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Growth Hormone Regulation of the Cytochrome P-450IIC Subfamily in the Rat: Inductive, Repressive, and Transcriptional Effects on P-450f (IIC7) and P-450$_{PB1}$ (IIC6) Gene Expression

STEFAN WESTIN, ANDERS STRÖM, JAN-ÅKE GUSTAFSSON, and PETER G. ZAPHIROPOULOS

Department of Medical Nutrition, Huddinge University Hospital, F60 Novum, Karolinska Institute, S-141 86 Huddinge, Sweden

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SUMMARY

The effects of growth hormone (GH) on two constitutively expressed rat liver cytochrome P-450 (P-450) enzymes, namely P-450f (IIC7) and P-450$_{PB1}$ (IIC6), have been investigated. Hypophysectomy of both male and female rats results in low expression of P-450f. Single daily injections of GH cannot restore expression of P-450f but further repress it. Continuous administration of GH, on the other hand, increases expression of P-450f to levels comparable to those of the normal male and female rat. P-450$_{PB1}$ is minimally affected by hypophysectomy or GH treatment, although a weak but significant repression by continuous administration of GH can be detected. Run-on analysis shows that continuous GH treatment increases transcription of P-450f. In addition, the sequence of the 5′ flanking region of this gene reveals DNA segments that might have a putative role in the transcriptional regulation of P-450f by GH.

P-450 enzymes are membrane-bound monooxygenases that catalyze the oxidative metabolism of both endogenous and exogenous compounds, including carcinogens, drugs, steroids, fatty acids, and prostaglandins (1, 2). P-450 enzymes have recently been assigned a new nomenclature based on amino acid sequence alignment data (3). In this system P-450s that are >40% identical constitute a family and those that are >60% identical, a subfamily. The P-450IIC subfamily has at least five members that are expressed in the rat, with three of these known to be sexually differentiated: P-450$_{16\alpha}$ (IIC11) and P-450g (IIC13) are male specific, whereas P-450$_{15\beta}$ (IIC12) is female specific (4–8). The sex difference in expression of P-450$_{15\beta}$ and P-450$_{16\alpha}$ depends on the plasma pattern of GH (9). The female secretory pattern, with its rather constant levels of GH, activates expression of P-450$_{15\beta}$ and the male secretory pattern, characterized by high peaks and low troughs, induces P-450$_{16\alpha}$ (10). On the other hand P-450g is turned on by the absence of GH rather than the pulsatile male-specific secretion pattern (7, 11).

Two additional IIC P-450s, namely P-450$_{PB1}$ (IIC6) and P-450f (IIC7), have recently been found to be transcriptionally activated before puberty (12). Expression reaches a maximum in adult animals, with P-450f being 2-fold higher in females than in males, whereas P-450$_{PB1}$ has no detectable sex difference. Because all these five P-450 enzymes share a high degree of structural identity and three of them are known to be regulated by GH, it was felt important to investigate whether GH had any effect on the expression of P-450$_{PB1}$ and P-450f.

Experimental Procedures

Materials. Osmotic minipumps were from Alza (Palo Alto, CA), GenescreenPlus membranes from NEN (Boston, MA), restriction enzymes, DNA-modifying enzymes, and the Erase-a-Base system from Promega (Madison, WI), the Sequenase sequencing kit from USB (Cleveland, OH), and the multiprime labeling kit, [α-^{32}P]dCTP (3000 Ci/mmol), [γ-^{32}P]ATP (5000 Ci/mmol), and [α-^{35}S]-dATP (1000 Ci/mmol) from Amersham (Arlington Heights, IL).

Animal experiments. Sprague-Dawley rats hypophysectomized at 6 weeks of age and control rats of the same age were purchased from Mollegårds Avlslaboratorium (Skensved, Denmark). Completeness of hypophysectomy was determined by monitoring the weight of the animals for 1 week before hormone treatment. Recombinant human GH (Somatonorm, 2 IU/mg), a generous gift from Kabi AB (Stockholm, Sweden), was administered to hypophysectomized rats at a daily dose of 120 μg, either by continuous infusion from osmotic minipumps at a rate of 5 μg/hr or by single daily injections. Animals were treated with GH for 3 or 6 days and sacrificed at 8 weeks of age. Minipumps were placed subcutaneously on the backs of the animals under ether anesthesia.

