Interaction of a Novel Sex-dependent, Growth Hormone-regulated Liver Nuclear Factor with CYP2C12 Promoter*

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CYP2C12 is a steroid hydroxylase cytochrome P450 whose female-specific expression in adult rat liver is transcriptionally activated by the continuous plasma growth hormone (GH) profile characteristic of adult female rats. DNase I footprinting and gel mobility shift analysis of the 5'-flank of the CYP2C12 gene were carried out to identify cis-acting elements and trans-acting factors that may contribute to the GH-regulated, sex-dependent transcription of this P450 gene. DNase I footprinting analysis revealed sex- and GH-regulated DNase I hypersensitivity sites at the boundaries of several protein binding sites detected along a 1560-nucleotide upstream segment of CYP2C12. Five distinct sites bound a novel continuous GH-regulated nuclear factor, GHNF, which is enriched in adult female and continuous GH-treated male liver nuclear extracts compared to untreated male liver nuclear extracts. Two other footprinted sites correspond to binding sites for the liver transcription factors C/EBP and albumin D element-binding protein and a third to an HNF1 binding site. A specific binding site for GHNF was also found in the 5'-proximal promoter of CYP2C11, an adult male-specific liver P450 gene, suggesting that GHNF may contribute to the down-regulation of that gene by continuous GH. GHNF was distinguished from the nuclear factors that bind to a GH response element upstream of the rat SpI 2.1 gene and is also distinct from the GH-activatable latent cytoplasmic transcription factors STAT 1, STAT 3, and STAT 5. These findings support the hypothesis that continuous GH-activated transcription of CYP2C12 in adult female rat liver (a) involves the activation of a novel GH-regulated nuclear factor which binds to multiple sites along the 5'-flank of this cytochrome P450 gene, and (b) proceeds via a signaling pathway distinct from the GH pulse-activated STAT5 pathway proposed to induce CYP2C11 and other male-expressed liver genes.

Liver cytochrome P450 (CYP) enzymes play a central role in several important metabolic processes that have a major impact on liver physiology, including cholesterol and steroid hormone hydroxylation, drug metabolism, and carcinogen activation. Steroid hormones are metabolized by liver P450 enzymes with a higher degree of regio- and stereoselectivity than many foreign compound substrates (1), suggesting that these endogenous lipophiles serve as physiological P450 substrates. The physiological requirements with respect to liver steroid hormone hydroxylation differ between the sexes, and, accordingly, several steroid hydroxylase liver P450s belonging to gene families CYP2 and CYP3 (2) are expressed in a sex-dependent manner (3). CYP2C11, the major male-specific androgen 16α- and 2α-hydroxylase in adult rat liver, is induced at puberty in males but not females (4, 5) under the influence of neonatal androgenic imprinting (programming) (6). The female-specific steroid sulfate 15β-hydroxylase CYP2C12 is induced at puberty in female rat liver (6, 7). Other male-specific rat liver P450s include the steroid 6β-hydroxylase CYP3A2 (6, 8–10), the testosterone 15α-hydroxylase CYP2A2 (11), and the fatty acid ω-hydroxylase CYP4A2 (12). Corresponding patterns of sex-dependent liver gene expression have also been reported for several mouse liver steroid hydroxylase P450s (13, 14) and for several non-P450 liver enzymes (e.g. Refs. 15–17).

The imprinting effect of neonatal androgen on the expression of these liver-specific steroid hydroxylase P450 genes is mediated by the hypothalamus and its regulation of pituitary GH secretory patterns (17). Three distinct responses of liver P450s to plasma GH profiles can be discerned (18): (a) continuous plasma GH, a characteristic of adult female rats, stimulates expression of female-specific liver enzymes, such as CYP2C12; (b) intermittent plasma GH pulsation, associated with adult male rats, induces the expression of male-specific liver enzymes, such as CYP2C11; and (c) continuous GH markedly suppresses liver expression of the male-specific P450s. The underlying mechanisms whereby GH regulates expression of these sex-dependent liver P450s are only partially understood. Continuous GH can act directly on the hepatocyte to regulate liver P450 expression, but in a manner that cannot be mimicked by insulin-like growth factor I (19), a mediator of GH's effects on extrahepatic tissues. Discrimination by the hepatocyte between male and female plasma GH profiles is likely to occur at the cell surface and may involve the activation of distinct intracellular signaling pathways by a chronic (female) as compared to an intermittent (male) pattern of plasma GH stimulation. This hypothesis is supported by the recent demonstration that intermittent, but not continuous, plasma GH activates the latent cytoplasmic transcription factor liver STAT 5 by a mechanism that involves both Jak2 kinase-catalyzed tyrosine phosphorylation and serine or threonine phosphorylation followed by nuclear translocation of the STAT protein (20, 21). GH pulse frequency is the most critical determinant for intermittent GH stimulation of male liver P450 expression, which is characterized by a requirement for a well-defined minimum recovery period that is not met in the case of female rat hepatocytes exposed to GH continuously (22). This recovery period may serve to reset the Jak2/STAT 5 intracellular sig-

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1 The abbreviations used are: CYP, cytochrome P450; GH, growth hormone; GHNF, growth hormone nuclear factor; STAT, signal transducer/activator of transcription; PCR, polymerase chain reaction; HNF, hepatocyte nuclear factor; C/EBP, CCAAT-enhancer binding protein; DBP, albumin D element-binding protein; nt, nucleotide(s); SIE, sex-inducible element.
naling apparatus, or perhaps may provide time needed for replenishment of cell surface GH receptors following GH pulse–induced receptor internalization.

