J. Steroid Biochem. Molec. Biol. Vol. 43, No. 8, pp. 1055–1072, 1992
Printed in Great Britain.

0960-0760/92 $5.00 + 0.00

REGULATION OF LIVER-SPECIFIC STEROID METABOLIZING CYTOCHROMES *P*450: CHOLESTEROL 7α-HYDROXYLASE, BILE ACID 6β-HYDROXYLASE, AND GROWTH HORMONE-RESPONSIVE STEROID HORMONE HYDROXYLASES

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Summary—The hydroxylation of cholesterol, bile acids, and steroid hormones by liver cytochrome *P*450 (CYP) enzymes proceeds with a high degree of regiospecificity, and contributes to both biosynthetic and catabolic pathways of sterol metabolism. CYP 7-catalyzed cholesterol 7α-hydroxylation, a key control point of bile acid biosynthesis, is regulated at a pretranslational step, probably transcription initiation, by multiple factors, including liver bile acid and cholesterol levels, thyroid hormone status, and diurnal rhythm. Hydrophobic bile acids, such as lithocholic acid, are converted to less cholestatic derivatives by 6β-hydroxylation carried out by CYP 3A *P*450s, which also catalyze steroid hormone 6β-hydroxylation reactions. Complex, gender-dependent developmental patterns characterize the expression of steroid 5α-reductase and several rat liver steroid hydroxylase CYPs. Multiple pituitary-dependent factors regulate the expression of these enzymes; of greatest importance are the gonadal steroids and the sex-dependent secretory patterns of growth hormone (GH) that they impart. The continuous presence of GH in circulation, a characteristic of adult female rats, positively regulates expression of the female-specific steroid disulfate 15β-hydroxylase CYP 2C12, while expression of the male-specific steroid 16α- and 2α-hydroxylase CYP 2C11 is stimulated by the intermittent pituitary secretion of GH that occurs in adult male rats. Intermittent GH can stimulate CYP 2C11 gene expression even when the hormone presents to the hepatocyte at a non-physiological pulse amplitude, duration, and frequency, provided that an interpulse interval of no GH (obligatory recovery period) is maintained for at least 2.5 h. GH regulates the expression of the CYP 2C11 and CYP 2C12 genes at the level of transcription initiation. This process is probably mediated by sex-dependent and GH-regulated protein–DNA interactions, such as those observed in the 5′-flank of the CYP 2C12 gene. Thyroid hormone is a second major regulator of liver steroid hydroxylase *P*450 activity. It regulates these enzymes directly, at a pretranslational step, and indirectly, through its stimulation of pituitary GH secretion and by its positive effects on the expression of the flavoenzyme NADPH-*P*450 reductase, which catalyzes electron transfer that is obligatory for all microsomal steroid hydroxylation reactions.

OUTLINE

Proceedings of the First International Symposium on A Molecular View of Steroid Biosynthesis and Metabolism, Jerusalem, Israel, 14–17 October 1991.

Abbreviations: CYP, cytochrome *P*450; GH, growth hormone; MSG, monosodium glutamate.

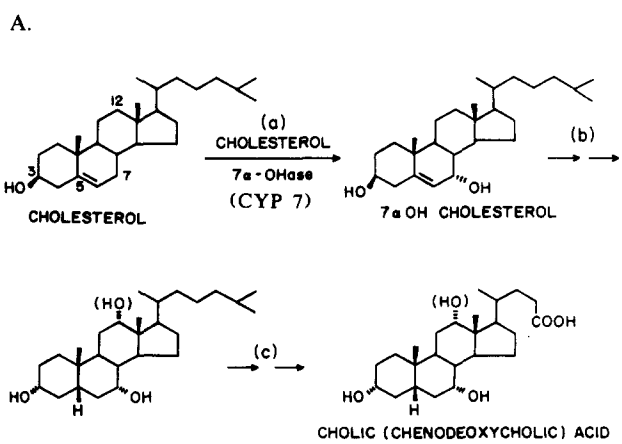
Of the ten mammalian cytochrome *P*450 (CYP) gene families described as of 1991 [1], eight encode enzymes that catalyze steroid hydroxylations. Four families include the key *P*450 enzymes required for steroid hormone biosynthesis from cholesterol (CYP 11, CYP 17, CYP 19, CYP 21) and two participate in the

conversion of cholesterol to bile acids in the liver, where they contribute in a major way to cholesterol homeostatis (CYP 7, CYP 27). Members of two other steroid hydroxylase families, CYP 2 and CYP 3, are expressed at high levels in liver, where they catalyze a broad range of hydroxylation reactions utilizing steroid hormones and bile acids, in addition to drugs and other foreign compounds. Liver expression of these steroid hydroxylase *P*450s is under endocrine control, with growth hormone (GH) and the thyroid hormones (T3, T4) playing major roles in regulating enzyme expression. This article reviews the biochemistry and regulation of several liver-specific steroid hydroxylase cytochrome *P*450 enzymes, with particular emphasis placed on *P*450s involved in bile acid biosynthesis and metabolism, and on the sex-specific steroid hydroxylase *P*450s that are under endocrine control.

1. REGULATION OF CHOLESTEROL 7α-HYDROXYLASE (CYP 7) AND ITS CONTROL OF BILE ACID BIOSYNTHESIS

The synthesis of bile acids from cholesterol requires the sequential action of several liver-specific *P*450 hydroxylases [2, 3] [Fig. 1(A)]. These include (a) cholesterol 7α-hydroxylase (CYP 7), which catalyzes the first and rate-limiting step of the overall biosynthetic pathway, (b) 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase, which is at the branchpoint leading to synthesis of cholic acid (3α, 7α, 12α-trihydroxy-5β-cholanoic acid) versus chenodeoxycholic acid (3α, 7α-dihydroxy-5β-cholanoic acid), and (c) *P*450-linked enzymes required for oxidative cleavage of the side chain of the C27 steroid to yield the C24 bile acid carboxylate; this latter sequence of reactions is initiated by a mitochondrial *P*450 enzyme, CYP 27, whose cDNA has been cloned, sequenced, and expressed [4, 5]. Of these three *P*450 enzymes, cholesterol 7α-hydroxylase (CYP 7) has received the most attention, owing to the major role that it plays in regulating the overall conversion of cholesterol to bile acids [Fig. 1(B)] [6, 7]. Studies carried out primarily in rat liver have established that cholesterol 7α-hydroxylation is regulated by multiple physiological factors. These include (a) down-regulation by bile acids, which return from the gut to the liver via the enterohepatic circulation, where they feedback inhibit liver microsomal cholesterol 7α-hydroxylase. This effect can take up to several days to be fully established, and is viewed as a form of long-term enzyme regulation; (b) mid-term regulation in response to diurnal factors, with enzyme activity maximal at midnight and at a minimum during the day, when the demand for bile acids is low; and (c) short-term responses to factors such as stress, other modulators of circulating corticosteroid levels, mevalonate injection (stimulation of liver cholesterol synthesis), and changes in thyroid hormone status [6, 8].

