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Interpulse interval in circulating growth hormone patterns regulates sexually dimorphic expression of hepatic cytochrome P450

(liver gene expression/pulsatile pituitary hormone secretion)

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ABSTRACT **Plasma growth hormone (GH) profiles are sexually differentiated in many species and regulate the sex-dependence of peripubescent growth rates and liver function, including steroid hydroxylase cytochrome P450 expression, by mechanisms that are poorly understood. By use of an external pump to deliver to hypophysectomized rats pulses of rat GH of varying frequency and amplitude, a critical element for liver discrimination between male and female GH patterns was identified. Liver expression of the male-specific steroid 2α(or16α)-hydroxylase P450, designated CYP2C11, was stimulated by GH at both physiological and nonphysiological pulse amplitudes, durations, and frequencies, provided that an interpulse interval of no detectable GH was maintained for at least 2.5 hr. This finding suggests that hepatocytes undergo an obligatory recovery period after stimulation by a GH pulse. This period may be required to reset a GH-activated intracellular signaling pathway or may relate to the short-term absence of GH receptors at the hepatocyte surface after a cycle of GH binding and receptor internalization. These requirements were distinguished from those necessary for the stimulation by GH of normal male growth rates in hypophysectomized rats, indicating that different GH responses and, perhaps, different GH-responsive tissues recognize distinct signaling elements in the sexually dimorphic patterns of circulating GH.**

Episodic secretion is a general characteristic of many hormones, including pituitary-dependent hormones, pancreatic islet, and parathyroid hormones (1), and is often crucial to the triggering of hormone-dependent responses in target cells. For growth hormone (GH), secretion by the pituitary is not only intermittent, or pulsatile, but also the frequency of pulsation is sex-dependent. In many species, including the rat, chicken, and human (2–5), pituitary GH secretion is more frequent in females than in males. In the case of the female rat, a high pulse frequency results in GH present in circulation continuously, at levels ≥ 10–20 ng/ml of plasma. This situation contrasts with the intermittent presence of GH in plasma of male rats (2, 6). Studies in rats (7–10) and mice (11–13) have demonstrated that the expression of a number of sexually differentiated hepatic proteins, including cytochrome P450 (P450)-linked steroid hydroxylases and drug-metabolizing enzymes, is primarily determined by plasma GH profiles and only secondarily regulated by the gonadal hormones through their effects on the hypothalamo–pituitary axis and its control of GH secretion (14–16).

The plasma GH profile in a male rat is characterized by a pulse of 200–250 ng of GH per ml every 3–4 hr followed by a 2- to 2.5-hr period when circulating GH is nearly undetectable (≤2 ng/ml). It is unclear, however, just what features in this pattern are recognized as male by the hepatocyte. This critical question is addressed by the present study, which uses a recently developed external pump apparatus (17) for generation of periodic GH pulses of various frequency and amplitude in hypophysectomized rats. Our findings lead us to conclude that neither the peak height, width, nor frequency of circulating GH pulses is critical, but rather a minimum length of trough period is key to the recognition of a GH pulse as masculine by adult rat hepatocytes. The significance of this observation is discussed in the context of possible mechanisms by which GH secretory patterns regulate liver gene expression.

MATERIALS AND METHODS

Animals. Adult intact and hypophysectomized male and female Sprague–Dawley rats were obtained from Charles River Breeding Laboratories. The animals were housed under conditions of regulated temperature (20–23°C) and photoperiod (12 hr of light/12 hr of darkness; lights on at 0800 hr) to minimize interanimal variation in GH secretory profiles, which are light entrained (2). Rats were hypophysectomized by the supplier at the age of 8 weeks and were maintained for 4–5 weeks before doing the GH-replacement experiments. The effectiveness of hypophysectomy was verified by the absence of weight gain over this period. Hormone-replacement experiments with rat GH (rGH) (1.8 international units/mg; National Hormone and Pituitary Program) used three different protocols: (*i*) continuous s.c. infusion with an osmotic minipump (model 2001, Alza) delivering 20 ng of rGH per g of body weight (BW) per hr for 7 days; (*ii*) s.c. injection at 12-hr intervals at 115–123 ng of rGH per g of BW per injection, as specified in the text; (*iii*) periodic injection via a chronic indwelling right atrial catheter implanted and controlled by an external syringe pump, as described (17). For this last protocol, injections were applied as 3-min pulses at intervals ranging from 3 hr and 25 min to 12 hr and at doses of 16–118 ng of rGH per g of BW per injection, as specified in the text. Eight-hour plasma GH profiles were determined by using an RIA with a sensitivity of 2–3 ng/ml (18). Repetitive blood sampling (0.025 ml) at 15-min intervals was done on unrestrained, unstressed, and completely conscious rats outfitted with a mobile catheterization apparatus (19). Plasma GH profiles monitored on days 3 and 6 of the 7-day GH treatment period were indistinguishable.