GH regulates steroid hydroxylase P450 expression at a pre-translational level (18) with a requirement for ongoing protein synthesis in the case of 2C12 mRNA (23). Unprocessed nuclear 2C12 heterogeneous nuclear RNA responds to circulating GH profiles in a manner that is indistinguishable from the corresponding mature, cytoplasmic mRNA (24). Consequently, transport of 2C12 mRNA to the cytoplasm and cytoplasmic 2C12 mRNA stability are unlikely to be important control points for GH-regulated P450 expression. Nuclear run-on transcription analyses have further established that GH regulates the sex-specific expression of CYP2C12, as well as CYP2C11, at the level of gene transcription initiation (24, 25). Studies of other CYP2C genes have identified promoter elements involved in basal or liver-specific gene expression (26–30); however, DNA elements and nuclear factors involved in continuous GH regulation of CYP2C12 or other CYP genes have not been identified. Attempts to elucidate the mechanisms underlying the transcriptional response of CYP2C12 to continuous GH, including promoter analysis to identify continuous GH-response elements and their cognate binding factors, have been greatly hampered by the absence of a suitable stable cultured cell model that responds to the pattern-dependent effects of GH, and by the unresponsiveness of in vitro transcription systems to the sex-dependent regulation that is evident in vivo (24). Preliminary in vitro investigation of the CYP2C12 gene has suggested that 2C12s 5′-flanking DNA may interact with nuclear factors which are differentially expressed in male versus female rat liver in a GH-regulated manner (24, 31). In the present study, we identify a novel GH-regulated nuclear factor (GHNF) that is present in female but not male rat liver and binds to multiple sites along the CYP2C12 promoter. In contrast to the GH pulse-responsive STAT proteins (20, 21), GHNF is shown to be activated by continuous plasma GH, thus establishing the occurrence in hepatocytes of multiple GH pattern-responsive nuclear signaling factors.

MATERIALS AND METHODS

Animals—Immature (4-week-old) and adult male and female Fischer 344 rats (8–10 weeks of age) were purchased from Taconic, Inc. (Germantown, NY). GH (National Hormone and Pituitary Program, NIDDK) was administered to intact adult male rats by continuous infusion using an Alzet osmotic minipump delivering 1.25 μg/day/n (24). Preliminary in vitro investigation of the CYP2C12 gene has suggested that 2C12s 5′-flanking DNA may interact with nuclear factors which are differentially expressed in male versus female rat liver in a GH-regulated manner (24, 31). In the present study, we identify a novel GH-regulated nuclear factor (GHNF) that is present in female but not male rat liver and binds to multiple sites along the CYP2C12 promoter. In contrast to the GH pulse-responsive STAT proteins (20, 21), GHNF is shown to be activated by continuous plasma GH, thus establishing the occurrence in hepatocytes of multiple GH pattern-responsive nuclear signaling factors.

RESULTS

DNase I Footprinting Analysis of CYP2C12 Gene 5′-Flank—Our earlier analysis of the CYP2C12 proximal promoter by DNase I footprinting revealed several sex- and GH-dependent differences in DNase I cleavage patterns in the first 230 nt (24), suggesting that GH can regulate specific protein-DNA interactions in the 5′-flank of the 2C12 gene. To further characterize these sites, and to ascertain whether the 2C12 gene contains additional binding sites further upstream that may interact with GH-regulated nuclear factors, we analyzed by in vitro DNase I footprinting a series of six overlapping DNA fragments extending from 2C12 nt to −560 to +60 on both the sense strands and the antisense strands. (2C12 DNA fragments spanning nt −1560 to −1220, −1258 to −987, −1028 to −774, −809 to −434, −473 to +60, and −231 to −117). Gel mobility shift experiments were performed using two 2C12 −5′-flank regions C to I (Table I), and each of the 2C11 promoter fragments used in this study (Table II) were prepared in a similar manner. 32P-labeled PCR products were purified on nondenaturing polyacrylamide gels (5% acrylamide, 0.13% bisacrylamide) run for 1 h at 200 V in 0.5 × TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0). DNA probes corresponding to each strand of 2C12 5′-flanking regions A and B, as well as the consensus binding site sequences for transcription factors, and for the liver-restricted transcription factors HNF-1, HNF-3, HNF-4, C/EBP, (24), and DBP (25) were synthesized, labeled on one strand as required, heated to 65 °C, and annealed at room temperature and then gel-purified prior to use. Consensus binding site oligonucleotides for the following transcription factors used in gel shift competition assays (see below) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Sp1, CREB, NFkB, E2F, Myc-Myb, Oct-1, AP-1, AP-2, MEF-1, NFB-2, Egr, GATA, NF-E2 (YY HNF-1, HNF-3, HNF-4, C/EBP, and DBP (24)). Gel mobility shift analyses were carried out as follows. Liver nuclear protein, 5 μg dissolved in 5 μl of nuclear extract buffer (25 mM Hepes [pH 7.6 at 4 °C], 10 mM NaCl, 0.5 mM phenylmethanesulfonyl fluoride, 0.1 mM EDTA, 1 μg dithiothreitol, 10% glycerol), was preincubated for 10 min at room temperature with 9 μl of electrophoretic mobility shift assay buffer (10 mM Tris·HCl, pH 7.5, 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl containing 2 μg of poly(dI·dC)). In some experiments, the poly(dI·dC) was increased to 5 μg. Double-stranded oligonucleotide probe (1 μl, 10 fmol), 32P-end-labeled on one strand using T4 polynucleotide kinase, was then added and further incubated for 30 min at room temperature. For competition experiments, unlabeled competitors were included at a 20- to 100-fold molar excess over the 32P-labeled DNA probe. Gels were pre-electrophoresed at 100 V for 30 min at room temperature. Electrophoresis through nondenaturing polyacrylamide gels (4% acrylamide, 0.05% bisacrylamide) in 0.5 × TBE buffer was for 2.5 h at 100 V. Gels were exposed to x-ray film or PhosphorImager plates to visualize the 32P-labeled bands.