RNA isolation and Northern blot analysis. RNA was isolated from rat livers by the method of Chomczynski and Sacchi (13). Total RNA was electrophoresed on a 1.2% agarose gel in the presence of 2.2 M formaldehyde and transferred to GenescreenPlus membranes, essentially as described by Maniatis *et al.* (14). The presence of an equal amount of RNA in each lane was verified by the equal intensities of the 18 S and 28 S ribosomal RNA bands after staining with ethidium bromide. The RNA extracted from animals receiving the same treatment was not pooled (6-day experiment) but applied separately in each lane. Hybridizations were performed with 10^6 cpm of labeled probe/ml

ABBREVIATIONS: P-450, cytochrome P-450; GH, growth hormone; SSC, saline sodium citrate; SDS, sodium dodecyl sulfate; bp, base pairs.

in 1 M NaCl, 50% formamide, 1% SDS, 100 μg/ml salmon sperm DNA, for 20 hr at 42°. Filters were washed twice in 2× SSC (1× SSC = 0.15 M NaCl, 0.15 M sodium citrate, pH 7.0) at room temperature, twice in 2× SSC/1% SDS at 65°, and twice in 0.1× SSC at 55°. A full length cDNA for P-450f has been isolated before (7) and the 3′ P-450f probe used in these experiments starts at nucleotide 1330 (*Sph*I site) and extends to the 3′ end (15). The probe used for detection of P-450$_{PB1}$ is a 50-bp oligomer of the anticoding strand (position 618 to 667) (15), synthesized at the Center for Biotechnology, Huddinge University Hospital. The hybridization conditions described above were used with both probes, which were chosen because of their low similarity to other P-450s in the IIC subfamily. The P-450f probe is 54, 53, 56, and 60% identical to P-450$_{15\beta}$, P-450g, P-450$_{16\alpha}$, and P-450$_{PB1}$, respectively, whereas the P-450$_{PB1}$ probe is 58, 60, 66, and 64% identical to P-450$_{15\beta}$, P-450g, P-450$_{16\alpha}$, and P-450f, respectively. The specificity of the hybridization and washing conditions was also indicated by the dramatically different pattern observed when the same or similar blots were probed under these conditions with P-450$_{15\beta}$, P-450$_{16\alpha}$, or P-450g-specific sequences.

Nuclear transcription run-on analysis. P-450f (a full length clone and a clone containing the same 3′ region as used in the Northern analysis experiments) and β-actin in pGEM plasmids were immobilized on GenescreeenPlus filters, using a Schleicher & Schuell slot blot apparatus. Five micrograms of plasmid DNA were used. Nuclei from livers of single rats were isolated essentially as described (16) and elongation of preinitiated RNA chains was performed according to the method of Greenberg and Ziff (17), with the addition of an RNase-free DNase I incubation at 37° for 10 min, followed by phenol/chloroform extraction and ethanol precipitation. The labeled RNA chains were then hybridized with the filters for 24 hr at 42°, under the same conditions as described for Northern analysis. For all filters, 1.5 × 10^8 cpm of radioactivity were used. Washes were for 30 min each, twice in 2× SSC at room temperature, twice in 2× SSC/1% SDS at 65°, twice in 0.5× SSC/1% SDS at 65°, and once in 2× SSC including 10 μg/ml RNase A at 37°. Autoradiography was carried out for up to 2 weeks.