Early studies of microsomal cholesterol 7α-hydroxylase established that this *P*450 enzyme is immunochemically and biochemically distinct from 9 other rat liver *P*450s, including CYP 2A1, a highly regiospecific steroid hormone 7α-hydroxylase [9]. Studies in this area were given a major boost by the successful purification of rat liver cholesterol 7α-hydroxylase in 1987 [10]. This development facilitated preparation of antibody probes useful for monitoring changes in levels of enzyme protein, and was also an important factor leading to the cDNA



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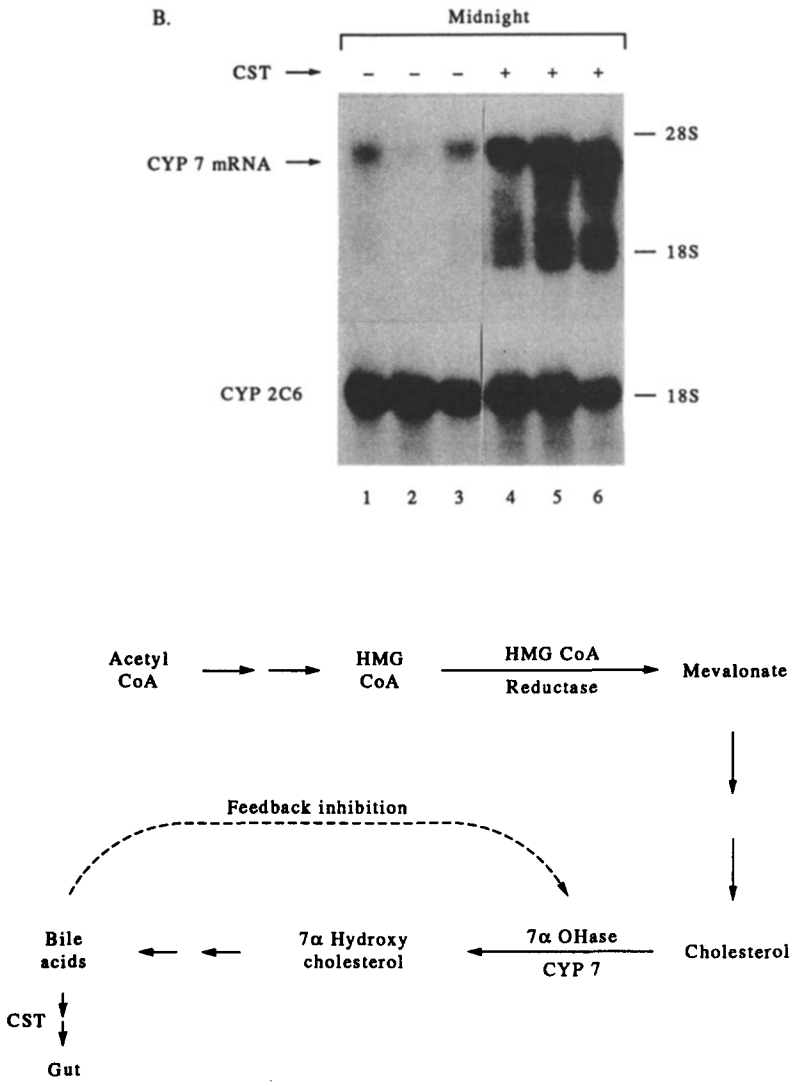

Fig. 1. Role of cholesterol 7α-hydroxylase (CYP 7) in conversion of cholesterol to bile acids. (A) Pathway leading from cholesterol to the major bile acids includes cholesterol 7α-hydroxylation (a), additional modifications to the steroid nucleus (b), and oxidative cleavage of the side chain, with introduction of a carboxyl group (c). (B) Feedback "inhibition" of CYP 7 gene expression is relieved in animals fed a diet containing cholestyramine (CST). This dietary treatment effectively blocks the return of bile acids from the gut to the liver via the enterohepatic circulation, and induces liver CYP 7 mRNA levels, as seen in the Northern blot analysis of CYP 7 mRNA in rat liver RNA samples isolated from normal diet (lanes 1–3) as compared to cholestyramine-fed rats (lanes 4–6). Size heterogeneity of CYP 7 mRNA reflects utilizing of multiple polyadenylation signals. CYP 2C6 mRNA levels serve as an internal standard for RNA load consistency. Data shown in (B) are from [17].

cloning of this *P*450 from rat [11–13] and subsequently human liver [14]. Cholesterol 7α-hydroxylase belongs to a unique *P*450 gene family, CYP 7. Genomic clones, including 5′-flanking DNA sequences, have been described [15, 16]. With the availability of these immunochemical and DNA probes, important questions relating to the mechanisms by which cholesterol 7α-hydroxylase gene expression is regulated could be addressed.

Changes in microsomal cholesterol 7α-hydroxylase activity have been directly correlated with changes in CYP 7 protein and mRNA levels under a wide range of physiological conditions [13, 17, 18]: (a) CYP 7 mRNA, protein, and activity are induced to similar extents over a period of days by dietary cholestyramine (interruption of bile acid feedback inhibition) [Fig. 1(B)] or by feeding cholesterol, the substrate of CYP 7. Apparent discrepancies in bile

fistula rats between the fold-increases in hepatic microsomal cholesterol 7α-hydroxylase activity as compared to the associated increases in CYP 7 protein level have been reported by some [19] but not other investigators [20]. Such discrepancies may arise if the anti-CYP 7 antibody used to quantitate CYP 7 protein mass is crossreactive with other microsomal proteins. (b) CYP 7 mRNA and protein both vary diurnally, and are maximal in rat liver at midnight. (c) CYP 7 mRNA was shown to be rapidly induced (3- to 6-fold increase within 2.5 h) by the cholesterol precursor mevalonate [17]. This increase can fully account for the associated increase in microsomal cholesterol 7α-hydroxylase activity, and indicates that this short-term response of enzyme activity to mevalonate does not result from increases in the local concentration of cholesterol substrate, as was originally proposed [21]. Thyroid hormone can also rapidly induce CYP 7 mRNA, albeit at doses that are supraphysiologic [22]. Although the above studies establish that both long- and short-term regulation of CYP 7 is primarily at a pretranslational level, other potential regulatory mechanisms, including translational regulation, regulated enzyme degradation, allosteric effects of bile acids on CYP 7 protein, changes in cholesterol availability [23, 24], and reversible dephosphorylation/phosphorylation of a putative CYP 7 phosphoprotein [25, 26] could, conceivably, come into play under some circumstances (cf. multifactorial regulation of HMG-CoA reductase, the key regulatory enzyme of cholesterol synthesis) [27].

Important questions remain regarding the underlying mechanisms for this multifactorial regulation of CYP 7 mRNA levels. The apparent short half-life of CYP 7 mRNA, evidenced by its diurnal rhythm, has been proposed to be a function of its long 3′-untranslated sequence (~2 kb), which contains multiple AUUA, AAU, and UAA motifs that might contribute to mRNA degradation [18]. Evidence for transcriptional regulation has been provided by nuclear run-on analyses, which suggest that transcript initiation can be stimulated up to 2- to 3-fold by long-term cholesterol feeding or by bile acid withdrawal [20]. This suggests that cholesterol, or one of its metabolites (perhaps an oxysterol), positively regulates gene transcription by directly or indirectly modulating the DNA binding affinity or activity of a transcription factor that interacts with controlling elements within the CYP 7 gene or its flanking DNA. Sterol response elements (SREs) similar to those present in the 5′-flanking DNA of HMG-CoA reductase and the low density lipoprotein (LDL) receptor genes [27] are, however, not apparent in the 5′-flanking 600 nucleotides of the rat CYP 7 gene [15].