Gel mobility supershift analysis was carried out by including a 10-min preincubuation with antibody to STAT1 (Transduction Laboratories, catalog number G96290), STAT3 (Santa Cruz, catalog number sc-482xs), STAT5b (Santa Cruz, catalog number sc-835xs), C/EBPα (Santa Cruz, catalog number sc-61x), or C/EBPβ (Santa Cruz, catalog number sc-150x) prior to addition of the 32P-labeled DNA probe. In control experiments, each of the anti-STAT antibodies was shown to be effective in supershifting specific protein-DNA complexes involving the corresponding STAT protein using either SIE probe (anti-STAT 1 and anti-STAT 3) or β-casein promoter probe (anti-STAT 5) and liver nuclear extract protein from hypophysectomized rats sacrificed 15 min after a single injection of rat GH, as reported elsewhere (21).
upstream DNA regions that contain one or more distinct nuclear protein binding sites were thus identified (Figs. 1–3). In addition to these seven regions, designated A–G, an extended region of the proximal 2C12 promoter contains several DNase I footprint and DNase I hypersensitivity sites (nt –231 to –36) (24). This latter piece of DNA includes region H, which contains at least two DNase I hypersensitivity sites that are preferentially cleaved in the presence of male nuclear proteins (nt –231 to –149), and the adjacent region I, which includes a DNase I hypersensitivity site that is preferentially cleaved in the presence of female nuclear protein (nt –159 to –112) (24).

At four sites along the 2C12 5′-flank, namely 2C12 regions A (nt –1483 to –1447), B (nt –1456 to –1412), D (nt –942 to –898), and E (nt –904 to –862), the DNase I footprinting patterns obtained in the presence of male rat liver nuclear extract were indistinguishable from the corresponding patterns of female or GH-treated male liver nuclear extracts. Region A includes a consensus binding site for the liver-enriched transcription factor HNF1 (37), at nt –472 to –1460 (Fig. 1), and regions B and D include potential binding sites and factors for the liver factor C/EBP (TTTCTCAA), suggesting that these sites and factors may contribute to the liver-specific expression of 2C12. In contrast, at three other upstream DNA regions, C, F, and G, clear differences in footprinting or DNase I hypersensitivity site cleavage were observed with male as compared to female or continuous GH-treated male liver nuclear extracts. Thus, 2C12 region C (nt –1388 to –1322) included a footprint (–1382 to –1364) that was not sex-specific, but was bordered by a DNase I hypersensitivity site, at nt –1382, which was significantly enhanced in the presence of female or continuous GH-treated male nuclear extract, but not untreated male liver nuclear extract (Fig. 1). Region F (nt –728 to –669) also contained a footprint (–722 to –710) and a sex-dependent hypersensitivity site, at nt –697, but in this case preferential DNase I cleavage was observed in the presence of male nuclear extract (Fig. 3A). Region G (nt –592 to –533) included a partial footprint (–559 to –538) that was observed in the presence of male but not female nuclear extract, as well as a hypersensitivity site, at nt –579, that was detectable in the presence of female nuclear extract (Fig. 3B). The sex-dependent, GH-regulated differences in DNase I hypersensitivity sites observed in the upstream regions C, E, and G are analogous to those seen in our earlier studies of the proximal promoter of 2C12 (regions H and I) (24).

Characterization of Nuclear Protein Binding to 2C12 Regions

–1560 (panel B). Double-stranded fragments corresponding to CYP2C12 5′-flanking DNA –1560 to –1220 (numbering relative to the transcription start site) were synthesized by PCR with a 5′-32P-label on one strand and then subject to DNase I footprinting in the presence of rat liver nuclear extract protein as described under “Materials and Methods.” Shown are autoradiograms of portions of the DNA sequencing gels containing the resulting DNase I cleavage patterns. Panel A, 32P-labeled “sense” (upper) strand, nt –1560 to –1220; panel B, 32P-labeled “antisense” (lower) strand, nt –1220 to –1560. DNA I hypersensitivity sites (HS) and footprints, designated A, B, and C from the 5′-end of the fragment, are shown at the side of each panel, and the DNA sequence calibrations, determined from a partial cleavage of each probe at A + G residues by the Maxam-Gilbert method (lane marked A + G). DNase I cleavages were carried out in the absence of liver nuclear protein (lanes 1 and 9) or in the presence of liver nuclear extract prepared from adult male (M; lanes 2 and 5) or adult female rats (F; lanes 3 and 6) or from male rats treated with GH given as a continuous infusion over 7 days (lanes 4, 7, and 8). GH, human GH infusion at 25 ng/g body weight/h; GH*, rat GH infusion at 20 ng/g body weight/h. Shown adjacent to footprint region A are the boundaries of an HNF1 consensus binding site (37) identified by computer analysis. Hypersensitivity site at nt –1382 was cleaved more extensively in the presence of female, or continuous GH-treated male liver nuclear extract (*F > M), as seen on both strands. Footprint C is seen more clearly on the antisense strand DNase I cleavage reaction shown in panel B.