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Abbreviations: GH, growth hormone; rGH, rat GH; BW, body weight; P450, cytochrome P450.

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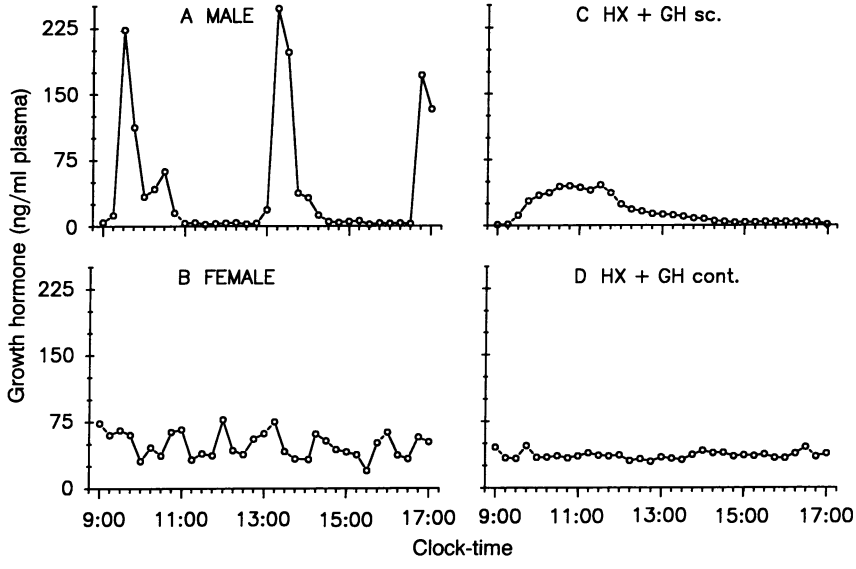

FIG. 1. Plasma GH profiles in adult male (*A*) and female rats (*B*) and in hypophysectomized (Hx) male rats treated with rGH by s.c. injection twice daily (*C*) or by continuous infusion (*D*). These plasma GH profiles are representative for individual intact rats and for hypophysectomized rats administered rGH for 7 days by s.c. injection, twice daily at 115 ng per g of BW per injection, or by continuous s.c. infusion with an osmotic minipump.

P450 Activity and mRNA Determinations. Liver microsomes were isolated, and testosterone hydroxylase and 5α-reductase activities were assayed, as described (10). P450 mRNA levels were monitored by Northern (RNA) blotting using ^{32}P-labeled oligonucleotide probes specific for individual P450s with methods described in detail elsewhere (20, 21).

RESULTS

As an initial approach to elucidating the cellular mechanisms that enable hepatocytes to discriminate between male and female GH secretory profiles, we undertook to identify which of the salient features of a GH pulse—namely, its amplitude, duration, and/or interpulse interval—are the most crucial for stimulating the expression of a male-specific P450 steroid 2α(or16α)-hydroxylase, designated CYP2C11 [according to the recently adopted systematic nomenclature (22)]. Eight-hour plasma GH profiles of intact adult male and female rats are compared (Fig. 1 *A* and *B*) with GH profiles determined for hypophysectomized adult male rats treated with rGH by using either an intermittent or a continuous hormone replacement regimen (Fig. 1 *C* and *D*). GH infusion using an osmotic minipump yielded a continuous presence of GH in plasma at levels comparable to those found in untreated adult female rats (Fig. 1*D*). This treatment stimulates expression of several female-predominant hepatic enzymes (23–25). By contrast, intermittent GH injection (twice daily s.c.), commonly used to mimic the pulsatile GH pattern present in untreated male rats, stimulates the expression of the male-specific CYP 2C11 (refs. 7–10; also see below). Fig. 1*C* shows, however, that the resultant plasma GH profile is nonphysiological, not only with respect to the frequency of the pulse but also the duration of GH exposure, which continued for at least 4 hr per injection.