As noted above, bile acid feedback "inhibition" of liver cholesterol 7α-hydroxylase activity is a major physiological control mechanism for bile acid biosynthesis, and is operative at the level of CYP 7 mRNA. This feedback regulation of CYP 7 mRNA expression could conceivably be mediated by specific receptor proteins/transcription factors that suppress CYP 7 gene transcription when bound by bile acids. However, while bile acids clearly effect a long-term down-regulation of CYP 7 expression *in vivo*, direct effects of bile acids on cholesterol 7α-hydroxylase activity or bile acid synthesis rates have been difficult to demonstrate in cultured cells (e.g. [24, 28]; see, however, [29]). Moreover, chronic biliary obstruction in rats (4 weeks bile duct ligation), which is associated with a significant increase in bile acids both in liver (2-fold increase) and in serum (25-fold increase), does not decrease hepatic cholesterol 7α-hydroxylase activity, but actually elevates it ~2-fold [30]. These findings raise the question as to whether the induction of CYP 7 expression following bile acid withdrawal *in vivo* might actually be driven by the associated changes in intracellular cholesterol levels, rather than removal of bile acids *per se* [31]. An alternate hypothesis is that the feedback inhibitory effects ascribed to bulk bile acids result from the action of specific (perhaps minor) bile acids not tested in the cell culture experiments and not present at sufficient levels to be inhibitory in the bile duct ligation studies. In support of this proposal is the finding that specific hydrophobic bile acids exhibit enhanced potency at down-regulating bile acid biosynthesis and cholesterol 7α-hydroxylase activity [8, 32]. Further clarification of these and related questions will require the identification of *cis*-acting regulatory elements in the CYP 7 gene and any *trans*-acting factors with which they interact to control *P*450 cholesterol 7α-hydroxylase gene expression. Preliminary studies suggest that cell lines such as human HepG2 cells [33], conditionally transformed, hepatocyte-derived rodent cell lines (G. Gil, personal communication), and a recently developed 25-hydroxycholesterol-resistant cell line that stably expresses CYP 7 mRNA and activity [34] may prove useful in this regard.

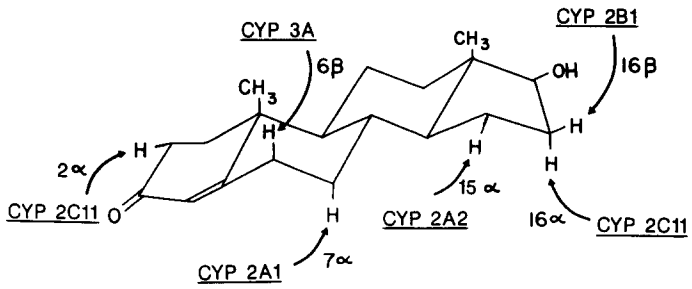

Fig. 2. Site-specificity of testosterone hydroxylation catalyzed by rat liver *P*450 enzymes. See Table 1 and Ref. [40] for additional details.

2. LIVER *P*450s ACTIVE IN STEROID HORMONE AND BILE ACID HYDROXYLATION

In addition to cholesterol, numerous other lipophilic compounds, including steroid hormones, bile acids, and fatty acids serve as important endogenous substrates of liver *P*450s [35]. Testosterone, progesterone, and related steroid hormones are hydroxylated in liver at multiple sites, principally by *P*450s belonging to gene families 2 (CYP 2) and 3 (CYP 3) (Fig. 2) (Table 1). The physiologic importance of many of these steroid hormone hydroxylation reactions is not fully understood, but may include one or more of the following: (a) hormone deactivation, (b) synthesis of novel steroids with unique biological properties or endocrine activities (e.g. CYP 2A1-catalyzed formation of 7α-hydroxytestosterone, which may help regulate testosterone production and metabolism in the testis [36, 37]), and (c) targetting of the steroid for conjugation and elimination. It is also possible that some steroid hydroxylations may be incidental activities of liver *P*450s whose primary function is the hydroxylation of other endogenous substrates, or perhaps even xenobiotic metabolism. The broad substrate specificities that are inherent to many liver *P*450s [38, 39], including those active in steroid hormone hydroxylation, support this latter suggestion. Regardless of their biological functions, the unique regio- and stereo-specificities of steroid hydroxylation exhibited by individual

Table 1. Sex-dependence of rat liver *P*450s and steroid 5α-reductase

CYP designation[a]	Trivial names[b]	Testosterone hydroxylase activities[c]	Hormonal regulation[d]	
			Androgenic imprinting[e]	Thyroid hormone[f]
1. Male-specific				
2A2	RLM2, M2	15α	++	±
2C11	2c, h, UT-A, M1, RLM5	2α, 16α	++	±
2C13	g, RLM3, M3	6β[g], 15α	++	ND
3A2	PCN2, 6β-1	6β, 2β	++	−−
4A2	IVA2, K-5	−[h]	ND	−
2. Female-specific				
2C12	2d, i, UT-I, F1	15β[i]	−−	±
3. Female-predominant[j]				
2A1	3, a, UT-F	7α	ND	−
2C7	f, RLM5b	16α	ND	++
5α-Reductase	5αR	−	−−	++

[a] Ref. [1].
[b] Designations given by various investigators to purified *P*450 protein preparations. See Refs [38, 40] for more complete listings and references.
[c] Shown are the major sites of testosterone hydroxylation catalyzed by the individual *P*450 proteins in purified, reconstituted enzyme systems. Testosterone metabolites specific to the *P*450's activity in rat liver microsomal incubations are underlined. Based on [38, 40, 62] and references therein.
[d] Influence of GH secretory patterns is summarized in Fig. 4 and is not reiterated here. "++" indicates a positive effect on adult enzyme expression, while "−−" indicates a suppressive effect. "−" indicates a lesser degree of suppression, while "±" indicates no major effect. ND—not determined in a definitive manner.
[e] Based on Refs [57, 60, 62, 73].
[f] Based on Refs [79, 82, 83, 89, 90].
[g] Although purified CYP 2C13 exhibits high testosterone hydroxylase activity, this enzyme makes marginal contributions to liver microsomal testosterone hydroxylation [125].
[h] *P*450 4A2 does not catalyze testosterone hydroxylation.
[i] 15β-Hydroxylation of steroid sulfates. CYP 2C12 also catalyzes weak testosterone 15α- and 1α-hydroxylase activities.
[j] Liver expression readily detectable in both male and female rats, but at a 3- to 10-fold higher level in females as compared to males.

liver *P*450s have proven very useful for characterizing these enzymes and for assessing the purity of isolated enzyme preparations [40]. These site-specific hydroxylations also provide catalytic monitors that differentiate between closely related liver *P*450s and can be used to assay for changes in their relative microsomal levels in response to drug exposure [40, 41] or changes in hormonal status (see below). Corresponding hydroxylation reactions in human liver microsomes [42] can also provide useful, non-invasive *in vivo* monitors [43] for hepatic monooxygenase capacity attributable to specific *P*450s. Finally, steroid hydroxylation reactions can facilitate studies on the active sites of these cytochromes, with dramatic alterations in the activity and/or site-specificity of hydroxylation sometimes occurring with changes of only 1–3 amino acid residues [44–46, 46a].

Bile acids comprise another important class of steroidal substrates of the liver *P*450s. Bile acids serve several important biological functions, including solubilization of cholesterol and phospholipids secreted into bile, and emulsification of dietary fats in the intestine, which facilitates their digestion. These functions require that the ratio of hydrophobic bile acids (those with only one or two hydroxyl groups on the steroid nucleus; e.g. lithocholic acid) to hydrophilic ones (three hydroxyl groups; e.g. cholic acid) be maintained within relatively narrow limits [35]. The formation of hydrophobic secondary bile acids by bacterial 7α-dehydroxylation in the intestine (e.g. conversion of cholate to deoxycholate, and chenodeoxycholate to lithocholate) can lead to toxicity and cholestasis unless counteracted by bile acid hydroxylation, which is liver *P*450-catalyzed. These hydroxylations are distinct from those required for the synthesis of bile acids from cholesterol, and are carried out in a species-specific manner. In rodents, bile acid 6β-hydroxylation is the most important reaction, and the resultant muricholic acids are major constituents ($\sim$20%) of the total bile acid pool [47]. Lithocholic acid is hydroxylated by rat liver microsomes predominantly at the 6β position (Fig. 3), with smaller amounts of 7α and 6α hydroxylated products also formed [48]. A hamster lithocholic acid 6β-hydroxylase, CYP 3A10, has recently been cloned and shown to be expresed in a male-specific manner in hamster liver [49]. This bile acid hydroxylase is inducible in young animals by cholic acid feeding. The same *P*450 can catalyze steroid hormone 6β-hydroxylation at a similar efficiency, despite the major differences in polarity and overall shapes of the two steroids (Fig. 3) (Chang, Teixeira, Gil, and Waxman, submitted for publication). In humans, the steroid hormone 6β-hydroxylase CYP 3A4 [42, 50]) can also metabolize lithocholic acid, but in this case the bile acid is hydroxylated at the 6α position (the major site of lithocholic acid metabolism in human liver [51]), followed by 6-*O*-glucuronidation and excretion [52]. By contrast, lithocholic acid 6β-hydroxylation in rat liver is catalyzed by *P*450(s) that are distinguishable from the major microsomal CYP 3A catalysts of steroid hormone 6β-hydroxylation [53]. Further studies are required to identify the structural features of the CYP3A enzymes that determine their site specificities for bile acid hydroxylation, and in the case of the rat enzyme, the requirements for recognition of bile acid versus neutral steroid substrates.