Fig. 1. DNase I footprint analysis of 32P-labeled CYP2C12 5′-flanking fragments –1560 to –1220 (panel A) and –1220 to
A-H by Gel Mobility Shift Analysis—

32P-End-labeled DNA fragments corresponding to 2C12 regions A-H were prepared and then used in gel mobility shift assays to further characterize their cognate DNA-binding nuclear factors. Our primary goal was to ascertain whether one or more of these DNA sequences could be identified as (a) binding sites for liver-enriched transcription factors that might contribute to the liver-specific expression of 2C12, or (b) binding sites for GH-regulated nuclear factors, which might contribute to the female-specific expression of the 2C12 gene. Each of the eight gel shift probes, corresponding to 2C12 regions A-H (Table I), yielded one or more specific, well-defined gel shift bands when incubated with nuclear protein extracted from adult female rat liver. In each case, the specificity of DNA binding was established by the ability of a large molar excess of unlabeled probe to compete with the corresponding 32P-labeled probe for complex formation (e.g., Fig. 4, lanes 2 and 3).

To identify potential binding sites for liver transcription factors, competition experiments were carried out using unlabeled DNA fragments whose sequences correspond to consensus binding sites for the liver-enriched transcription factors HNF1, HNF3, HNF4, and C/EBP (34). An albumin D-binding protein (DBP) probe (35) was also evaluated. Fig. 4A shows that 2C12 region A probe yields two specific gel shift complexes, A1 and A2, and that formation of complex A1 is selectively competed by the HNF1 binding site probe. When 32P-labeled, this HNF1 probe formed a gel shift complex with rat liver nuclear extract that comigrated with 2C12 complex A1. Complex A1 was also selectively supershifted by an antibody to HNF1α, further supporting the identification of complex A1 with HNF1 (data not shown). An AP1 binding site probe (34) tested as a control also competed with complex A1; however, AP1 competition could not be verified using a second AP1 probe (Santa Cruz Biotechnology) which contains the same AP1 core consensus sequence (TGACTCA) but differs in its flanking DNA (data not shown). By contrast, the gel shift complexes formed by region B (Fig. 4B) and region D (data not shown) were selectively inhibited by binding site probes for the liver-enriched factors C/EBP and DBP. This suggests that the same or a closely related C/EBP- or DBP-like factor binds to these two sites. Indeed, gel mobility supershift analysis using antibody to C/EBPα verified the presence of this liver factor in both complexes (data not shown). None of the liver factor DNA-binding fragments inhibited gel shift complexes formed by 2C12 probe H (Fig. 4C) or probes C, E, or F (data not shown).

To test for the possible presence of sex-dependent or GH-regulated nuclear factor(s) in these 2C12 promoter DNA-nuclear protein complexes, we compared the gel shift complex formation activity of female rat liver nuclear extracts to that of untreated male and continuous GH-treated male nuclear extracts. Several independent preparations of nuclear extract proteins were examined to eliminate from consideration any differences that might reflect preparation to preparation variation in nuclear DNA binding activity rather than true sex differences. Sex-dependent differences in gel shift complex formation were not observed with 2C12 regions A (Fig. 5A) or regions B or D (data not shown), in agreement with the sex-independent DNase I footprint and hypersensitivity patterns shown in Figs. 1 and 2. Similarly, no sex difference was observed for the single gel shift complex formed by probe G (data not shown). By contrast, 2C12 regions C, F, and H, which exhibited sex-dependent DNase I cleavage patterns (Figs. 1 and 3 (24)), each yielded either one or two gel-shifted bands that were substantially enriched in the female as compared to the male nuclear extracts (Fig. 5, B–D). A sex-dependent gel shift band was also observed with a region E probe (data not shown).
shown), even though no sex-dependent DNase I footprinting or hypersensitivity sites could be detected in this DNA region (Fig. 2). GH treatment of adult male rats by continuous infusion induced the DNA-binding activity detected in liver nuclear extracts with all four DNA probes (i.e., 2C12 regions C, E, F, and H), indicating that GH, and in particular the plasma GH pattern, can regulate the sex-dependent expression or DNA binding activity of these liver nuclear factor(s) (Fig. 5, B–D versus A; lanes marked M/GH versus M). A further correlation between the expression of 2C12 and the expression of the GH-regulated nuclear factor(s) is provided by the finding that at 4 weeks of age, when 2C12 protein (6) and 2C12 mRNA (data not shown) are expressed in both sexes, male and female rats both express the GH-regulated liver nuclear factor (Fig. 5, lanes marked Imat).

Relationship between Nuclear Proteins Bound to 2C12 Regions C, E, F, and H—Competition experiments were carried out to test for possible relationships between the GH-regulated nuclear factor(s) that bind to 2C12 regions C, E, F, and H. We first investigated whether unlabeled DNA fragments corresponding to these four regions could inhibit nuclear protein binding to 32P-labeled region F. As seen in Fig. 6A, the female liver-specific gel shift complexes formed by region F were fully shown, even though no sex-dependent DNase I footprinting or hypersensitivity sites could be detected in this DNA region (Fig. 2). GH treatment of adult male rats by continuous infusion induced the DNA binding activity detected in liver nuclear extracts with all four DNA probes (i.e., 2C12 regions C, E, F, and H), indicating that GH, and in particular the plasma GH pattern, can regulate the sex-dependent expression or DNA binding activity of these liver nuclear factor(s) (Fig. 5, B–D versus A; lanes marked M/GH versus M). A further correlation between the expression of 2C12 and the expression of the GH-regulated nuclear factor(s) is provided by the finding that at 4 weeks of age, when 2C12 protein (6) and 2C12 mRNA (data not shown) are expressed in both sexes, male and female rats both express the GH-regulated liver nuclear factor (Fig. 5, lanes marked Imat).

