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Complete cDNA sequence of a cytochrome P-450 inducible by glucocorticoids in human liver

(dexamethasone/regulation/mRNA/gene family/Hep G2)

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ABSTRACT **HLp is a human liver cytochrome P-450 that is immunochemically related to the glucocorticoid-inducible liver cytochrome P-450p in the rat and its homologue in the rabbit, P-450 LM3c. To investigate the structure and regulation of HLp, we used a monoclonal antibody that recognizes purified HLp to screen a human liver cDNA library in λgt11. We isolated and sequenced two overlapping cDNA clones that span the entire 2011 bases of an mRNA that codes for a protein of 504 amino acids. The predicted amino-terminal amino acid sequence of this protein is identical to the first 20 residues determined from purified HLp. HLp mRNA shares more than 70% sequence homology with related proteins from the rat and rabbit but less than 40% homology with other published cytochrome P-450 genes. Moreover, Southern blot analysis of human and rat genomic DNA revealed 50 and 60 kilobases of DNA, respectively, hybridizable to the HLp cDNAs. Blot analysis of human liver RNA from five patients revealed major (2.2 kilobase) and minor (3.0 kilobase) bands that hybridized to HLp cDNAs. The apparent concentration of these hybridizable mRNAs as well as the amounts of immunoreactive HLp protein in microsomes from the same liver were increased in a dose-dependent relationship in three patients who received dexamethasone, a potent glucocorticoid. Furthermore, in samples of RNA and of microsomes isolated from cultures of a human hepatoma cell line (Hep G2) incubated for 120 hr in medium containing dexamethasone, there was a 6-fold induction of the two mRNA species hybridizable to HLp cDNAs and a 3-fold induction of immunoreactive HLp protein as compared to the values for cultures incubated in steroid-free medium. We conclude that HLp is a human representative of a conserved glucocorticoid-inducible cytochrome P-450 gene family whose mechanism of induction involves accumulation of HLp mRNA.**

The liver cytochromes P-450 are a supergene family of microsomal hemoproteins that catalyze the oxidative biotransformation of numerous endogenous substrates, such as steroids, as well as xenobiotics, such as drugs. Extensive studies in many species of laboratory animals indicate that there are multiple polypeptide forms of these cytochromes that can be distinguished by differences in their structure, substrate specificity, or responses to various types of inducers (1–3). These hemoproteins appear to have been conserved in evolution. For example, in rats treated with pregnenolone 16α-carbonitrile, a "catatoxic" steroid that protects animals from stress and from the lethal (4) or carcinogenic (5) effects of many toxic substances, P-450p is the major form of liver cytochrome P-450 (6). We have found that there are forms of hepatic cytochrome P-450 in the rabbit (LM3c), the hamster, the gerbil, and the mouse that (*i*) cross-react with anti-P-450p antibodies; (*ii*) catalyze oxidation of the same model substrates; (*iii*) are inducible by glucocorticoids, phenobarbital, and macrolide antibiotics; (*iv*) are encoded by mRNA species inducible by a potent glucocorticoid, dexamethasone (Dex); and (*v*) hybridize to a cloned P-450p cDNA (7). Others (8) have found a second rat liver protein, termed P-450/PCN, that resembles P-450p in many of these characteristics. This suggests that there are multiple genes related to P-450p both within and across species lines.

The question may be asked, to what extent is the rapidly accumulating information about the biochemistry, genetics, and regulation of cytochrome P-450 gene families in animals relevant to humans? A most encouraging finding in this regard is the demonstration that human liver contains HLp, a cytochrome P-450 that is both immunochemically and functionally related to P-450p and to rabbit LM3c (9). Furthermore, HLp and rat P-450/PCN share 57% homology in their amino-terminal amino acid sequences (9). Finally, the concentration of immunoreactive HLp protein and the amount of erythromycin *N*-demethylase activity were found to be highest in those patients who received Dex (9). Therefore, HLp appeared to be an inducible human liver cytochrome P-450 that resembled P-450p.