To address the question of whether CYP 2C11 gene expression in intact male rats is stimulated by the natural GH pulses characteristic of this sex (e.g., Fig. 1*A*), we used a timer-controlled external syringe pump to generate regular, physiological pulses of GH in hypophysectomized rats at intervals of 3 hr and 25 min, 4 hr, 6 hr, or 12 hr (i.e., seven, six, four, and two GH pulses per day). Analysis of the resultant plasma GH profiles in both male (Fig. 2) and female rats (data not shown) confirmed the effectiveness of the external pump for delivering reproducible amounts of GH at regular intervals. Physiological plasma GH levels (peaks of 200–250 ng of GH per ml) were achieved by administering a dose of 30–40 ng of rGH per g of BW per pulse; GH doses 2-fold lower yielded plasma GH peaks of 80–100 ng/ml, whereas 2-fold higher GH doses increased peak hormone levels to the 400–600 ng/ml range, without noticeable effect on pulse duration (data not shown). GH pulses administered in this manner were effective in stimulating normal male growth rates (i.e., weight

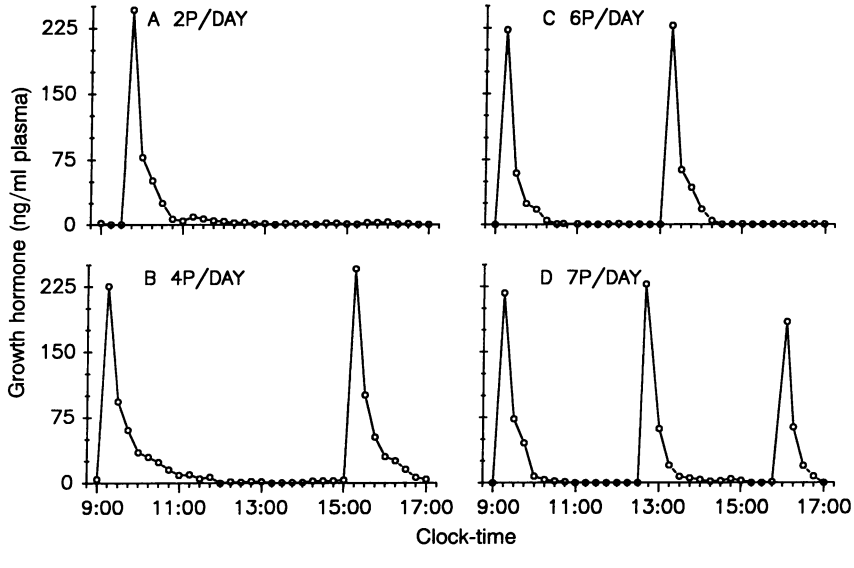

FIG. 2. Plasma GH profiles in hypophysectomized male rats administered GH by external pump. These GH profiles are representative for individual hypophysectomized male rats receiving GH pulses (P) (30–32 ng of rGH per g of BW per injection) two, four, six, or seven times daily for 7 days (*A–D*, respectively). Equivalent GH patterns were seen in GH-treated hypophysectomized female rats studied in parallel (data not shown).