Relationship between Nuclear Proteins Bound to 2C12 Regions C, E, F, and H—Competition experiments were carried out to test for possible relationships between the GH-regulated nuclear factor(s) that bind to 2C12 regions C, E, F, and H. We first investigated whether unlabeled DNA fragments corresponding to these four regions could inhibit nuclear protein binding to 32P-labeled region F. As seen in Fig. 6A, the female liver-specific gel shift complexes formed by region F were fully shown, even though no sex-dependent DNase I footprinting or hypersensitivity sites could be detected in this DNA region (Fig. 2). GH treatment of adult male rats by continuous infusion induced the DNA binding activity detected in liver nuclear extracts with all four DNA probes (i.e., 2C12 regions C, E, F, and H), indicating that GH, and in particular the plasma GH pattern, can regulate the sex-dependent expression or DNA binding activity of these liver nuclear factor(s) (Fig. 5, B–D versus A; lanes marked M/GH versus M). A further correlation between the expression of 2C12 and the expression of the GH-regulated nuclear factor(s) is provided by the finding that at 4 weeks of age, when 2C12 protein (6) and 2C12 mRNA (data not shown) are expressed in both sexes, male and female rats both express the GH-regulated liver nuclear factor (Fig. 5, lanes marked Imat).

**Table I:** Gel mobility shift competition analysis of CYP2C12 5’-DNA: identification of GHNF binding sites and binding sites for liver transcription factors

| 2C12 DNA region | 5’-Nucleotides | Binding site specificity |
|-----------------|----------------|-------------------------|
| A               | −1483 to −1447 | HNF1<sup>a</sup>         |
| B               | −1436 to −1412 | C/EBP, DBP              |
| C               | −1388 to −1322 | GHNF                   |
| B/C1           | (−1336 to −1360) | GHNF + C/EBP, DBP |
| C1<sup>b</sup> | (−1388 to −1360) | Inactive               |
| C2             | (−1394 to −1352) | Inactive               |
| C3             | (−1326 to −1281) | Inactive               |
| C4             | (−1297 to −1220) | GHNF                   |
| D               | −942 to −898  | C/EBP, DBP              |
| E               | −904 to −862  | GHNF                   |
| E1             | (−895 to −870) | Inactive               |
| F               | −728 to −669  | GHNF                   |
| F1             | (−728 to −708) | Inactive               |
| F2             | (−708 to −682) | Inactive               |
| F3             | (−728 to −682) | Active<sup>c</sup>     |
| G               | −592 to −533  | Not determined          |
| H               | −231 to −149  | GHNF                   |
| H1             | (−231 to −185) | Active<sup>c</sup>     |
| H2             | (−185 to −149) | Inactive               |
| I               | −159 to −112  | Unknown                |

<sup>a</sup> Numbering relative to 2C12 transcription start site. Nucleotide numbering is shown in parentheses for subfragments of 2C12 DNA regions A–I (e.g., B/C1, C1, etc.; indented in left column).

<sup>b</sup> Shown are binding site specificities determined by gel mobility shift competition using consensus binding site oligonucleotides and confirmed by gel supershift analysis, in the case of HNF1, C/EBP, and DBP, or by cross-competition analysis, in the case of GHNF binding to 2C12 regions C, C4, E, F, and H.

<sup>c</sup> HNF1 binding corresponds to gel shift complex A1 (Fig. 4A). Binding site specificity corresponding to complex A2 was not identified.

<sup>d</sup> Subfragments of regions C, E, F, and H (nt sequences shown in parentheses) which are effective with respect to competition for GHNF binding are designated “active.” Subfragments which do not retain gel shift competition activity are designated “inactive.”

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**Fig. 3.** DNase I footprint analysis: 32P-labeled CYP2C12 5’-fragments −809 to −434 (sense strand; panel A) and −434 to −809 (antisense strand; panel B). DNase I cleavage was carried out as described in Fig. 1. Shown is a DNase I hypersensitivity site adjacent to 2C12 footprint region F, at nt −697, that is more intense in the presence of male than in female or GH-treated male rat liver nuclear extracts (M > F<sup>−</sup>) (panel A), as well as two hypersensitivity sites in the vicinity of 2C12 footprint region G, at nt −516 and −579, that are more intense with the female extracts (F > M<sup>−</sup>) (panel B). Footprint G (nt −538 to −559) corresponds to a partial footprint that is evident in the presence of male but not female nuclear extract protein.
Growth Hormone-regulated Liver Nuclear Factor

Fig. 4. Gel mobility shift analysis of liver nuclear factor binding to 2C12 5'-DNA fragments. DNA probes for 2C12 regions A, B, and H (panels A-C, respectively) (10 fmol of 32P-labeled DNA probe/ lane) were incubated with adult female rat liver nuclear extract (5 μg of protein) alone (lane 1 of each panel) or in the presence of 20-fold molar excess (first sample of each pair) or a 100-fold molar excess (second sample) of unlabeled 2C12 5'-flank DNA fragment or unlabeled liver transcription factor consensus binding site DNA fragments (competitor DNA, as indicated). Panel A, region A gives two gel mobility shift complexes, designated A1 and A2; HNF1 consensus site DNA fragment selectively inhibits formation of complex A1. Panel B, gel shift complex formation by region B is inhibited by C/EBP and DBP consensus binding site DNA probes. An identical result was obtained using a probe for region D (not shown). Panel C, none of the consensus DNA fragments, except probe H itself, inhibited complex formation.