Isolation of the 5′ region of the P-450f gene. A rat genomic library in EMBL 3 vector, kindly provided by Dr. D. R. Colman (Departments of Anatomy and Cell Biology, and Pathology, Columbia University, New York) was screened, using as a probe a segment of the P-450$_{16\alpha}$ cDNA starting at the 5′ end and extending to the *Acc*I site (position −5 to 179) (4). This segment has an 82% nucleotide identity with the corresponding region of P-450f. In addition to the isolated clones that fit the P-450$_{16\alpha}$ gene structure, two independent clones had a unique restriction pattern, and sequencing of their hybridizing segments established their identity with P-450f. Unidirectionally deleted subclones generated by the Erase-a-Base system were used to obtain sequence information on the 5′ flanking region. The University of Wisconsin Genetics Computer Group program was used for sequence analysis (18).

Results

In our initial experiments, hypophysectomized rats of both sexes were treated with GH for 3 days and Northern analysis of total liver RNA was performed. P-450f mRNA dramatically decreased after hypophysectomy. Single daily injections of GH did not restore expression in hypophysectomized male or female rats. Continuous infusion of GH increased P-450f mRNA expression in females, whereas no such increase was detected in males (Fig. 1). P-450$_{PB1}$ mRNA was slightly increased after hypophysectomy, and this increase was unaffected by discontinuous treatment. Continuous GH administration reestablished the normal level of P-450$_{PB1}$ mRNA (Fig. 2). To further investigate the dramatic effects of the pituitary on P-450f, hormonal treatment of the animals was extended to 6 days (Figs. 3 and 4). As previously observed, hypophysectomy decreased P-450f mRNA. Discontinuous administration of GH to hypophysectomized animals for 6 days decreased P-450f mRNA even more, to almost nondetectable levels. Continuous GH infusion for 6 days significantly increased P-450f mRNA in hypophysectomized rats of both sexes. Interestingly, a sex difference in all these treatments is observed. Female rats always express more P-450f mRNA than males (compare Figs. 3 and 4).

To investigate whether P-450f is transcriptionally activated by GH, run-on analysis was performed on liver nuclei from controls, hypophysectomized rats, and hypophysectomized rats

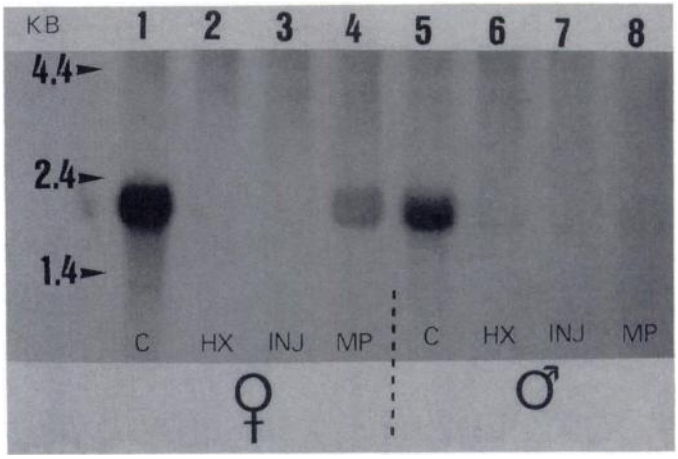

Fig. 1. Northern analysis of total liver RNA from control rats, hypophysectomized rats, or hypophysectomized rats treated with GH for 3 days, probed with P-450f-specific sequences (see Experimental Procedures). *Lane 1*, control female; *lane 2*, hypophysectomized female; *lane 3*, hypophysectomized female given GH by single daily injections; *lane 4*, hypophysectomized female given GH by osmotic minipumps; *lane 5*, control male; *lane 6*, hypophysectomized male; *lane 7*, hypophysectomized male given GH by single daily injections; *lane 8*, hypophysectomized male given GH by osmotic minipumps. Twenty micrograms of total RNA were used in each lane.