Table 1. Influence of hypophysectomy and GH replacement on body weight (BW) gains

	BW gain, g per rat per week	
	Male	Female
Intact*	19.6 ± 3.1	7.8 ± 1.3†
Hypox	(−)0.4 ± 1.8†	0.3 ± 0.5†
Hypox + catheterization	(−)2.9 ± 2.2†	(−)3.1 ± 2.1†
Hypox + 7 P per day	18.2 ± 3.6	21.6 ± 3.5
Hypox + 6 P per day	19.3 ± 4.8	17.3 ± 3.8
Hypox + 4 P per day	16.8 ± 3.7	21.5 ± 2.3
Hypox + 2 P per day	19.8 ± 4.1	16.7 ± 1.4
Hypox + 2 s.c. per day	14.2 ± 3.8	14.8 ± 3.2
Hypox + continuous infusion	9.4 ± 2.2†	10.0 ± 2.1†

The values are expressed as mean ± SD BW gain for at least 3 rats per group. Intact and hypophysectomized (Hypox) rats were untreated or were catheterized and then given two, four, six, or seven daily pulses (P) of rGH for 7 days at 30–40 ng per g of BW per pulse. Twice daily s.c. injections and continuous GH infusion were as described.

*With or without catheterization.

†$P < 0.01$ when compared with intact male rats. All groups were significantly different from intact females at $P < 0.01$, except for the Hypox + continuous infusion male and female groups. All groups were significantly different from the Hypox males and females (with or without catheterization) at $P < 0.01$.

gain) in both male and female hypophysectomized rats, independent of whether the hormone was applied two, four, six, or seven times per day (Table 1). In contrast, a lower, female-like growth rate was achieved when GH was administered as a continuous infusion with an osmotic minipump (Table 1).

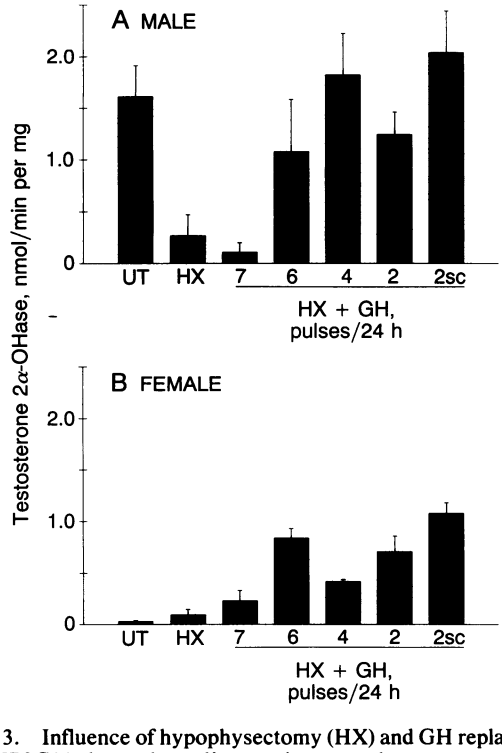

FIG. 3. Influence of hypophysectomy (HX) and GH replacement on CYP2C11-dependent liver microsomal testosterone 2α-hydroxylase activity. GH was administered to hypophysectomized male and female rats for 7 days by external pump at a frequency of two, four, six, and seven pulses per day or by s.c. (sc) injection twice per 24 hr, as indicated. Liver microsomal activities (mean ± SD) were determined for $n = 3$–4 male rats per group (*A*) or for $n = 2$–4 female rats per group (*B*), and are both in units of nmol of 2α-hydroxytestosterone formed per min per mg of microsomal protein. Indistinguishable profiles were obtained when CYP2C11-dependent testosterone 16α-hydroxylase activity (15) was assayed. UT, untreated rats.