competed by a 20-fold molar excess of DNA fragments corresponding to 2C12 regions F, H, and E (lanes 2, 4, and 8). DNA fragments covering regions H + I, as well as D + E (lanes 3 and 6) also inhibited formation of the gel shift complexes with 2C12 probe F, while fragments I and D alone were noninhibitory (lanes 5 and 7). Fragment G (cf. lane 10) partially inhibited complex formation, but only at a 100-fold excess of competitor DNA (data not shown). Fragments A and B were noninhibitory, as were HNF1, HNF3, and HNF4 consensus binding site fragments, confirming the specificity of the observed inhibitions.

Full-length fragment C (nt −1388 to −1322) was inhibitory to complex F formation (see below, Fig. 6A, lane 12), but a shorter region C fragment, corresponding to nt −1388 to −1360 (fragment C1), was not inhibitory (Fig. 6A, lane 9). Similar experiments carried out with 32P-labeled gel shift probes C and H demonstrated that all four 2C12 promoter fragments (i.e., C, E, F, and H) could also inhibit formation of the female-specific gel shift complexes detected using these two probes (Fig. 6, B and C, and data not shown). These findings strongly suggest that 2C12 promoter regions C, E, F, and H each bind the same, or closely related nuclear factors, designated GHNF.

Further Localization of DNA Binding Elements—To better localize the DNA binding elements within each promoter segment, we tested shorter DNA fragments derived from regions C, E, F, and H for their ability to competitively inhibit GHNF DNA binding activity (Table I). Division of region H into two shorter fragments, H1 (nt −231 to −185) and H2 (nt −185 to −149), demonstrated that subregion H1 largely retains GHNF competition activity assayed using either probe H (Fig. 6C, lanes 3 and 4) or probe F (data not shown). Shortening the inhibitory fragment corresponding to region E from 43 nt (−904 to −862) to 36 nt (−895 to −870; fragment E1) led to a loss of this activity (Fig. 6C, lane 10). Gel shift complex competition activity was also lost upon division of region F (−728 to −669) into two subfragments selected on the basis of the footprinting and DNase I hypersensitivity pattern shown in Fig. 3B (F1, nt −728 to −708; F2, nt −708 to −682) (Fig. 6C, lanes 11–13), but was retained within the slightly shortened F3 (nt −728 to −682) (data not shown).

Subdivision of region C (nt −1388 to −1322) using the same approach revealed a somewhat more complex picture. Fragment C1 (−1388 to −1360) and the somewhat longer fragment C2 (−1394 to −1354) were both inactive (Fig. 6 and data not shown), despite the inclusion in these fragments of both the protein binding site and the sex-dependent DNase I hypersensitivity site detected by footprint analysis. This suggests that the key DNA binding sequences are localized further down-
FIG. 6. 2C12 DNA regions C, E, F, and H bind the same or a closely related GHNF cross-competition gel mobility shift analysis. Gel mobility shift analysis was carried out using adult female liver nuclear extract and 2C12 DNA probes F, C, and H (panels A–C, respectively) in the presence of a 20-fold molar excess of the indicated unlabeled competitor DNAs using methods described under Fig. 4. Unlabeled DNA probes corresponding to 5'-flanking regions of the CYP2C12 promoter are indicated at the top of each lane (see text and Table I for details). Unlabeled liver transcription factor consensus DNA fragments (HNF1, HNF3, and HNF4) are included in panel A, lanes 13–15, and panel B, lanes 9–13.

stream, between nt −1360 and −1322. On the other hand, extension of the inactive fragment C1 in the 5'-direction, to incorporate sequences not present in the original fragment C, such as the adjacent binding site B (fragment B/C1, nt −1436 to −1360), yielded a strong inhibitory fragment (Fig. 6B, lane 2). This suggests that the extended region between nt −1436 and −1322 may contain two GHNF binding sites, one between nt −1412 and −1360 and one between −1360 and −1322. The proposed presence of a GHNF binding site toward the 5'-end of region C is supported by the female-specific, GH-regulated gel shift complex observed using 32P-labeled fragment B/C1 as probe (Fig. 7A, upper gel shift band) and by its specific inhibition by 2C12 fragments E, F, and H (Fig. 7B). The lower gel shift complex formed with probe B/C1 corresponds to that of binding site B, insofar as it migrates equivalent to complex B (data not shown) and is not sex-dependent (Fig. 7A). Furthermore, formation of this gel shift complex is selectively inhibited by fragment B and by the C/EBP and DBP consensus binding site fragments that compete for binding to site B (Fig. 7B, lanes 8 and 9). Partial inhibition of gel shift complex B formation by fragment H was observed in these experiments (Fig. 7B, lane 5), suggesting that fragment H may also contain a C/EBP or DBP binding site. Fragment H's GHNF binding activity per se does not include C/EBP or DBP, however, since consensus DNA fragments for these two liver factors do not inhibit complex H formation (Fig. 4C). The selective inhibition of the upper, female-specific nuclear complex C1 by 2C12 fragments E and F (Fig. 7B, lanes 6 and 7) and that of the lower, sex-independent complex B by C/EBP and DBP consensus fragments (lanes 8 and 9) evidence the independent binding of the corresponding nuclear factors to 2C12 sites B and C1.