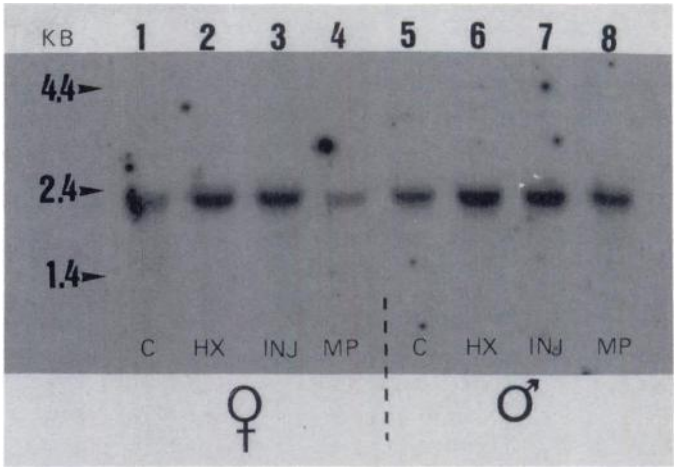

Fig. 2. Northern analysis of total liver RNA from control rats, hypophysectomized rats, or hypophysectomized rats treated with GH for 3 days, probed with a P-450$_{PB1}$-specific oligomer (see Experimental Procedures). *Lane 1*, control female; *lane 2*, hypophysectomized female; *lane 3*, hypophysectomized female given GH by single daily injections; *lane 4*, hypophysectomized female given GH by osmotic minipumps; *lane 5*, control male; *lane 6*, hypophysectomized male; *lane 7*, hypophysectomized male given GH by single daily injections; *lane 8*, hypophysectomized male given GH by osmotic minipumps. Twenty micrograms of total RNA were used in each lane.

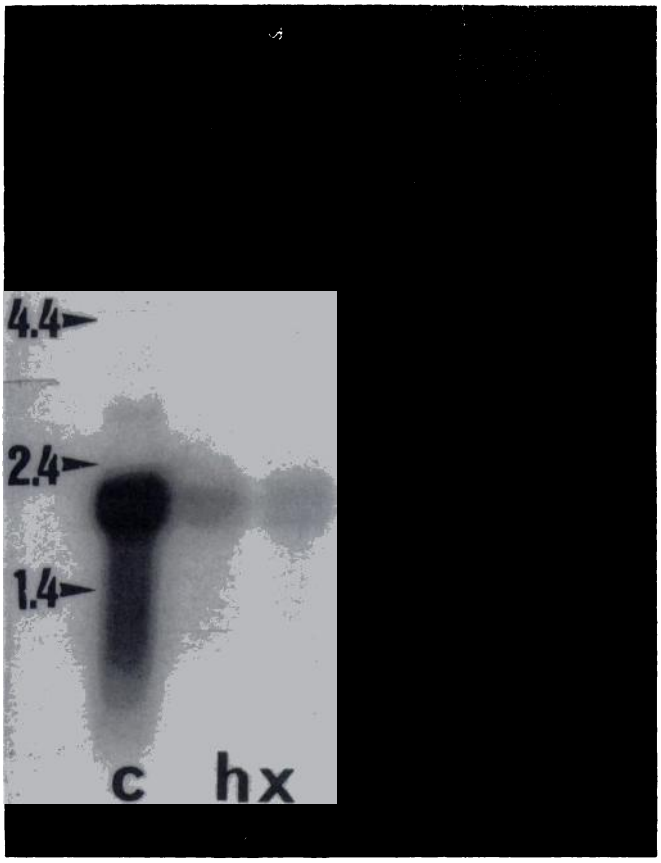

Fig. 3. Female liver RNA from control rats, hypophysectomized rats, or hypophysectomized rats treated with GH, for 6 days, subjected to Northern analysis with P-450f-specific sequences (see Experimental Procedures). *Lane 1*, control female; *lanes 2* and *3*, hypophysectomized females; *lanes 4* and *5*, hypophysectomized females given GH by single daily injections; *lanes 6* and *7*, hypophysectomized females given GH by osmotic minipumps. Twenty micrograms of total RNA were used in each lane. *Arrowheads*, size, in kb, of molecular weight markers.