To further investigate the structure and control of HLp, we employed recombinant DNA techniques to isolate and characterize cloned cDNAs to HLp. We have used these clones to determine the complete nucleotide and deduced amino acid sequences of HLp, to estimate the amount of human genomic DNA related to HLp, and to determine the amounts and size(s) of hybridizable HLp mRNA in RNA samples from human liver and from cultured Hep G2 cells, a human hepatoma cell line (10). We conclude that HLp is a human homologue of the "P-450p gene family" that is induced by glucocorticoids through a mechanism involving accumulation of HLp mRNA.

MATERIALS AND METHODS

Library Screening. Meloy Laboratories (Springfield, VA) provided a library of cDNAs constructed from liver RNA from a single adult human cloned in the expression vector λgt11 (11). Approximately 10^5 recombinants were screened (12) with the use of monoclonal antibody 13-7-10 raised against a human liver cytochrome P-450 (13) that recognizes purified HLp (unpublished observations). Colonies expressing immunoreactive protein were visualized by sequentially applying goat anti-mouse IgG and monoclonal mouse peroxidase anti-peroxidase (Miles–Yeda, Rehovot, Israel) followed by 3,3′-diaminobenzidine tetrachloride in hydrogen peroxide (7). Strongly positive colonies were identified and

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Abbreviations: Dex, dexamethasone; bp, base pair(s); kb, kilobases.

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were purified by multiple screening of isolated plaques (14). The phage DNA from each was isolated by the plate lysate method (14), and the cloned cDNA inserts were excised by *Eco*RI digestion and purified from 5% polyacrylamide gels with the use of a D-Gel apparatus (Epigene, Baltimore, MD).

The longer of the two purified cDNA inserts, Hp55b, was radiolabeled with [α-^{32}P]dCTP (ICN) by nick-translation (Bethesda Research Laboratories) and was used to rescreen approximately 10^5 recombinant plaques from the same cDNA library replicated onto nitrocellulose filters (14). Positive plaques were isolated, and the phage harboring the longest cDNA, termed Hp12-9, was used in further studies.

Nucleotide Sequence Analysis. Restriction fragments generated by *Eco*RI or *Sst* I digestion of the cDNA inserts were inserted in both orientations into M13mp19 to determine the nucleotide sequence using the method of Sanger *et al.* (15). (Reagents supplied by Amersham.) In some instances rapid deletion sequencing (International Biotechnologies, New Haven, CT) was utilized. Results were confirmed by sequencing each nucleotide position at least three times.

Southern Blots. The excised inserts were subjected to electrophoresis in 0.8% agarose and transferred onto a nitrocellulose filter (16). The filters were then baked, prehybridized, hybridized, and washed according to the specification of the manufacturer (Schleicher & Schuell). For genomic blots, human and rat genomic DNA was isolated from leukocytes (17) and digested overnight with either *Eco*RI or *Hin*dIII (Bethesda Research Laboratories) (3 units/μg of DNA) under conditions recommended by the supplier. The digested DNA was subjected to electrophoresis in an 0.8% agarose gel for 44 hr at 15 mA. The DNA was denatured, neutralized, and transferred onto a nitrocellulose filter (16). The filter was air dried, baked *in vacuo* for 2 hr, and prehybridized in 1% bovine serum albumin/1 mM EDTA/0.5 M $NaHPO_4$, pH 7.2/7% (wt/vol) $NaDodSO_4$ at 65°C for 5 min (18). The filters were then hybridized as described except the hybridization temperature was 50°C. Low stringency washes consisted of two 15-min periods with 2× SSC, 0.1% $NaDodSO_4$ at 25°C followed by two 15-min periods with 0.1× SSC, 0.1% $NaDodSO_4$ at 25°C. For high stringency, a 15-min wash with 0.1× SSC, 0.1% $NaDodSO_4$ at 42°C was added. (1× SSC = 0.15 M NaCl, 0.15 M sodium citrate, pH 7.0.) Hybridized bands were visualized by autoradiography for 2 days at −80°C with two intensifying screens.