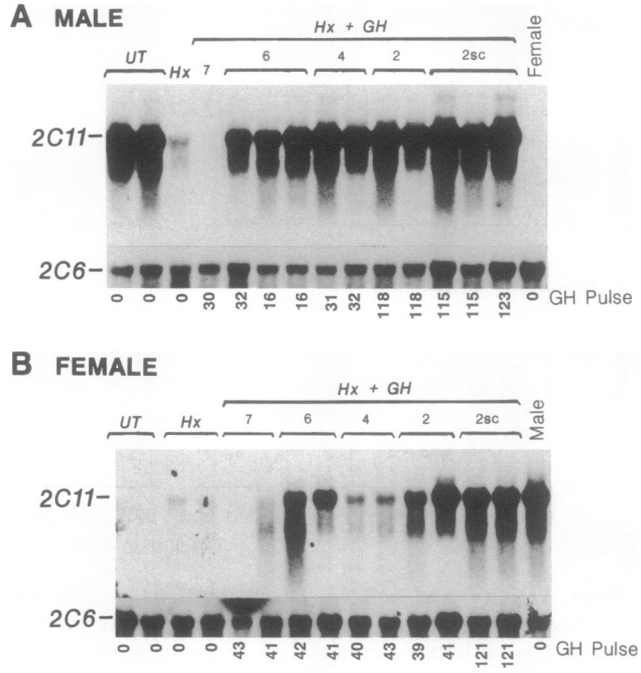

FIG. 4. Northern blot analysis of CYP2C11 mRNA. Total liver RNA isolated from individual male (*A*) and female (*B*) rats was analyzed on Northern blots probed with ^{32}P-labeled oligonucleotide probes specific for CYP2C11 and for CYP2C6, which served as a positive control for RNA integrity and loading. Hypophysectomized rats (Hx) were treated with two to seven GH pulses per day, as indicated, and at the indicated GH doses (ng of rGH per g of BW per pulse or per s.c. injection).

The effects of these GH treatments on liver CYP2C11 expression were analyzed by monitoring CYP2C11-catalyzed liver microsomal testosterone 2α-hydroxylation (Fig. 3). Several individual liver samples were also analyzed for CYP2C11 mRNA levels (Fig. 4), which in all cases paralleled the activity values. As reported (7–10), CYP2C11 expression diminished greatly after hypophysectomy of male rats and was nearly undetectable in intact and hypophysectomized female rat liver. CYP2C11 expression in the hypophysectomized males was markedly stimulated by GH pulses delivered at the *in vivo*-like frequency of six times daily, as well as by the less frequent, nonphysiological frequencies of two and four times daily. Similar responses were obtained in hypophysectomized female rats, although the magnitude of the increase was somewhat lower than that seen in the males (cf. Figs. 3*A* and 3*B*). Comparable stimulatory responses were achieved over a 7-fold range of GH doses per injection and over a 3-fold range of total daily GH exposure in experiments done at two, four, and/or six GH pulses per day (Fig. 4 and data not shown). Surprisingly, however, GH pulsation at a frequency of seven per day was ineffective or only marginally effective (Figs. 3 and 4) when tested in five independent experiments, despite the fact that both six and seven daily GH pulses stimulated normal male growth rates in the same groups of animals (Table 1). The dramatic difference in the responsiveness of CYP2C11 to six vs. seven GH pulses per day (differing by only 35 min in interpulse interval) strongly suggests that to signal a male liver response, the minimal plasma GH trough time is highly restrictive, and in the seven pulses per day hypophysectomized animals the trough may simply be too short to masculinize the liver with respect to CYP2C11 expression.

Because seven regular GH pulses per day were not recognized by the hepatocyte as masculine, we examined

whether this pulsation pattern presents to the liver signaling elements that are recognizable as feminine. Two responses that are conferred by a female (i.e., continuous) GH pattern were monitored: (*i*) induction of the female-predominant liver enzyme steroid 5α-reductase and the female-specific CYP2C12 (23–25) and (*ii*) suppression of CYP3A2 and CYP2A2, adult male-specific P450s distinguished from CYP2C11 by their apparent lack of dependence on GH pulsation for full expression (10). In these studies, seven daily GH pulses increased steroid 5α-reductase expression in hypophysectomized male rat liver much less effectively than did continuous GH infusion (Fig. 5*A*). Moreover, seven daily GH pulses were unable to induce CYP2C12 mRNA in the hypophysectomized rats, even though continuous GH treatment effected a substantial elevation of this mRNA (data not shown). GH pulsation at seven per day was also much less effective than continuous GH treatment with respect to suppression of CYP3A2 and -2A2 activities and mRNAs (Fig. 5*B* and data not shown). Thus, the full feminizing effects of GH on hepatic gene expression are not imparted by the profile of seven daily GH pulses and are only realized when GH is present in circulation on a continuous basis.