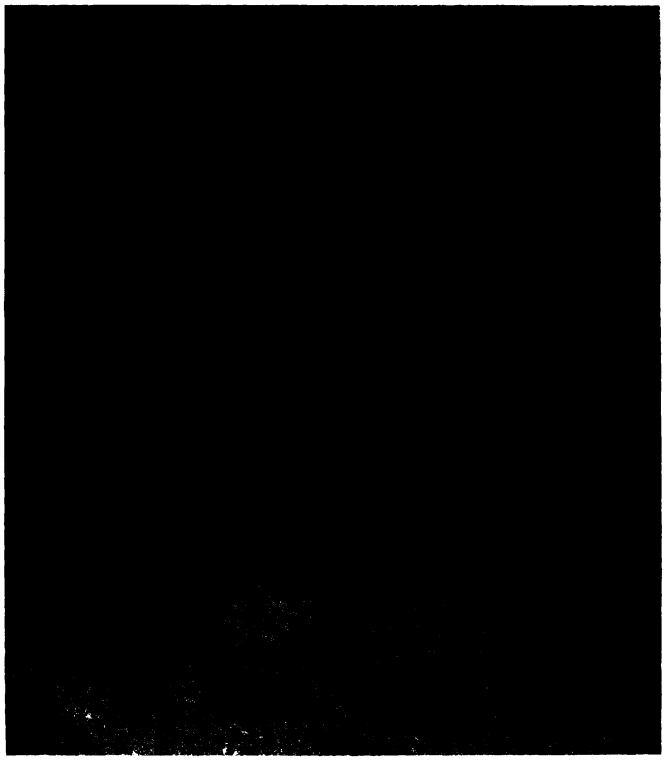

Fig. 4. Male liver RNA from control rats, hypophysectomized rats, or hypophysectomized rats treated with GH, for 6 days, subjected to Northern analysis with P-450f-specific sequences (see Experimental Procedures). *Lane 1*, control male; *lanes 2* and *3*, hypophysectomized males; *lanes 4* and *5*, hypophysectomized males given GH by single daily injections; *lanes 6* and *7*, hypophysectomized males given GH by osmotic minipumps. Twenty micrograms of total RNA were used in each lane. *Arrowheads*, the size, in kb, of molecular weight markers.

treated with GH for 3 days (Fig. 5). Females had a high transcription rate, which was reduced significantly after hypophysectomy. Continuous GH administration to hypophysectomized females increased transcription to a level comparable to that of the normal female. Males had a transcription rate that was approximately 2-fold lower than that in females. Hypophysectomy reduced transcription and continuous GH administration increased it back to the normal male level. In addition to the full length cDNA of P-450f, a 3′ cDNA segment was also used in the experiments with nuclei from females. The similar results obtained with this 3′ end segment indicate that no significant cross-hybridization with other IIC P-450s apparently occurred.

Because human GH was used in all these studies, a control experiment with hypophysectomized rats treated with bovine GH was performed, to unambiguously prove that the effects seen are somatogenic and not lactogenic. The levels of P-450f expression, as determined by Northern analysis, were similar to those of rats treated with human GH, indicating that the effects of GH recorded in this investigation were of somatogenic and not lactogenic nature (data not shown).

Tissue distribution analysis with total RNA from liver, lung, prostate, olfactory tissue, brain, and hypothalamus was also performed. Other than in the liver, a faint signal for P-450f mRNA was detected only in the female lung (data not shown).

In order to obtain further insight into the transcriptional activation of P-450f by GH, the 5′ flanking region of this gene was isolated. About 1000 bp of the flank were sequenced, and a comparison with the 5′ flank of the gene for the GH-inducible cytochrome P-450$_{15\beta}$ (19) revealed a 21-bp segment (position −720 to −700 in P-450f, position −1287 to −1267 in P-450$_{15\beta}$) having only three base changes between the two genes (Fig. 6). In addition to a putative TATA box (position −52 to −49), two CAAT sequences forming a stem loop structure are present at −239 to −227 bp.