RNA Blots. The human liver specimens were obtained at surgery from five patients under protocols approved by the Committee for the Conduct of Human Research (Medical College of Virginia). Code numbers refer to individual patients. Patient 9 was a 36-yr-old white male organ donor. Incidental drugs received during the 24 hr prior to surgery included an acetaminophen suppository, ampicillin, and codeine. Patient 10 was a 36-yr-old white female who underwent elective removal of a hepatic hemangioma. Incidental drugs received included lorazepam, morphine sulphate, and cephazolin. Patient 11 was a 38-yr-old white female organ donor. She received 24 million units of penicillin G on the day of surgery. Patient 17 was a 56-yr-old white male organ donor. He was previously healthy but had a blood ethanol concentration of 3010 mg/liter when admitted. Patient 19 was a 69-yr-old white male who underwent elective hepatic lobectomy for metastatic sigmoid cancer. He received Corgerol, aspirin, Demerol, and triazolam in the 24 hr prior to surgery. Rats and rabbits were pretreated as described elsewhere (7). Total RNA was extracted with the use of guanidine thiocyanate (19) from rat, rabbit, or human liver and from cultures of Hep G2 cells. Poly(A)$^+$ RNA was isolated by one cycle of chromatography on oligo(dT)-cellulose (Collaborative Research, Waltham, MA). The RNA (0.2–1.0 μg) was subjected to electrophoresis in 1.0% agarose gels in 10 mM sodium phosphate buffer containing 1.1 M formaldehyde and transferred to nitrocellulose (20). The hybridization conditions were those described above for cDNA Southern blots.

Immunoquantitation. The concentration of HLp in human liver microsomes was determined as described (7) by quantitation of immunoblots developed with monoclonal antibody 13-7-10 followed sequentially with goat anti-mouse IgG peroxidase conjugated and 3,3′-diaminobenzidine tetrahydrochloride in hydrogen peroxide. Purified HLp (0.5–4.0 pmol) (7) served as the standard. The density and total area of the immunostained bands was determined with the use of a Zeiss integrated scanning densitometer (7).

Hepatoma Cells. Hep G2 cells were seeded in 60-mm culture dishes and grown to confluency in Earle's minimal essential medium containing 10% (vol/vol) fetal calf serum (GIBCO) in a humidified chamber with 5% CO_2/95% air. Cells were then maintained in standard medium or in medium supplemented with 0.1 mM Dex. After 120 hr cells were harvested for protein (21) and RNA (19) determinations.

RESULTS AND DISCUSSION

Isolation and Characterization of HLp cDNA Clones. The human liver cDNA library cloned in the expression vector λgt11 was screened with monoclonal antibody 13-7-10 that recognizes purified HLp. Two positive clones were obtained harboring cDNA inserts of 900 (Hp55) and 615 base pairs (bp) (Hp51), respectively. Digestion of clone Hp55 with *Eco*RI to release the cDNA insert from the phage vector produced fragments of 170 bp (Hp55s) and 730 bp (Hp55b). The latter fragment of clone Hp55 was purified, nick-translated, and used to rescreen the human liver cDNA library. Of 10 positive colonies identified, clone Hp12-9 contained the largest cDNA insert of 1750 bp, which upon digestion with *Eco*RI yielded 520- and 1230-bp fragments. Southern blot analysis of the cloned cDNAs demonstrated that Hp55b hybridized only with the 520-bp fragment of clone Hp12-9, whereas Hp55s failed to hybridize with either fragment of clone Hp12-9 (data not shown). pDex12, a cloned cDNA that encodes the 3′ half of rat P-450p mRNA (or a closely related mRNA) (7), hybridized to neither fragment of Hp55 and to only the 1230-bp fragment of Hp12-9 (not shown). These results suggested that the 5′–3′ order of the fragments was Hp55s, Hp55b, 520 bp of Hp12-9, 1230 bp of Hp12-9. Subsequent nucleotide sequence analysis of these cloned cDNAs confirmed this alignment as is indicated by symbols in Fig. 1.