DISCUSSION

GH secretory patterns are sexually differentiated in rats and other species and confer sex-dependent patterns of liver gene expression. These effects are particularly striking in rodent liver (7–13), where they play a major role in the sexual differentiation of several important physiological pathways and other processes, including steroid hormone biotransformation, drug metabolism and pharmacokinetics, and chemical carcinogen bioactivation (14, 15, 28, 29). The cellular and molecular mechanisms by which GH secretory patterns regulate liver gene expression are, however, largely unknown. The fact that in male rats the pituitary secretes GH in regular pulses separated by undetectable baselines, in contrast to the more constant GH secretion characteristic of females, has led to the reasonable, but unproven, hypothesis that the discrete pulses of GH are responsible for expression of the male phenotype (6, 14). In this study, we established that regular GH pulses generated in hypophysectomized rats markedly stimulate liver expression of the male-specific CYP-

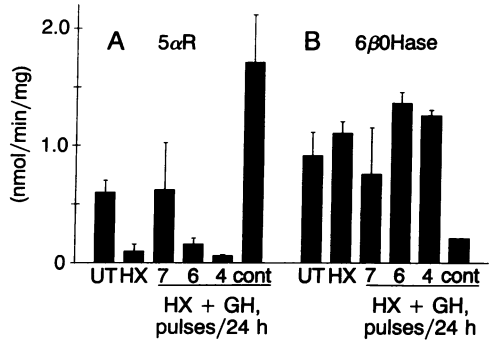

FIG. 5. Incomplete feminization of hepatic enzyme expression in hypophysectomized male rats by seven GH pulses per day. Feminization was monitored in isolated liver microsomes by the elevation of the female-predominant testosterone 5α-reductase activity (5αR) (*A*) and by the suppression of CYP3A2-dependent testosterone 6β-hydroxylase (6βOHase; male-specific activity) (*B*). Rat treatments and analysis of microsomal enzyme activities were as for Fig. 3. cont, continuous GH treatment, as in Fig. 1*D*. UT, HX, untreated and hypophysectomized male rat liver microsomes. Activities shown can be compared to those of intact female liver microsomes: 7.9 ± 3.7 and 0.13 ± 0.04 nmol/min per mg of protein for testosterone 5α-reductase and 6β-hydroxylase, respectively ($n = 4$). The incomplete feminization of these activities in the continuous GH treatment group shown in this figure reflects the additional requirement of thyroxine for the full effect of GH (26, 27).

2C11. However, this masculinization of liver gene expression does not require a physiological GH pulse amplitude or pulse duration. This fact is indicated by the effectiveness (Figs. 3 and 4) of both high and low peaks of GH generated by external pump, by the effectiveness of broad GH "pulses" generated by s.c. GH injections (Fig. 1*C*), and by our earlier finding that GH peak heights can be decreased by up to 90% in monosodium glutamate-treated rats without impairing the expression of CYP2C11 (18, 30). The effectiveness of low-amplitude GH pulses may be understood in terms of the high affinity of the GH receptor for GH, $K_d = 10^{-10}$ M (31), which corresponds to half-maximal saturation of the receptor at a plasma GH concentration of only 2 ng/ml.

Masculinization of liver P450 expression also does not require the specific GH pulse frequency of six or seven per day that occurs in intact, adult male rats (2, 6, 18) but, rather, appears dependent on a minimum interpulse trough interval. The present studies show that in the case of a hypophysectomized rat model, the interpulse trough interval must be at least 2.5 hr in length. Accordingly, interpeak trough times of no detectable circulating GH (≤2 ng/ml) that differ by as little as 35 min were found to give rise to distinct patterns of liver gene expression. Interestingly, in the mouse, which also expresses male-specific and female-specific P450s and other liver proteins under the influence of plasma GH patterns (11–13), GH secretion is pulsatile and occurs at regular intervals in both males and females. In male mice, however, plasma GH pulses occur each 2.5 hr, giving rise to trough periods of undetectable GH almost 2 hr in length, whereas in females a shorter GH cycling time of 1.4 hr results in trough times of <1 hr (32). Those findings support the present conclusion that a critical minimum trough period, which may vary from one species to the next, is obligatory for liver expression of GH-regulated, male-specific proteins, such as CYP2C11.