3. SEX-SPECIFIC EXPRESSION OF STEROID HYDROXYLASE LIVER *P*450s

Early studies on the purification and characterization of steroid hydroxylase *P*450s from rat liver led to the discovery that several of these enzymes are expressed in a sex-specific manner, subject to developmental regulation, and under endocrine control (Table 1; for reviews, see [40, 54]). For instance, CYP 2C11, the major male-specific androgen 16α- and 2α-hydroxylase of adult rat liver, is induced at puberty in male but not female rats [55, 56], as are two other male-specific steroid hydroxylase *P*450s, CYP 2A2 [57, 58] and CYP 2C13 [59, 60]. In

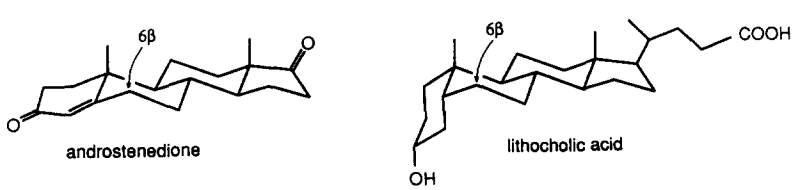

Fig. 3. Structural comparison of androstenedione and lithocholic acid. Both steroids can undergo 6β-hydroxylation reactions catalyzed by CYP 3A *P*450s, despite significant differences in the polarity and overall shape of the two steroids. Figure is from [53].

contrast, the female-specific steroid sulfate 15β-hydroxylase CYP 2C12 is induced at puberty in female rat liver [61, 62], as are the female-predominant liver enzymes CYP 2C7 (a weak steroid 16α-hydroxylase) [59, 63] and steroid 5α-reductase [62, 64], which is not a cytochrome *P*450. Other sex-dependent rat liver *P*450s exhibit somewhat more complex postnatal profiles. CYP 3A2 is an adult male-specific steroid 6β-hydroxylase that is expressed in both sexes shortly after birth, but is suppressed at puberty only in females [62, 65]. CYP 2A1 is a female-predominant steroid 7α-hydroxylase (female–male CYP 2A1 ratio = 3–4:1 at adulthood) that is expressed in both sexes shortly after birth and is suppressed at puberty to a greater extent in males than in females [62].

Studies on the endocrine regulation of these liver *P*450s have been directed toward resolution of three general questions: (1) Which hormones regulate their sex-dependent expression? (2) How do these hormones interact with each other and with the hepatocyte? By which mechanisms do they regulate *P*450 enzyme expression? These questions are not only important for our understanding of the regulation of liver steroid metabolic pathways, but also have implications with respect to the influence of hormonal status on a broad range of other reactions that can be catalyzed by these steroid hydroxylase *P*450s, including drug metabolism and carcinogen activation [64, 66, 67]. They also serve as a model system for elucidation of the endocrine axes that regulate hepatic gene expression. The following general conclusions have emerged from these studies:

(a) The sex-dependent steroid hydroxylase *P*450s are regulated at a pretranslational level, with parallel changes in liver microsomal steroid hydroxylase activity, *P*450 protein, and *P*450 mRNA generally occurring in response to changes in hormonal status (e.g. [68, 69]).

(b) The male-specific *P*450s are expressed in postpubertal animals in response to early (postnatal) androgen exposure, which *imprints* [70, 71], or programs, for later developmental changes [57, 62, 72] (Table 1). In contrast, adult androgen exposure contributes in a *reversible* manner to the maintenance of full enzyme expression at adulthood [73]. The mechanism by which neonatal androgen exposure imprints liver gene expression is still obscure, but probably involves the hypothalamus and its regulation of pituitary GH secretory patterns [74], which play a key role in regulating expression of the sex-specific *P*450 enzymes (see below). Direct effects of androgen on liver enzyme expression (i.e. effects of androgen in hypophysectomized rats) can be observed in some instances, but these are minor compared to the effects of GH.

(c) Continuous plasma GH, a characteristic of adult female rats, stimulates expression of the female-specific CYP 2C12, while intermittent GH pulsation, associated with adult male rats, induces the expression of CYP 2C11 [56, 61, 75, 76] (Fig. 4). Continuous GH can also stimulate the expression of several female-predominant enzymes, including steroid 5α-reductase, CYP 2A1, and CYP 2C7 [77–80]. The effect of intermittent GH exposure on other male-specific liver *P*450s (2A2, 2C13, 3A2, 4A2) is less clear. Expression of these CYP enzymes is not obligatorily dependent on GH pulses, when judged by their high level of expression in hypophysectomized rats of both sexes [57, 60, 81, 82]. On the other hand, the expression of these *P*450s in liver can be stimulated by intermittent GH pulses given to adult male rats depleted of circulating GH by neonatal monosodium glutamate treatment (D. J. Waxman, P. A. Ram, and B. H. Shapiro, unpublished experiments).

(d) GH can also have negative effects on liver steroid hydroxylase enzyme expression. Continuous infusion of GH markedly suppresses expression of the male-specific *P*450s (Fig. 5). This suppression cannot be attributed solely to the destruction of pulsatile plasma GH profiles that occurs when intact male rats are given GH by continuous infusion, since suppression of the male-specific *P*450s can also be observed in hypophysectomized rats given continuous GH infusion. GH suppression also occurs in the case of some of the female-predominant enzymes, in response to the male pattern of intermittent plasma GH [78, 83]. GH can also suppress the basal and/or induced levels of some of the xenobiotic inducible liver *P*450s (e.g. GH suppression of the phenobarbital-

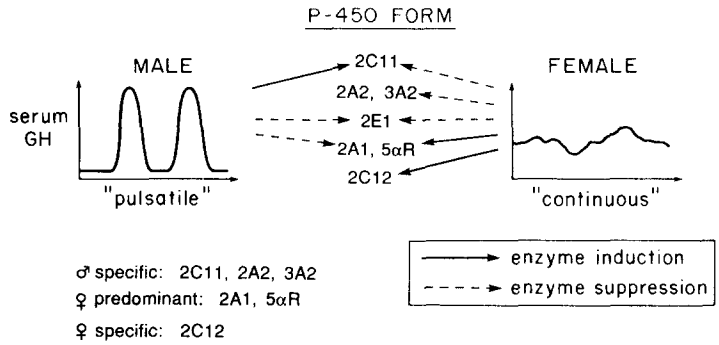

Fig. 4. Role of GH secretory profiles in the expression of rat hepatic *P*450 enzymes and steroid 5α-reductase. Influence of pulsatile vs continuous plasma GH on the expression of hepatic enzymes whose expression in adult rats is male-specific, female-predominant, or female-specific. Stimulation of enzyme expression is indicated by a solid line, and suppression of enzyme expression by a dashed line. Other pituitary-determined hormones (e.g. thyroid hormone) may be required for the full effects of GH on some of these hepatic enzymes (see text). 5αR—Steroid 5α-reductase. Figure is modified from [78].