Hp55 and Hp12-9 span the entire length of a 2011-bp mRNA with a continuous reading frame from nucleotide 61 to 1572. The 615-bp cDNA in the other clone (Hp51), initially identified by immunoscreening, was entirely encompassed in the large fragment (Hp55b) of clone Hp55. Since the sequences of Hp55 (bp 1–880), Hp51 (bp 279–895), and Hp12-9 (bp 381–2011) were identical in their regions of overlap, it is likely that these cDNAs are derived from a common mRNA. The protein encoded by this mRNA consists of 504 amino acids with a molecular weight of 57,566. Final proof of the identity of this (or any) cDNA will require complete comparison of the deduced amino acid sequence with that determined from the purified protein. Nevertheless, we have assembled compelling evidence that this protein is HLp. The first 20 amino-terminal amino acids predicted by this cDNA sequence are identical to those determined from purified HLp (except for a missing initial methionine) (9). Moreover, HLp cDNA clones Hp55 and Hp51 directed synthesis in *E. coli* of peptides recognized by monoclonal antibody 13-7-10 that reacts with purified HLp. Finally, HLp cDNA hybridized to glucocorticoid inducible mRNAs from rat and rabbit liver (Fig. 2) that encode proteins structurally and immunochemically related to HLp (9).

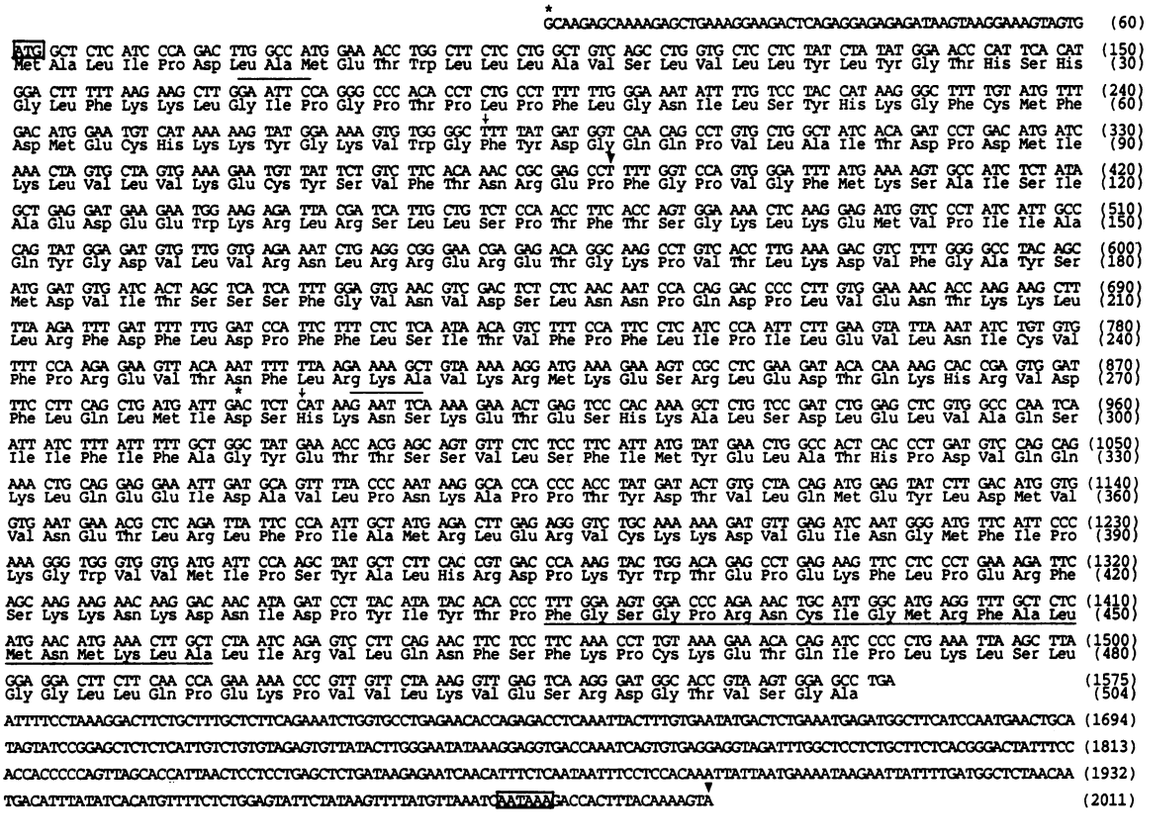

FIG. 1. Nucleotide and deduced amino acid sequence of HLp cDNA. The nucleotide sequences of the cloned cDNAs Hp55 (★–★), Hp51 (↓–↓), and Hp12-9 (▼–▼) were determined. No mismatches were identified in the regions of overlap. *Eco*RI restriction sites are overlined. The homologous carboxyl-terminal region found in other cytochromes P-450 is underlined. Boxed sequences denote the initiation codon and poly(A)$^+$ addition signal, respectively.

Comparison of HLp with Other Cytochromes P-450. Computer-assisted comparisons of the complete nucleotide and derived amino acid sequences of HLp with those reported for other cytochromes P-450 revealed that HLp represents a distinct gene family (Table 1) (23–31). HLp shares greater than 70% homology with the complete nucleotide sequence of rat P-450/PCN as well as with partial sequences of rabbit LM3c (Table 1). HLp was less closely related (less than 40% homology) to other mammalian cytochrome P-450 sequences published to date (Table 1). The only other completely sequenced human cytochrome P-450 cDNA (24) (human P_1-450) was prepared from a dioxin-inducible mRNA in a human breast carcinoma cell line and was found to share 63% nucleotide-sequence homology with the dioxin-inducible P_1-450 in the mouse (29). Retention of 60–70% sequence homology, particularly in translated regions of a gene, would be expected assuming man diverged from rodents approximately 80 million years ago (32). We noticed that the 5′ and 3′ noncoding portions of HLp mRNA retained 53% homology with the corresponding region of rat P-450/PCN (23), where-

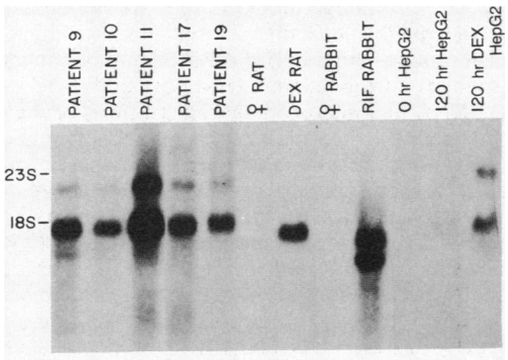

FIG. 2. Autoradiograph of RNA hybridization of various mRNAs with an HLp cDNA. Samples of poly(A)$^+$-selected liver RNA, prepared from humans (1.0 μg), female rats (0.25 μg), female rabbits (0.25 μg), and Hep G2 cells (1.0 μg) were subjected to electrophoresis in a denaturing agarose gel, transferred to nitrocellulose, and hybridized with [^{32}P]Hp55b.

Table 1. Comparisons between nucleotide and deduced amino acid sequences of HLp with other cytochromes P-450

Protein	Ref.	Nucleotide homology, %	Amino acid homology, %	Carboxyl-terminal cysteine region
Rabbit LM3c*	†	74		
Rat P-450/PCN	23	71	73	95
Human P_1-450	24	32	25	45
Human HpP-450 4*	25	35	32	40
Human pHp450(1)*	26	31	30	35
Rat P-450e	27	40	34	35
Rat P-450c	28	31	23	45
Mouse P_1-450	29	31	26	40
Rabbit P-450LM3b	30	38	32	40
P-450SSC	31	36	30	48

Global alignments of published sequences were made with NUCALN and PRTALN (22) running on a VAX computer. Homology of the region surrounding the conserved carboxyl-terminal cysteine is also indicated. The results are given as the percent homology with HLp (see Fig. 1).

*Partial sequences.

†P.S.G. and P. Maurel, unpublished observations.

as human P_1-450 retained only 34% homology in nontranslated portions of the equivalent mRNAs in rodents (24). Whether these portions of the HLp mRNA have been unusually well conserved for some reason cannot be adequately assessed because only partial sequences have been reported to date for other human cytochromes P-450 (Table 1).

