapid tail vein injection of large volumes of plasmid-containing saline, generating a high hydrodynamic pressure in the liver, leads to transfection of hepatocytes (Liu et al. 1999, Zhang et al. 1999). Indeed, using the rapid tail vein injection technique, we could show that 2.3 kb of the 5′ flanking region of the a1bg gene-directed sex-specific hepatic expression that was dependent on the pattern of GH exposure.

The picture of sex-dependent a1bg gene regulatory regions that emerged from expression analysis of the deletion constructs of 2.3 kb-Luc is very complex (Fig. 1). The non-sexually dimorphic expression of 1 kb-Luc, due to mitigated expression in females, indicates that there are elements in the 2.3–1 kb region of importance for enhanced expression in females. The fact that expression of 160 bp-Luc was also sexually dimorphic indicates that negative elements reside in the area of 1 kb to 160 bp, which is supported by the higher expression of (1 kb/160 bp)del-Luc than of 1 kb-Luc in females. Moreover, this could indicate that the putative sex-dependent element(s) within −2.3 to −1 kb is of importance for female-specific expression ‘only’ in the context of (a) negative element(s) within −1 kb to −160 bp. Despite the indicated complexity, we focused on identifying elements in the 160 bp promoter region driving the sex-dependent expression.

The presence of consensus sites for Stat5 and HNF6 in the proximal 160 bp of a1bg, activated and induced by GH respectively was not surprising, since these sites have been shown to be involved in the regulation of the female-specific and GH-dependent CYP2C12 gene (Lahuna et al. 1997, Sasaki et al. 1999, Helander et al. 2002). In vitro footprinting confirmed binding of liver nuclear proteins to the HNF6 consensus site, and EMSA supershift analysis verified binding of both Stat5 and HNF6 to respective site. Stat5b is clearly involved in transducing the effect of the male GH pattern (Waxman et al. 1995) and has been proposed from in vitro experiments to inhibit CYP2C12 expression (Delesque-Touchard et al. 2000). However, in vivo transfection experiments have shown that Stat5 sites are essential for the expression of CYP2C12 gene (Sasaki et al. 1999). Our results for the a1bg gene concur with the CYP2C12 in vivo data from Kamataki’s group (Sasaki et al. 1999), an intact 3′Stat5-binding element in a1bg was required for maximal expression of the transfected 2.3 kb-Luc. The proximal Stat5 site contributed to the luciferase expression in both sexes in accordance with the higher amount of activated Stat5 seen in male liver as compared with female liver (Tannenbaum et al. 2001). Our results show that the Stat5 site conveys expression of a1bg to higher extent in male than in female livers, thereby reducing the sex difference. Consequently, mutation of this site increased the sex difference. On the other hand, HNF6 is expressed at higher levels in female than in male rat liver (Lahuna et al. 1997). Indeed, following mutation of the HNF6-binding element, mutHNF6-Luc, the sex-differentiated expression was attenuated due to reduced expression in females. Thus, for a1bg, the sex-related difference in amount of HNF6 is likely to contribute to the sex-differentiated and female characteristic expression.

In contrast to the sex-differentiated expression of a1bg-luciferase constructs, in vivo transfection experiments with CYP2C12 reporter constructs have not revealed sex-specific mechanisms (Sasaki et al. 1999). Further studies by the same group show that chromatin condensation in male rats could be one underlying mechanism for the lack of expression of CYP2C12 in male liver; treatment of male rats with trichostatin A (TSA), a histone deacetylase inhibitor, enhanced the effect of continuous GH administration on CYP2C12 expression, TSA treatment alone did not induce CYP2C12 (Endo et al. 2005). We have treated male-derived primary rat hepatocytes with varying concentrations of TSA in the presence or the absence of GH, but have not been able to demonstrate an effect on a1bg expression (data not shown). This does not exclude that chromatin condensation has a role in sex-differentiated expression of a1bg.