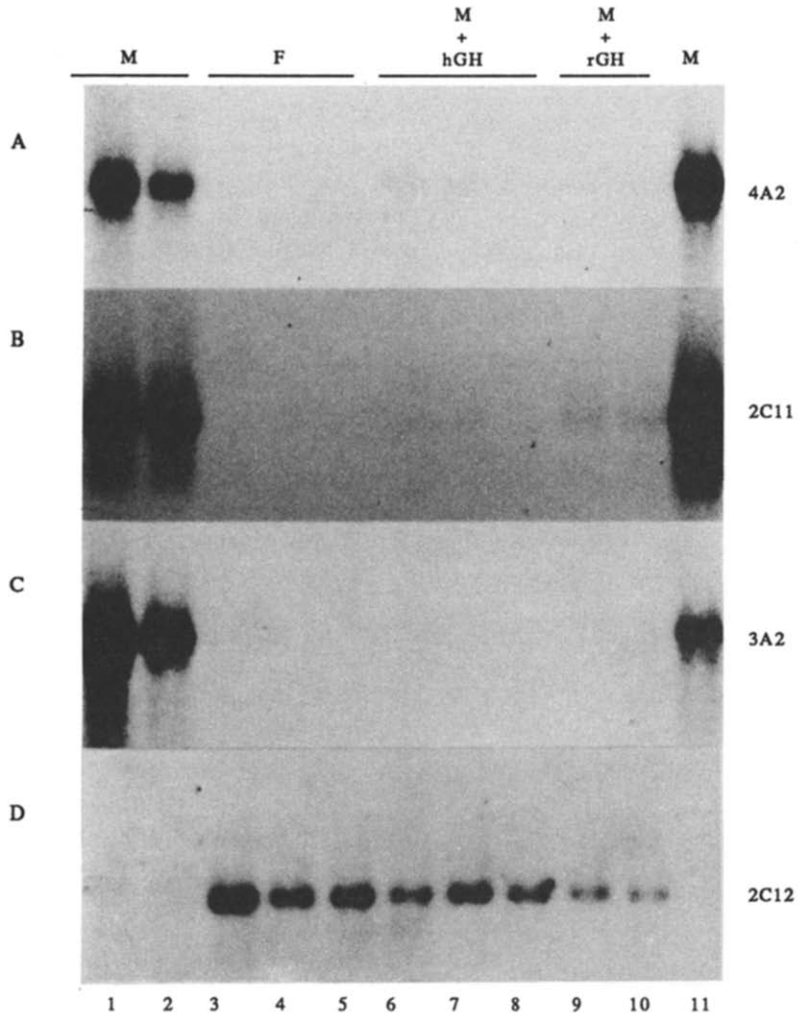

Fig. 5. Continuous GH treatment suppresses male-specific *P*450 mRNAs (A–C) while it induces the female-specific CYP 2C12 mRNA (D). Shown is a Northern blot of liver RNA samples isolated from individual adult male (M) and female (F) rats or from male rats treated with human GH (hGH) or rat GH (rGH) by continuous infusion. Blot was probed sequentially for the indicated *P*450 mRNAs. Data shown is from [82].

inducible CYP 2B1, which occurs both *in vivo* [84], and in hepatocyte cultures [85, 86]). In the case of CYP 2E1, both continuous and intermittent GH treatment suppress enzyme expression when administered to hypophysectomized rats [78, 87] (Fig. 4), albeit not when given to rats rendered GH-deficient by streptozotocin-induced diabetes [88].

(e) Although GH is the major regulator of these liver *P*450s, thyroid hormone also plays a critical role: it positively regulates some [79] but not all [83] of the female-predominant enzymes, and it negatively regulates some of the male-specific enzymes [89, 90] (Table 1). These effects of thyroid hormone, which can be demonstrated in both hypophysectomized and hypothyroid rats, are independent of the indirect effects that thyroid hormone has on liver *P*450 levels as a consequence of its effects on liver GH receptors [91] and its stimulation of GH gene transcription and GH secretion by the pituitary [92].

4. CELLULAR AND MOLECULAR MECHANISMS OF GH ACTION

4.1. Cellular actions

GH can act directly on the hepatocyte to regulate liver *P*450 expression, as demonstrated by the responsiveness of primary rat hepatocyte cultures to GH-stimulated expression of CYP 2C12 (Fig. 6) [93]. To date, however, it has not been possible to reproduce in these hepatocyte cultures the intermittent GH stimulation of CYP 2C11 expression that occurs *in vivo*. This raises the possibility that the effects of intermittent GH require the participation of additional endocrine factors not present in the cell cultures.

At the cellular level, it is highly likely that the plasma membrane-bound GH receptor [94, 95] transduces the effects of GH binding at the hepatocyte surface [95a]. This binding is associated with receptor dimerization [96], perhaps similar to that observed with several other polypeptide hormone receptors [97]. GH binding may also lead to phosphorylation on tyrosine of the GH receptor and/or other associated proteins [98], as occurs with several other members of the cytokine receptor superfamily. However, in view of the apparent absence of a tyrosine kinase domain in the GH receptor polypeptide chain, this phosphorylation is unlikely to be catalyzed by the GH receptor itself.

Studies in intact male rats have shown that the GH receptor internalizes to an intracellular compartment coincident with its stimulation by plasma GH pulses, and reappears at the cell surface at the time of the next hormone pulse [99, 100]. GH receptor immunoreactivity has also been observed in association with both the nuclear membrane and chromatin, leading to the speculation that GH might exert its action directly via these nuclear receptors [101]. Whether GH receptor internalization is ligand-driven, and the importance of this internaliz-

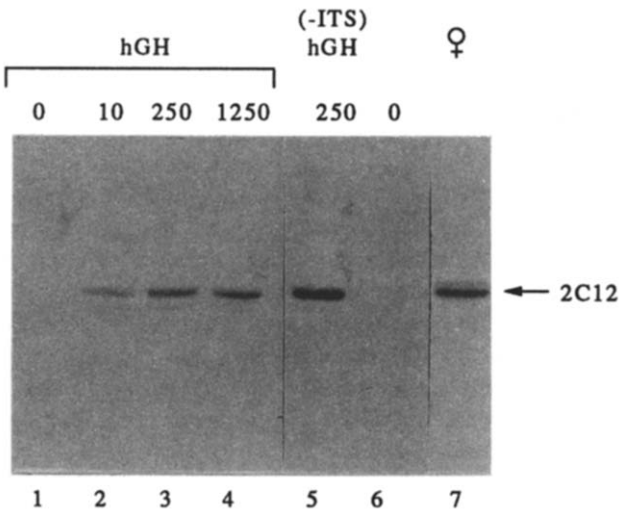

Fig. 6. GH induction of CYP 2C12 protein in primary hepatocytes cultured from an adult male rat. Detection of 2C12 was by Western blotting using an anti-CYP 2C12 antibody. Human GH (0–1250 ng/ml, as shown) was applied for 5 days to primary rat hepatocytes cultured as described [86]. Lane 7, parallel analysis of liver microsomes isolated from an untreated adult female rat. "-ITS"—Cells cultured in the absence of insulin, transferrin, and selenium.

ation for transduction of the effects of GH on liver *P*450 expression is, however, still undetermined. Studies of other GH responses indicate that activation of the GH receptor can lead to activation of protein kinase C [102–104]. Conceivably, activation of this pathway might also be important for the effects of GH on liver *P*450 expression [105]. Insulin-like growth factor I (IGF-I), which is produced in the liver in response to GH stimulation, does not mimic the effects of GH on liver *P*450s [93, 106, 107], suggesting that an autocrine mechanism does not apply.