The cytochromes P-450 share a conserved cysteinyl region in the carboxyl-terminal portion of their amino acid sequences that may play a critical role in binding of the heme prosthetic group (33). This region in HLp (cysteine-443) is 95% homologous when compared to the corresponding region in P-450/PCN, and is 35–48% homologous when compared to other cytochromes P-450 (Table 1). HLp contains no cysteine residue in the amino-terminal conserved cysteinyl region. Absence of a cysteine in this region also has been reported for adrenal cytochrome $P\text{-}450_{SCC}$ (31) and P-450/PCN (23) and, therefore, the importance of this "conserved region" is open to question.

Southern Blot Analysis of Human and Rat Genomic DNA Homologous to HLp. Human DNA isolated from the lymphocytes of a volunteer was digested with *Eco*RI or *Hin*dIII endonucleases and was analyzed on Southern blots. A mixture of [^{32}P]Hp55 plus [^{32}P]Hp12-9 served as the hybridization probes. Bands comprising nearly 50 kilobases (kb) of DNA (Fig. 3) were observed, suggesting that the human genome may contain multiple genes or pseudogenes related to HLp. The experiment was repeated using rat DNA hybridized to the HLp cDNA probes under less stringent conditions (Fig. 3), and multiple bands totaling approximately 60 kb of DNA were visualized. Assuming the genes are in the size range of 10–15 kb, our Southern hybridization analysis with HLp cDNA is consistent with that reported with the use of P-450/PCN cDNA as a probe (8) and suggests that there are three to five homologous genes in the P-450p family in each species.

Hybridization of HLp cDNAs to Human Liver RNA. RNA blot analysis of human liver poly(A)$^+$ RNA revealed two RNA species that hybridized to [^{32}P]Hp55b (Fig. 2, lanes 1–5). The apparent size of the most intensely hybridized band was 2200 nucleotides (≈18S). This value is in good agreement with the size of HLp cDNA if it is assumed that HLp mRNA has a typical poly(A)$^+$ tract of 100–200 bases. The apparent size of the minor RNA band was 2800 nucleotides (≈23S). The ratio of the hybridization signal of these mRNAs was ≈5:1 (short/long) and remained unchanged after washing under conditions of high stringency or when a similar blot was hybridized with [^{32}P]Hp12-9 at several conditions of increasing stringency. Based on calculated homologies (Table 1), HLp cDNA would be expected to hybridize to related mRNAs in rat and rabbit liver. This prediction was confirmed since [^{32}P]Hp55b hybridized to a Dex-inducible mRNA in rat liver (lanes 6 and 7) and to a macrolide antibiotic (rifampicin)-inducible mRNA in rabbit liver (lanes 8 and 9). Both animal mRNAs were similar in their apparent size to the major hybridizable band in human liver.

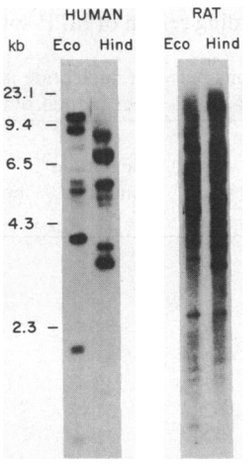

FIG. 3. Autoradiograph of Southern blot analysis of genomic DNA hybridized with HLp cDNAs. Human and rat genomic DNA (10 μg) was digested with either *Eco*RI (Eco) or *Hin*dIII (Hind). The fragments were separated by electrophoresis, transferred to nitrocellulose, and hybridized with a mixture of [^{32}P]Hp55 and [^{32}P]Hp12-9 followed by either low (rat) or high (human) stringency washings. Mobility of DNA standards (*Hin*dIII-digested λ phage DNA) is indicated at the left in kb.