Finally, examination of DNA segments adjacent to region C suggested the presence of an additional GHNF binding site between nt −1297 and −1220. This DNA region, designated C4, was identified by its competition for GHNF binding to region F (Fig. 8A, lanes 9 and 10) and region H (data not shown) and was separated from the region C GHNF binding site (−1388 to −1322) by a segment that did not contain binding activity (−1326 to −1281; region C3, Table I) (Fig. 8A, lane 11). Interestingly, the 5'-portion of 2C12 region C4 (nt −1297 to −1277) is conserved in the GH-regulated CYP2C7 (38); however, this sequence was not conserved in the other GHNF binding sites identified in the present study. Examination of the DNA segment upstream of region A (nt −1560 to −1447) did not reveal any additional GHNF binding sites (Fig. 8B, lane 7).

Binding Site for GH-regulated Nuclear Factor in Proximal Promoter of CYP2C11 Gene—The gel shift competition assay described above was used to investigate whether the 5'-flank of CYP2C11 contains DNA elements that might bind to the GHNF defined by 2C12 regions C, C4, E, F, and H. Conceivably, such DNA elements could be involved in the down-regulation of CYP2C11 transcription by continuous plasma GH (24). Initial experiments demonstrated that a DNA fragment covering the 2C11 promoter and transcription start site (2C11 nt −275 to +67) was highly inhibitory to formation of the GHNF DNA complex by 2C12 fragments F (Fig. 8B, lane 3) and H (data not shown). Subsequent experiments revealed that DNA fragments covering 2C11 nt −575 to −197, −275 to −197, and nt −64 to +67 did not specifically compete for GHNF binding activity, while a central fragment, 2C11 nt −200 to −62, retained this activity (Table II; Fig. 8B, lanes 4–6; and data not shown). This latter region of 2C11 was further subdi-
DNA fragments prepared from either the probe F and a 50-fold molar excess of the indicated unlabeled competitor DNA fragments were inactive (nt 147 to 102) of the Spi 2.1 gene (36).

**TABLE II**

**GHNF binding site within CYP2C11 5′-flank**

DNA fragments corresponding to the indicated regions of the CYP2C11 promoter were synthesized by PCR, then assayed for their ability to inhibit formation of the GHNF complex formed on CYP2C12 regions F and H (e.g. Fig. 8).

| 2C11 5′-nucleotides | GHNF competition activity |
|----------------------|--------------------------|
| −573 to −197         | −                        |
| −430 to −197         | −                        |
| −273 to +67          | +                        |
| −573 to −197         | −                        |
| −200 to −62          | +                        |
| −64 to +67           | (+)‡                     |
| −200 to −121         | +                        |
| −135 to −85          | +                        |
| −94 to −62           | +                        |

* Partial competition (see Fig. 8A, lane 5).

Further support for the absence of STATs 1, 3, and 5 in the GHNF/2C12 promoter gel shift complex was provided by gel shift competition studies using STAT response element oligonucleotides. GHNF complex formation with 2C12 fragment F was not inhibited by a 100-fold molar excess of unlabeled SIE oligonucleotide probe, which binds GH-activated STAT 1 and STAT 3 (21), or by β-casein promoter probe, which binds GH-activated liver STAT 5 (20) (Fig. 9, lanes 3 and 4). A double-stranded oligonucleotide probe derived from the rat Spi 2.1 gene (nt −147 to −102) (36), which corresponds to a functional GH response element and includes a functional STAT 5 binding site (40, 41), also did not specifically inhibit GHNF/2C12 complex formation (Fig. 8A, lane 13). Two other STAT proteins, STAT 2 and STAT 4, are not expressed at significant levels in liver and therefore are not likely to be present in our 2C12 promoter DNA-protein complex. The interleukin 4-activated liver STAT 5 (20) (Fig. 9, lanes 5 and 6) and H (data not shown). Similarly, a polyclonal anti-STAT 5 antibody, which strongly supershifted the female-specific GHNF DNA-protein complex when tested with 2C12 promoter region fragments F (Fig. 9, lane 7) and H (data not shown), further supported the absence of STATs 1, 3, and 5 in the GHNF-containing 2C12 DNA-protein complex (lane 7).

**DISCUSSION**

The present study was carried out to identify specific binding sites for liver-enriched transcription factors, as well as binding sites for GH-regulated liver nuclear factors that may contribute to regulated expression of CYP2C12. Eight specific regions that are footprinted by rat liver nuclear proteins were identified in
the 5′-1.5 kilobase DNA of the CYP2C12 gene, and three of these regions were shown to bind well-characterized liver-enriched transcription factors in binding site competition and gel supershift experiments (site A1, factor HNF1, and sites B and D, factors DBP and C/EBP). Five other regions of the 2C12 5′-flank (regions C, C4, E, F, and H) were shown to bind nuclear factor(s) that are highly enriched in female as compared to male rat liver nuclei. Continuous GH treatment of adult male rats feminized the pattern of liver nuclear DNA binding activity to each of these latter 2C12 promoter elements, just as this same hormone treatment stimulates 2C12 gene expression in vivo (3). Competition experiments demonstrated that the same, or a closely related, female-dominant liver nuclear factor, designated GHNF, binds to all five CYP2C12 DNA sites. Furthermore, although GHNF activity was low or undetectable in adult male liver nuclei, it was readily detectable in liver nuclei from immature male and immature female rats, which both express CYP2C12 at a low level (6). Together, these findings provide strong support for the hypothesis that one or more of the corresponding 2C12 DNA-binding regions serves as an important site for continuous GH activation of 2C12 gene expression and that GHNF may be an important mediator of this hormonal stimulatory effect in vivo.