Discrimination by the hepatocyte between the male and female plasma GH profiles is required to achieve the two dramatically different patterns of liver gene expression that GH can elicit. This discrimination may occur at the cell surface, which expresses a greater level of GH receptors in female as compared to male rats ([108]; see, however, [109]), or it may involve differences in the intracellular signalling pathways elicited by a chronic (female) versus an intermittent (male) pattern of GH stimulation. In order to address these issues, studies have been carried out to determine which of the three descriptive features of a GH pulse—namely, pulse height, duration, and frequency—is required for proper recognition of a GH pulse as masculine. Direct measurement of the actual plasma GH profiles achieved when GH is administered to hypophysectomized rats by twice daily s.c. GH injection (i.e. the intermittent GH replacement protocol most commmonly used to stimulate CYP 2C11 expression) has revealed broad peaks of circulating GH, which last as long as 5–6 h [76]. Since these GH "pulses" effectively stimulate expression of the male-specific CYP 2C11, it is apparent that GH pulse duration need not be tightly regulated to elicit this response from the hepatocyte.

Studies using a monosodium glutamate (MSG)-treated rat model have provided insight into the requirements with respect to GH pulse height. Neonatal treatment of rats with MSG destroys the neurons in the arcuate nucleus of the hypothalamus and their ability to stimulate secretion of GH-releasing factor, which normally triggers pituitary GH release [110, 111]. Consequently, neonatal MSG treatment eliminates circulating GH at adulthood and abolishes liver 2C11 expression [112], as well as the expression of several other male-specific liver *P*450s [113]. When MSG is administered at a sub-maximally effective dose, however, full expression of CYP 2C11 occurs, even though plasma GH peaks are reduced by up to 90% (intermittent GH peaks of 20–25 vs 200–250 ng/ml peaks in normal rats) (Fig. 7). Thus, GH pulse height is also not a critical factor for stimulation of CYP 2C11 expression. This finding can be understood in terms of the K_d of the GH–GH receptor complex, which at 10^{-10} M (2 ng/ml) [94], is only 1% of the usual peak plasma hormone level. The observation that GH-deficient dwarf rats (NIMR/AS) express normal levels of 2C11 and other sex-dependent liver *P*450s led some investigators to propose that GH might not be the pituitary factor responsible for regulation of these steroid hydroxylases [114]. However, a more likely explanation is that the level of circulating GH in these rats, while low (plasma peaks up to 15 ng/ml; [115]), is sufficient to sustain normal *P*450 profiles. This suggestion is given strong support by the findings of the MSG study discussed above [112].

Experiments carried out in MSG-treated female rats have led to an unexpected finding: elimination of detectable circulating GH (< 2 ng/ml) is not associated with any decrease in liver expression of the female-specific CYP 2C12 or steroid 5α-reductase [113]. This result contrasts with the hypophysectomy and GH-replacement experiments summarized above, which indicated that expression of these enzymes is obligatorily dependent on continuous GH exposure. One possible explanation of the MSG finding is that exceedingly low levels of GH are sufficient to maintain full expression of these female enzymes. Alternatively, additional factors that have yet to be identified play an important role in the regulation of these female-dominant enzymes.

The importance of GH pulse frequency for stimulation of a male pattern of liver *P*450 expression has been examined in hypophysectomized rats given defined GH pulses of regular frequency using an electrically controlled syringe pump [76]. Pulsations of 6 and 7 times/day, which approximate the physiological frequency, as well as pulsations of lesser frequencies, could be produced. Analysis of liver *P*450 expression after 7 days GH pulsation revealed that GH frequencies of 2, 4, and 6 pulses/day effectively stimulated male *P*450 gene expression, whereas the 7 pulse/day rats did not respond. Thus, interpeak trough times of no detectable circulating GH that differ by as little as 35 min (i.e. 6 vs 7 GH pulses/day) give

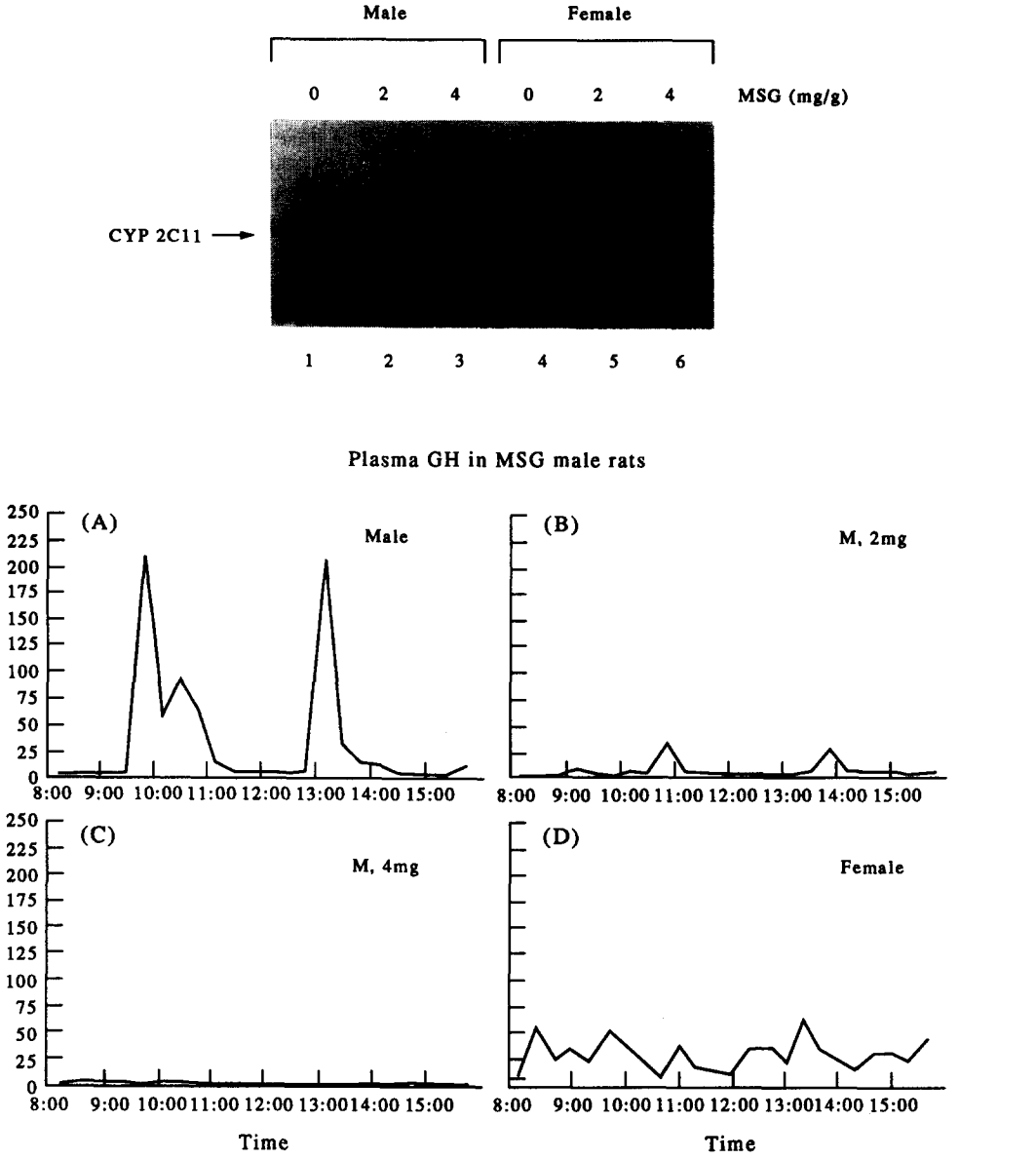

Fig. 7. Influence of neonatal MSG treatment on plasma GH levels (ng/ml, on *y*-axis) in adult male (A–C) and female rats (D) and on CYP 2C11 protein levels (Western blot). Neonatal treatment with MSG at a dose of 2 mg/g greatly reduces plasma GH peak levels at adulthood (B) without major effect on CYP 2C11 protein expression. MSG at a dose of 4 mg/g eliminates detectable plasma GH (C) and 2C11 protein expression. Based on data reported in [112].