Discussion

In this report the effects of GH on one sex-differentiated (P-450f; female > male) and one sex-nondifferentiated (P-450$_{PB1}$) P-450 of the IIC subfamily were investigated. Hypophysectomy of both male and female rats increased expression of P-450$_{PB1}$, whereas continuous GH administration to hypophysectomized animals reduced expression to levels found in intact rats. These repressive GH effects are of smaller magnitude than seen with the male-specific P-450$_{RLM2}$ (IIA2), -$_{2a}$ (IIIA2) (20), and -g (7, 11) and are more reminiscent of the effects seen with phenobarbital inducible P-450b (IIB1) and P-450e (IIB2) (21) and the ethanol-inducible P-450j (IIE1) (22).

In contrast to P-450$_{PB1}$, P-450f has a detectable sex difference; it is present in mature females at twice the level in males.

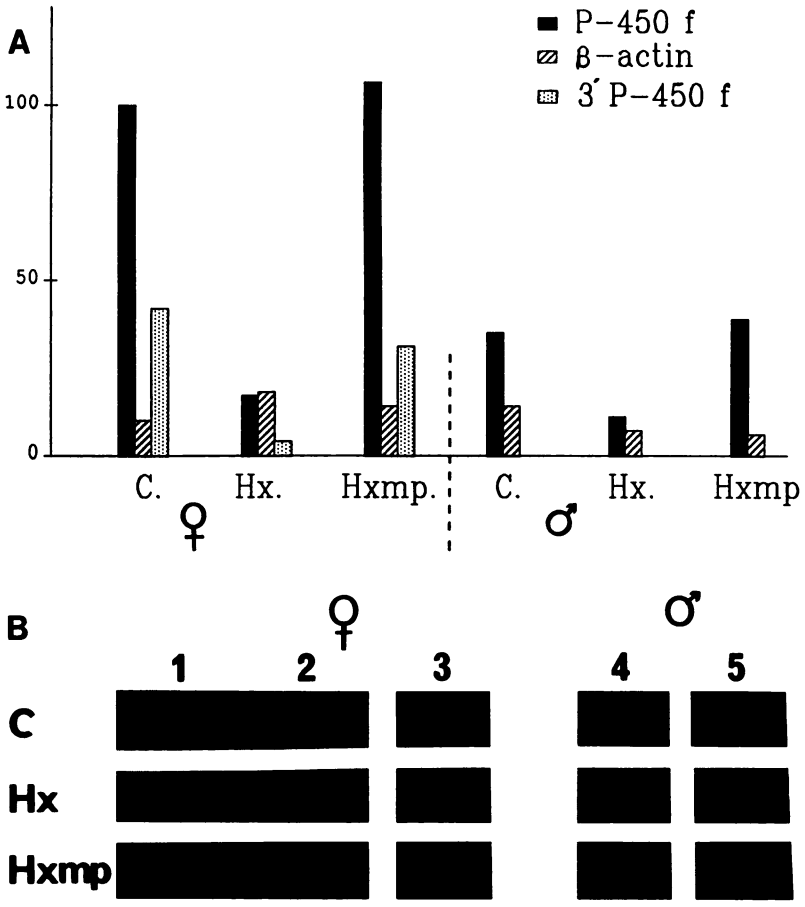

Fig. 5. P-450f transcription run-on analysis with liver nuclei from control rats (*C*), hypophysectomized rats (*Hx*), or hypophysectomized rats treated with GH by osmotic minipumps (*Hxmp*) for 3 days (see Experimental Procedures). A, *Bars* represent densitometric values from scanning of the autoradiogram. The transcription rate of P-450f in normal female rats is given the arbitrary value of 100. B, The DNA bound to the filters is as follows: *lane 1*, full length P-450f; *lane 2*, 3′ P-450f; *lane 3*, β-actin; *lane 4*, full length P-450f; *lane 5*, β-actin.