That nuclear protein(s) binding to the a1bg −116/−89 region also contributes to the female characteristic expression of 2.3 kb-Luc was supported by the data showing a reduced expression in female liver and thereby a reduced sex difference following deletion of this region, similar to the effect of mutation of the HNF6 site. Expression of the construct lacking the −116/−89 region in combination with a mutated HNF6 site, (−116/−89)delmutHNF6-Luc, was markedly reduced and no sex difference prevailed, implying that the −116/−89 region together with the HNF6 site are regions of importance for the female characteristic expression of the a1bg gene. It is noteworthy that the level of expression of (−116/−89)delmutHNF6-Luc was significantly reduced also in male livers. Thus, the lack of effect of obliteration of the individual −116/−89 area or HNF6 site was not coherent with the effect when both were abrogated. However, the impact of combined deletion of −116/−89 and mutation of the HNF6 site was much higher on the sex-differentiated expression of the a1bg gene. It is not known how NF1 modulates transcriptional activity, and both activation and repression of transcription have
been reported (Gronostajski 2000). Co-factors such as CBP/p300 and HDAC have been shown to interact with NF1 proteins suggesting modulation of chromatin structure (Chaudhry et al. 1999). NF1 factors have also been shown to interact directly with the basal transcription machinery as well as with other transcription factors, including Stat5 (Kim & Roeder 1994, Mukhopadhyay et al. 2001) and synergistic effects with HNF4 have been reported (Ulvila et al. 2004). In addition to the HNF6, Stat5 and NF1/Oct sites, the a1bg promoter harbours an imperfect HNF4 site at −51/−39 with two mismatches compared with the HNF4 consensus site. HNF4 is clearly important for the expression of CYP2C12 (Sasaki et al. 1999), however, the −51/−39 region in a1bg was not protected in the footprinting analysis and was therefore not analysed further. Like NF1, Oct proteins have been reported to be involved in activation as well as repression of gene expression (Phillips & Luisi 2000). Stat5 has been shown to form a stable complex with Oct-1 (Magne et al. 2003). Moreover, NF1 and Oct-1 have been shown to, reciprocally, facilitate each other’s binding (O’Connor & Bernard 1995, Belikov et al. 2004). Interestingly, administration of GH to early pregnant rabbits has been shown to increase binding of NF1 and Oct-1 in EMSA experiments using mammary gland extracts (Malewski et al. 2002). We performed footprinting analysis of the 160 bp a1bg region also with liver nuclear extracts from male rats but found no indication of sex-differentiated binding to the −116/−89 region, neither did western blot experiments unveil any sex differences in NF1 or Oct-1 levels in rat liver (data not shown).

The female pattern of GH exposure is more efficient than the male pattern in inducing albumin expression (Norstedt & Palmiter 1984) and in the albumin promoter, a composite enhancer element-binding HNF5α and NF1 in a ternary complex has been demonstrated (Jackson et al. 1993). At the time of the studies on the albumin promoter, HNF6 had not yet been identified and it is conceivable that HNF6 can bind to the HNF3 site in the albumin promoter. In line with our results for the a1bg promoter, binding of additional proteins to the HNF3/6-NF1 enhancer element in the albumin promoter has been indicated (McPherson et al. 1993). Furthermore, two HNF6 consensus sites in the CYP2C12 promoter are adjacent to putative NF1 sites. Based on our present results, we propose that adjacent binding sites for NF1 and HNF6 constitute a gene regulatory unit of importance for transducing the female-specific effect of GH in rat liver.

The in vivo system used in this study does not reflect the true in vivo situation, but represents a valuable model whereby sex differences in hepatic gene expression can be explored, being an intermediate in vitro/in vivo situation. The in vitro techniques used, in vitro footprinting and EMSA, are not sufficient for revealing the factors that bind and interact to convey the GH-mediated and female-specific expression of the model gene. Techniques such as ChIP analyses and the use of probes for enrichment of bound factors in combination with mass spectrometry will be needed to further delineate what factors that are involved.

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