GHNF was distinguished from the GH-regulated nuclear factor that binds to and regulates the GH-inducible rat liver Sp1 2.1 gene (42, 43), as shown by the inability of a GH response element/binding site oligonucleotide derived from that gene (36) to compete for GHNF binding to the 2C12 promoter sites. Recently, the latent transcription factor STAT 5 was identified as a major liver factor which binds to the Sp1 2.1 GH response element used in our competition studies (44). In agreement with these findings, antibody to STAT 5 did not supershift GHNF-containing complexes formed on 2C12 promoter fragments under conditions where a strong supershift was obtained with a bona fide STAT 5 complex formed by male liver nuclear extract on the rat β-casein promoter (see Ref. 21). STAT 5 is activated in adult male but not female rat liver by intermittent GH pulses (20), and it likely contributes to the male-specific expression of liver P450 genes, such as CYP2C11, which are activated by GH pulsation (20, 39). Two other STAT proteins, STAT 1 and STAT 3, also are activated by GH in rat liver (21, 45, 46), albeit with a dependence on GH dose and plasma profile that is distinct from that of STAT 5 (21). However, neither of these STATs is a component of GHNF, as demonstrated by gel mobility supershift studies. GHNF was also distinguished from 20 other well-characterized transcription factors, including 5 liver-enriched factors, by gel mobility shift competition experiments. GHNF is thus a novel, GH-activated nuclear factor that is distinct from many other general and GH-responsive factors. Our attempts to further characterize GHNF with respect to definition of its core target DNA sequence were hampered by the apparent absence of a readily identifiable core consensus sequence that is shared by the six GHNF sites identified in the present study (five in CYP2C12 and one in CYP2C11) and by the loss of GHNF activity that we encountered when GHNF binding sites shorter than 43–47 nt were used as DNA probes in gel shift analysis. These findings, together with our observation that GHNF migrates as a large, somewhat diffuse complex in gel shift studies, suggest that GHNF may correspond to a large protein complex that makes multiple DNA contacts over an extended region. Further study will be required to identify the constituent components of GHNF and to elucidate the mechanisms whereby it is activated in female rat liver in response to continuous plasma GH stimulation.

Analysis of the 5′-flank of CYP2C11 led to our discovery of a GHNF binding site, located at 2C11 nt −135 to −85 in this male-expressed P450 gene. Although the male-specific expression of 2C11 is primarily regulated through the positive effects of GH pulses, continuous GH suppression in adult female rats is also likely to be an important control mechanism, as indicated by the basal expression of 2C11 in female rat liver that first becomes detectable following hypophysectomy, and by the complete suppression of 2C11 mRNA levels that follows continuous GH treatment of these rats (47). Conceivably, the binding of GHNF to 2C11 nt −135 to −85 detected in the present study (Fig. 8) may serve as a mechanism for shutting off CYP2C11 gene transcription in female rats. This hypothesis is supported by the observation that a corresponding DNA element has the capacity to suppress expression of a heterologous promoter when transfected into primary hepatocytes (29).

Continuous GH treatment in vivo stimulated liver nuclear GHNF activity, indicating that this nuclear factor is either induced by GH de novo or that continuous GH activates pre-existing GHNF protein by phosphorylation or perhaps by some other signaling mechanism. GHNF activity is greatly enriched in female as compared to male liver nuclei, indicating that the GH-dependent signaling pathway which leads to GHNF activation can readily discriminate between continuous and intermittent plasma GH profiles. Since, as noted above, GHNF does not contain any of the STAT factors that are presently known to respond to GH in rat liver, GHNF activation may proceed independent of the GH-activated Jak/STAT pathway, which in male rats leads to intermittent activation of liver STAT 5 coincident with a GH pulse (20). Further studies will be required to ascertain whether GHNF requires tyrosine phosphorylation for DNA binding activity, as do the GH-activated STAT proteins. The precise mechanisms for GHNF activation in response to continuous, but not intermittent, GH are still undefined, but could involve GH-stimulated Ca2+ signaling mechanisms, which may proceed independent of Jak2 kinase (48), or perhaps a phospholipase A2-dependent signaling pathway such as the one that may facilitate GH-stimulated expression of 2C12 in rat hepatocytes (49).

In addition to binding sites for GHNF, the CYP2C12 promoter was shown to contain multiple binding sites for the liver-enriched transcription factors HNF-1 and C/EBP, DBP. Presumably, one or more of these sites contributes to the liver specificity that characterizes CYP2C12 gene expression. HNF-1 plays a role in liver-specific expression and postnatal developmental activation of CYP2E1 (50). C/EBP and/or DBP have been shown to contribute to the expression of multiple liver-expressed P450s, including C26 (28), 2D6 (51), 7A1 (52), and 2C12 (53). Although no significant sex differences in C/EBP-DBP binding to 2C12 sites B and D were detected in the present study, these sites, as well as a C/EBP-DBP-like site adjacent to site H, could still contribute to the GH-regulated expression of this P450 gene, as suggested by the finding that GH can rapidly activate two isoforms of C/EBP in preadipocytes (54). Further studies are required to clarify whether GH stimulates a comparable activation of C/EBP in liver and what role it might play in CYP2C12 gene expression.

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