rise to strikingly different patterns of liver gene expression. Accordingly, while GH pulse height and duration are not critical, if GH pulsation becomes too frequent, the hepatocytes loose their ability to recognize the pulse as "masculine". The cells thus require a minimum GH off-time (~2.5 h in this hypophysectomized rat model), which implies a need for an obligatory recovery period, a condition not met in the case of hepatocytes exposed to GH continuously (female profile). This recovery period may be required to reset an intracellular signalling apparatus, or perhaps may allow for replenishment of cell surface GH receptors. Interestingly, different pulse frequency requirements were observed for GH stimulation of normal male growth rates in these same hypophysectomized rats, a response which probably involves GH action at one or more extrahepatic sites [76]. Thus, distinct GH-responsive tissues, and conceivably even different GH responses within the same cell, may recognize distinct signalling elements in the sexually dimorphic patterns of circulating GH.

4.2. Molecular mechanisms

As noted above, GH regulates steroid hydroxylase *P*450 expression at the level of steady-state mRNA (pretranslational regulation). In the case of CYP 2C12 mRNA, induction by continuous GH exposure requires ongoing protein synthesis, as indicated by the inhibitory effects of cycloheximide on CYP 2C12 mRNA accumulation in primary hepatocyte cultures [107]. Reports in the literature are conflicting with respect to whether this regulation involves transcriptional control of the *P*450 genes by GH, or whether posttranscriptional mechanisms also play a role (cf. [116] vs [68] and [117]). These reports, however, base their conclusions on nuclear run-on transcription analyses, which can be subject to erroneous interpretation if the hybridizations are not carried out under conditions of DNA excess [117], if background radioactivity contributes significantly to the hybridization signals, or if cross-hybridization of the DNA probes to related RNA sequences occurs. The question of whether GH regulates CYP 2C11 and 2C12 gene transcription was recently examined in our laboratory by analyzing nuclear RNA precursors of the CYP 2C11 and 2C12 mRNAs using exon/intron junction probes in an S1 nuclear protection assay [118]. These studies established that the unprocessed, nuclear 2C11 and 2C12 RNAs (hnRNA) are responsive to circulating GH profiles in a manner indistinguishable from the corresponding mature, cytoplasmic mRNAs: no 2C12 mRNA precursors were detected in untreated male or hypophysectomized female liver nuclei (Fig. 8), and no

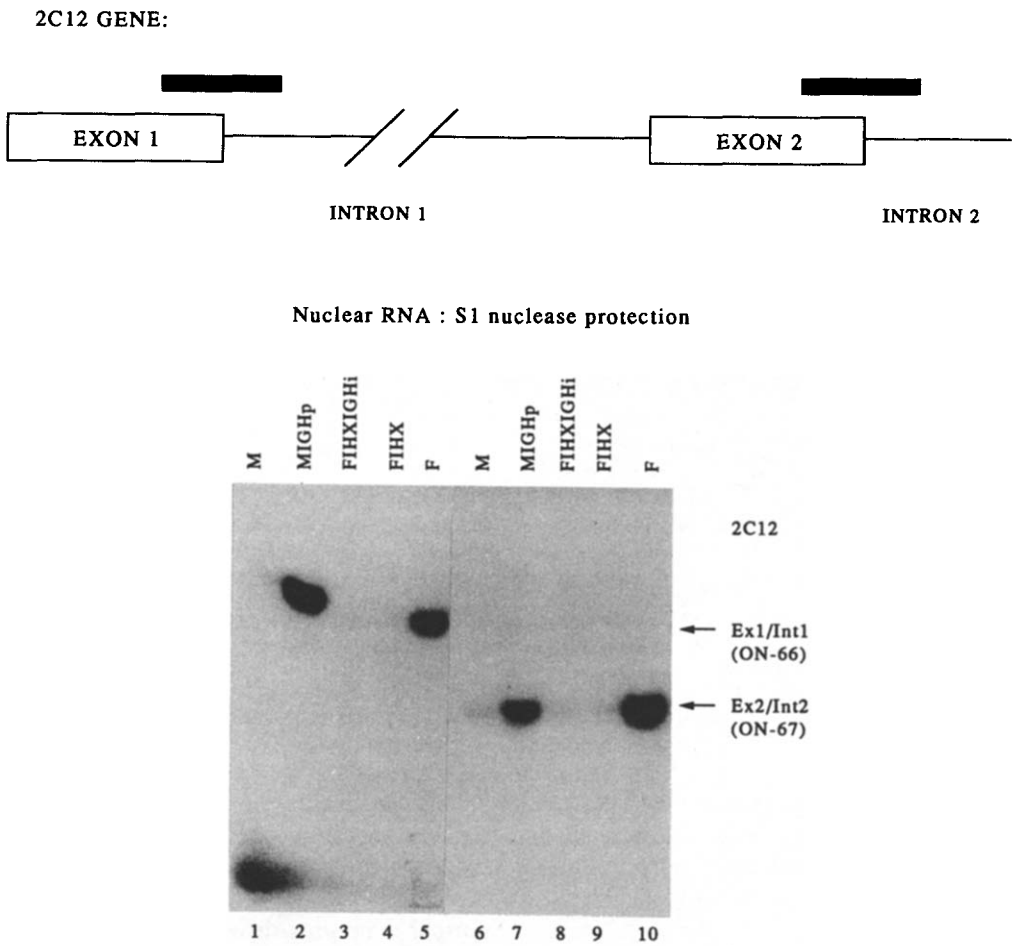

Fig. 8. Analysis of unprocessed CYP 2C12 nuclear RNA (hnRNA) by S1 nuclease protection using exon/intron junction oligonucleotide probes. Data shown are from [118] and evidence a female-specific, GH-dependent expression of these unprocessed transcripts of the 2C12 gene. M/GHp, GH given to intact male rats by continuous infusion using an osmotic minipump. F/HX/GHi, GH given by intermittent injection to a hypophysectomized female rat. The representation of the CYP 2C12 gene shown at the top of the figure is not drawn to scale. Horizontal bars correspond to the oligonucleotide probes. Hybrids shown in the autoradiograph at the bottom are of the lengths expected for uncleaved probes ON-66 and ON-67 (26 and 20 nucleotides, respectively).

2C11 mRNA precursors were found in untreated female or hypophysectomized male liver nuclei. Thus, transport of 2C11 and 2C12 mRNA to the cytoplasm, and cytoplasmic mRNA stability are unlikely to be important GH-regulated control points for sex-specific *P*450 RNA expression. These conclusions were supported by nuclear run-on transcription analyses, which, additionally, provided firm evidence that GH regulates the sex-specific expression of the CYP 2C11 and CYP 2C12 genes at the level of transcript initiation [118]. Transcription was also shown to be the major step for regulation of CYP 2A2 RNA, whose male-specific expression appears to be primarily a consequence of the suppressive effects of continuous GH exposure in adult female rats [57]. Transcription initiation is thus the step at which three distinct effects of GH are operative: stimulation of 2C11 expression by pulsatile GH, suppression of 2A2 (and 2C11) expression by continuous GH, and stimulation of 2C12 expression by continuous GH [118] (cf. Fig. 4).