Whereas hypophysectomy decreased expression of P-450f in both sexes, the sex difference remained approximately 2-fold. It, therefore, does not seem inconceivable that males produce a factor repressing basal expression of P-450f, the synthesis of which is not eliminated after hypophysectomy. Alternatively, a female factor, refractory to hypophysectomy, might stimulate basal expression of P-450f. These postulated basal factors could well explain the different effects of a similar GH treatment to male and female hypophysectomized rats. Continuous GH administration for 6 days in hypophysectomized rats results in P-450f levels that are approximately 2 times higher in females than males. The fact that no major increase was seen in the male hypophysectomized rat after 3 days of treatment indicates that the GH effects are slow and that synthesis of protein factors mediating the GH action might be required. These experiments suggest that the female-specific GH pattern establishes normal male levels of P-450f expression when simulated in hypophysectomized males. On the other hand, discontinuous GH treatment (male-specific pattern) of hypophysectomized rats of both sexes reduces expression of P-450f even further, with males having lower levels than females. Therefore, the pattern of GH administration is extremely critical for the effects of the hormone. Discontinuous GH treatment may sometimes repress P-450 gene expression (7, 11, 20), but in all these cases continuous GH administration results in a much more dramatic repression. Furthermore, the male-specific GH pattern does not restore P-450f expression to the level of intact males. This suggests either that this pattern is not critical for the expression of P-450f in males or that the discontinuous mode of administration used (injections once daily) does not accurately mimic the male pattern, which is characterized by secretory bursts of GH every 3.3 hr. Clearly, however, this discontinuous administration of GH has repressive effects on P-450f and it is known that similar treatment induces the male-specific P-450$_{16\alpha}$.

The mechanisms through which GH elicits its effects are not well established. The GH receptor levels are apparently not the determining factor, because normal, hypophysectomized, and GH-treated hypophysectomized rats of both sexes have similar levels of the receptor (23). In the same context, P-450$_{15\beta}$, which responds to continuous GH administration in a similar way as P-450f (significant response only after 6 days of GH treatment in hypophysectomized rats) (8), has been shown to be directly induced by this peptide hormone in hepatocytes maintained in a culture system on Matrigel (24). In addition, using this Matrigel culture system, GH has been shown to directly repress phenobarbital-induced and isosafrole-induced expression of P-450b/e and P-450p (IIIA1), respectively (25).

Obviously, GH dramatically affects the expression of a number of P-450s. According to their GH regulation, one might tentatively categorize them into five groups, 1) P-450$_{16\alpha}$, activated by the discontinuous GH pattern, repressed by continuous GH administration, and characterized by higher expression in males; 2) P-450$_{RLM2}$, -$_{2a}$, and -g, also characterized by higher expression in males but minimally affected by discontinuous administration, although strongly repressed by continuous GH infusion; 3) P-450b, -e, -j, and -$_{PB1}$, also repressed by continuous GH administration but without a dramatic sex differentiation;