Our finding that hepatocytes require a minimum GH "off time" to express the masculinizing effects of pulsatile GH suggests that after stimulation by a GH pulse, male rat hepatocytes become refractory to subsequent GH pulses until a minimum recovery period has elapsed. Persistent stimulation, such as occurs in female rats due to the continuous presence of GH in circulation, precludes such a recovery, and leads to a distinct—i.e.—feminine pattern of liver gene expression. The biochemical events underlying this obligatory recovery period are still undefined but may involve resetting of the intracellular signaling pathways triggered by GH (33–35) or, alternatively, may relate to the temporary unavailability of cell-surface GH receptors. In adult male rats, hepatocyte GH receptors are apparently internalized after a plasma GH pulse and subsequently reappear at the cell surface with an overall cycling time of ≈3 hr (36, 37), a period that approximates the minimum interpulse interval seen in the present study. It is not known whether liver GH receptor cycling occurs in the absence of ligand or whether it is directly driven by the appearance of GH pulses at the cell surface. Although the adaptation of GH-receptor cycling to GH pulses applied less frequently than *in vivo* (e.g., two or four GH pulses per day) would not pose a problem under a ligand-driven receptor internalization model, hepatocytes may be unable to respond to GH pulses that recur more frequently than the endogenous male rhythm. Conceivably, in the seven-pulse-per-day experiments described in this report, the interpulse interval may be shorter than the minimum time needed for reappearance of unoccupied GH receptor at the cell surface, a process that probably requires new receptor-protein synthesis (38).

A recent analysis of liver P450 enzyme profiles in the dwarf rat strain NIMR/AS, which is characterized by a defect in GH synthesis that reduces circulating GH by 90–95% (40), revealed essentially normal patterns of both female- and

male-specific P450 enzymes, including CYP2C11 (41), despite the great reduction in plasma GH levels in this strain. This led Bullock *et al.* to propose that GH may not regulate the sexual differentiation of liver P450 expression (41). They further suggested that the apparent actions of GH indicated by earlier studies (7–12) may have, in part, resulted from nonsomatogenic properties of the bovine and human GH preparations used (41). This interpretation now appears unlikely because in the present study rGH is shown to be fully effective, both in masculinizing liver CYP2C11 expression when given in a suitable pulsatile manner (Figs. 3 and 4) and in feminizing hepatic CYP2C12 and steroid 5α-reductase levels when given to intact male rats as a continuous infusion (data not shown). The possibility that NIMR/AS rats have low, but sufficient, levels of circulating GH [plasma peaks up to 15 ng/ml; ref. 40], as presented by Bullock *et al.* (41), seems the more likely explanation for their finding of normal P450 expression in these rats. Indeed, the somewhat elevated CYP2C11 levels in this strain, as well as the actual GH profiles described by Charlton *et al.* (40), are strikingly similar to those achieved after induction of GH deficiency in adult rats by treatment with monosodium glutamate neonatally at a dose of 2 mg/g of BW, where near-normal liver P450 patterns are also observed (18, 30). Although low, GH levels in both models of GH deficiency are still significant when compared to the K_d of the GH–GH receptor complex (31). The finding of normal P450 profiles in NIMR/AS rats is thus supportive of the present conclusion that it is the pattern of circulating GH, rather than the absolute magnitude of individual GH pulses, that is key to the sexual differentiation of liver phenotype.

GH secretory profiles not only regulate hepatic-gene expression but also play a critical role in the peripubescent stimulation of somatic growth, which is more rapid in males under the influence of GH pulsation (6, 39). In our study, stimulation of normal male weight gain in hypophysectomized rats (a measure of somatic growth) was achieved in both males and females when physiological GH pulses were given two, four, six, or seven times per day, whereas continuous GH exposure stimulated the lower growth rate characteristic of female rats. The effectiveness of seven GH pulses per day in restoring male growth but not male liver P450 expression leads us to conclude that these two GH-stimulated responses have distinct GH pulse requirements and that this may conceivably derive from a tissue-dependent recognition of signaling elements in the sexually dimorphic circulating GH patterns. Further study is required for a better understanding of the responses of cell-surface GH receptors to male and female plasma GH patterns, as well as the intracellular signaling mechanisms and subsequent molecular events that lead to the major changes in gene expression that follow GH stimulation of target tissues.

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