In order to further explore the molecular mechanisms that control the GH responsiveness of the CYP 2C11 gene [119] and the CYP 2C12 gene [117], cloned 5′-flanking DNA fragments of these genes have been analyzed by DNase I footprinting for a differential interaction of either gene with nuclear proteins (putative transcription factors) present in male vs female rat liver [118]. Several footprints were observed in the upstream few hundred nts of each gene, but without major differences between the male and female extracts. However, sex- and GH-dependent differences in DNase I cleavage patterns ("hypersensitivity" sites) were observed at several sites, indicating that GH can regulate specific protein–DNA interactions in the 5′-flanking regions of both genes. These differential binding interactions were hypothesized to contribute to the sex-specific transcription of the CYP 2C11 and CYP 2C12 genes [118] (cf. GH-regulated binding of a liver nuclear factor to 5′-flanking DNA of the GH-inducible rat serine protease inhibitor (Spi) 2.1 gene; [120]). *In vitro* transcription assays employing CYP 2C11 and 2C12 5′-flanking DNA sequences did not, however, faithfully mimic the sex-specific transcription of the 2C11 and 2C12 genes, indicating that additional *cis*-elements, *trans*-acting factors, or perhaps a higher-order chromatin structure may be required to achieve the transcriptional regulation of these genes that occurs *in vivo* [118]. More detailed molecular studies will be required to identify the full range of positive and negative components of the 2C11 and 2C12 transcription machinery and their responsiveness to plasma GH patterns in order to fully elucidate the mechanisms by which GH regulates the sex-dependent expression of these liver *P*450 genes.

5. THYROID HORMONE REGULATION OF NADPH *P*450 REDUCTASE

Liver steroid hydroxylase activity is not only dependent on the level of expression of individual steroid hydroxylase *P*450s, but also is influenced by the activity of NADPH *P*450 reductase; this obligatory, and often rate-limiting, electron-transfer flavoprotein is required for all microsomal *P*450-catalyzed steroid hydroxylations. Studies on the endocrine regulation of liver microsomal NADPH *P*450 reductase have established that thyroid hormone (T4, thyroxine) plays a major role in regulating *P*450 reductase enzyme expression,

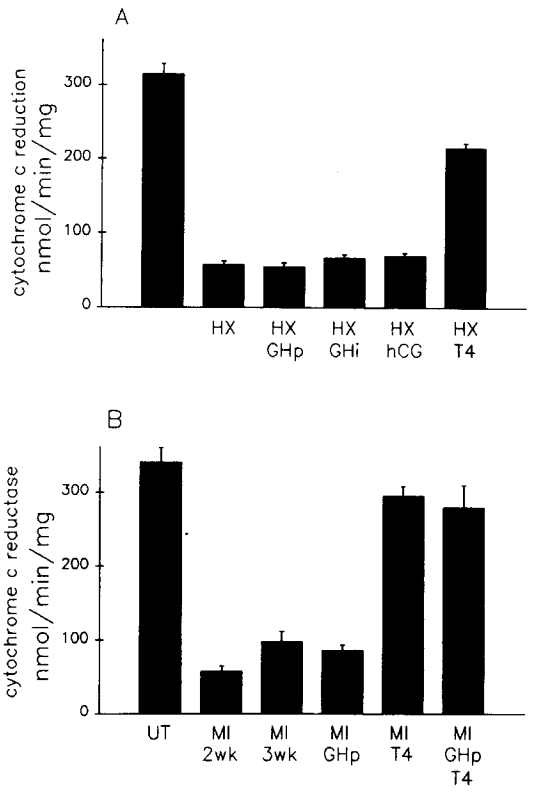

Fig. 9. Influence of thyroid hormone status on liver NADPH *P*450 reductase activity, as judged by hypophysectomy (HX) (A) or methimazole-induced hypothyroidism (MI) (B) (treatment for a total of 2 or 3 weeks, as indicated), followed by replacement of thyroxine (T4) or other pituitary-dependent hormones. GHp, GH given by continuous infusion using an osmotic minipump. GHi, GH given by intermittent s.c. injection, twice daily. Figure based on data presented in [121, 122].

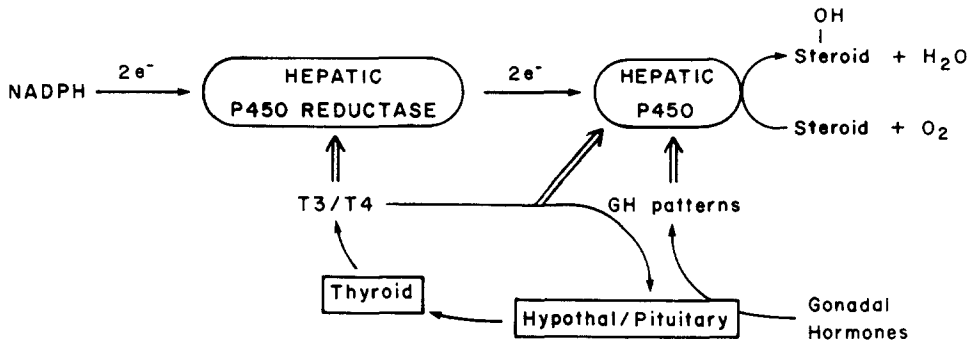

Fig. 10. Regulation of liver *P*450-catalyzed steroid hydroxylation by multiple endocrine factors acting in an interactive and interdependent fashion.

with a major decrease (≥80%) in liver microsomal *P*450 reductase activity effected by hypophysectomy or by methimazole-induced hypothyroidism. This activity loss is substantially reversed by physiological replacement of T4, but not by replacement of GH or other pituitary-dependent factors (Fig. 9) [121, 122]. Moreover, T4-stimulated restoration of liver *P*450 reductase activity *in vivo* substantially increases microsomal steroid hydroxylase activities, and restores indvidual liver *P*450s to their initial specific activities. A similar effect can be achieved by supplementation of isolated liver microsomes with exogenous, purified *P*450 reductase, which preferentially stimulates steroid hydroxylation catalyzed by microsomes prepared from thyroid-deficient animals [121].

Further studies have revealed that *P*450 reductase protein and mRNA are also decreased significantly in the hypothyroid state, and are restored by thyroxine treatment, providing evidence for pretranslational regulation [122]. Interestingly, 1 h after thyroid hormone injection of hypothyroid rats, liver *P*450 reductase protein and activity are elevated *prior to* any detectable increase in the underlying mRNA. This suggests that thyroid hormone enhances the translatability of *P*450 reductase mRNA (cf. [123]), or, alternatively, that it stabilizes preexisting *P*450 reductase protein. This latter effect could be due to an increase in the availability of the reductase's FMN and FAD cofactors, whose levels are decreased in hypothyroid rat liver [124]. Further complications have been observed when a hyperthyroid state is induced by triiodothyronine (T3)-treatment of euthyroid rats. In this case a 12-fold elevation of *P*450 reductase RNA is achieved, but this does not translate into significant induction of *P*450 reductase protein or activity [122]. Since *P*450 reductase mRNA can be readily induced by thyroid hormone treatment of primary hepatocyte cultures (Ram and Waxman, unpublished experiments), it may be possible to use this cellular system to study in greater detail some of the complex regulatory effects that thyroid hormone confers on *P*450 reductase.

In conclusion, steroid hydroxylation by liver *P*450s contributes in a major way to a number of important metabolic pathways, including cholesterol metabolism, bile acid synthesis and metabolism, and steroid hormone hydroxylation. Each of these enzymes and pathways is subject to unique regulatory controls. In the case of the sex-dependent steroid hormone hydroxylase *P*450s, GH secretory patterns and thyroid hormone levels are the most important endocrine regulators, but an underlying role for gonadal imprinting of adult hypothalamopituitary function is also evident (Fig. 10). GH regulates the sex-specific expression of liver *P*450s through transcriptional mechanisms, while thyroid hormone exerts a multiplicity of effects: on *P*450 reductase, which is a rate-limiting component of the overall hydroxylation pathway, at the level of the pituitary, through its positive effects on GH secretion, and through its direct effects on expression of individual cytochrome *P*450 genes.

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