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-950  AATGTCCTCCTTAGCCTCAGACATTTGAACACTAGGTACCCAGTTGGTAC  -901
-900  TGCTGTTTGGGAACACTTAGCTGGTATCAGTTATTGTTATTTTCTCTGTA  -851
-850  CAGAATCATGCTTAACCAGTTTCATATGTGCATTAGTGGAAAATGGAGAA  -801
-800  TCATGAAACTTTTCTTCAAGAATCTATAATAAAGTCAGGGTAATAAGCAT  -751
-750  GCTCGTTAGATATTCACAGTGGATTTTAAATTCTTCCAACTATGATCTGT  -701
-700  GAGATACTGACAGTTCTTTTTGTTTCACCACCCTTAGAAAAAGAATAATG  -651
-650  GTCTTTTATAATTGAGTAAAGATGTGTGACTGACATGTATGTTTTCTGTT  -601
-600  CTTTGTTTTGTATTGGACAAGAGTAAAATAAGGAGCTATAAAGCATATGT  -551
-550  TATTGAACAAAATCTGTTGGAATGTTTAGTATAGAAAATAAACATTTTAA  -501
-500  GTATGCATGATCTTTGTGGTATGTAAATATATTTCTGGTTATTGGGGCAA  -451
-450  TGTAGCAGAAGAGCTGTCAGTTAGAGACCTGCCTATGTTATATAGTATGA  -401
-400  TTTTGTTAAAAATAAACATATTTCATTGAGTATATAAATATCATAAGTAA  -351
-350  TATATGACTTAAGTCATATATGGAGGAGTTTTCTTGAATCTTAAAATTAA  -301
-300  TATATTAATTCTGAAGAATTACCACACATTAGAAAATATTTGTTTACTTT  -251
-250  TATTATCAGTAATTGGGCACCAATCCTTAGTATTAGCCAGAAATTCAAAT  -201
-200  GGGCACTGTATAAAGGTCAAAGTAAATGATATCTTATATTTATCTCTATC  -151
-150  AAAGGACAAAGATCACTTGATAGAGAGCAAGTGGGATTAAAGATTCCCTA  -101
-100  ATGGACTAATCAATGATGCTCAAAGAGGTGCATGTTGTTTCAGCATGTTA   -51
 -50  TAAAATCTCTGTGCTGCCTGATCACAACTGCATCAGTAAGGAGGGTCTCC    -1
   1  ATGGATCTGGTCACTTTCCTGGTACTTACTCTCTCCTCTCTCATTCTCCT    50
      MetAspLeuValThrPheLeuValLeuThrLeuSerSerLeuIleLeuLe
  51  CTCACTCTGGAGACAGAGCTCTAGGAGAAGGAAGCTCCCTCCTGGCCCCA   100
      uSerLeuTrpArgGlnSerSerArgArgArgLysLeuProProGlyProT
 101  CTCCTCTCCCAATTATTGGTAATTTCCTCCAGATAGATGTGAAGAACATC   150
      hrProLeuProIleIleGlyAsnPheLeuGlnIleAspValLysAsnIle
 151  AGCCAATCCTTAACCAAGGTAAGTATTGTCGGTGATCCTGTAATAACTTG   200
      SerGlnSerLeuThrLys
 201  ATAAGGAAGATAAATTAG  218
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Fig. 6. Nucleotide sequence of the 5′ flanking region of P-450f. The nucleotide sequence of the flank and the deduced amino acid sequence of first exon are shown in the *upper* and *lower lines*, respectively. *Underlined* are: a 21-bp segment having significant sequence identity with the 5′ flanking region of P-450$_{15\beta}$ (base changes only at positions −712, −705, and −704), a putative TATA box, and two CAAT sequences forming a stem loop structure.

4) P-450$_{15\beta}$, induced by the continuous GH pattern and characterized by higher expression in females; and 5) P-450f, also induced by the continuous GH pattern but without a dramatic sex difference. It, therefore, appears likely that many pathways are involved in the mechanism of action of GH. For example, the constant presence of high levels of GH might initiate both an activating (P-450$_{15\beta}$ and -f) and a repressive (P-450$_{RLM2,\ -2a,}$ and -g) pathway. Furthermore, the discontinuous presence of GH might initiate two additional pathways, an activating one (P-450$_{16\alpha}$) and a repressive one (P-450f). Interplay of these alternative pathways, the molecular nature of which constitutes an exciting focus of our current research, would result in the specific expression of individual P-450 enzymes.

The observed increase of P-450f mRNA by continuous GH administration appears to occur primarily at the transcriptional level, as indicated by the run-on experiments. Furthermore, the 5′ flanking region of this gene has segments of significant sequence identity to the 5′ flanking region of P-450$_{15\beta}$, the other known P-450 that increases its expression in response to continuous GH administration. Whether these segments have a function in relation to the GH mechanism of action remains to be investigated.

After submission of this manuscript, a report describing the GH effects on P-450f mRNA levels appeared (26). These results are quite similar to the ones reported here, except that discontinuous GH administration increases the levels of P-450f mRNA, although not to the same extent as continuous GH administration. A careful examination of the discontinuous treatment protocols reveals that Sasamura *et al.* (26) use higher amounts of GH than we do in a twice daily injection scheme, and this may account for the different effects observed.

Acknowledgments

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Send reprint requests to: Peter G. Zaphiropoulos, Department of Medical Nutrition, Huddinge University Hospital, F60 Novum, Karolinska Institute, S-141 86 Huddinge, Sweden.