Even though HLp cDNAs clearly hybridize to two human liver mRNA species, only a single immunoreactive HLp band has been detected in analysis of multiple samples of human liver microsomes on immunoblots developed with polyclonal antibodies directed against P-450p or P-450LM3c (9) or with monoclonal antibody 13-7-10 (data not shown). The smaller of these mRNAs likely encodes for HLp. The larger mRNA may encode for a similar protein that cannot be readily resolved from HLp. However, failure of the two mRNAs to preferentially hybridize to one of the HLp cDNAs under various conditions of stringency suggests that the larger mRNA may represent HLp mRNA with an extended 3′-nontranslated region formed by the use of alternate polyadenylylation signals as has been reported for other genes (34). The latter possibility may extend to other human cytochromes P-450 inasmuch as human clone PhP(1) hybridizes to two human liver mRNAs that also differ in size by 600–800 bp and are present in the same ratio (5:1, short/long) (35).

Our results establish that the structural homology of the P-450p gene family has been conserved. However, it cannot be assumed that the regulation of these genes is the same. Indeed, a dramatic example to the contrary is that pregnenolone 16α-carbonitrile, a strong inducer of P-450p, does not at all induce the homologous protein (LM3c) in the rabbit or in cultures of rabbit hepatocytes (unpublished observations), while rifampicin, the most potent inducer of LM3c in rabbits, does not at all induce P-450p in rats (7) or in cultures of rat hepatocytes (unpublished observations). However, in the present study, the amounts of human liver mRNA hybridizable to HLp cDNA were increased (6-fold) in a dose-dependent relationship in the three patients who had received Dex as compared to two patients who received none of the drugs that induce P-450p in rats (Table 2). In addition, the relative amounts of hybridizable HLp mRNA were similar to the concentrations of HLp protein measured in liver microsomes by quantitative immunoblot analysis (r = 0.98) (Table 2). Although these findings are preliminary, due to the small number of subjects studied, they suggest that both HLp protein (9) and HLp mRNA may be inducible by Dex in man. To further examine this possibility, we prepared poly(A)$^+$ RNA and microsomes from cultures of the human hepatoma Hep G2 when the cells first reached confluency (0 hr) and also after the cultures had been incubated for 120 hr in medium without glucocorticoids (120 hr). Little, if any, mRNA hybridizable to HLp cDNA (Fig. 2, lanes 10 and 11) was detected in either specimen. Nevertheless, both samples contained small amounts (40 pmol of immunoreactive protein per mg of protein) of an immunoreactive protein with mobility identical to that of purified HLp on immunoblots developed with anti-P-450p IgG or with monoclonal antibody 13-7-10 (data not shown). In contrast, when companion cultures were exposed for 120 hr to medium containing Dex, there appeared two bands of mRNA hybridizable to HLp cDNA (Fig. 2, lane 12). The mobility of the hybridizable mRNAs in the cells was the same as those in RNA samples from human liver (Fig. 2). The estimated increases in hybrid-

Table 2. Quantitation of HLp protein and mRNA in human livers

Patient	Relative value: HLp protein	Relative value: HLp mRNA	Treatment
9	209	290	Dex (200 mg); Dph (200 mg); Amb (60 mg)
10	186	160	None
11	409	634	Dex (16 mg/day, 13 days) Dph (300 mg/day, 13 days)
17	109	214	Dex (10 mg); Dph (1 g)
19	100	100	None

HLp protein and mRNA was determined by scanning densitometry of immunoblots and RNA blots. The data are given as the percentage of the respective values for patient 19 that were as follows: immunoblots, 0.22 nmol/mg, and RNA blots, arbitrary units. All treatments were administered once in the 24 hr prior to surgery and represent potential inducers. All other medications are listed. Abbreviations: Dph, phenytoin; Amb, amobarbital.

izable mRNA in Dex-treated cultures was 6-fold over the minimum detectable value in control cultures. This was accompanied by a 3-fold increase in immunoreactive HLp protein in Dex-treated cultures (data not shown).

We conclude that HLp mRNA may be inducible by glucocorticoids in man, as is P-450p mRNA in rats (6). Accumulation of HLp mRNA hybridizable to HLp cDNA appears to account for the increased amount of immunoreactive HLp protein. We have proposed that induction of P-450p in rat hepatocytes is mediated by a nonclassical glucocorticoid receptor (36). The availability of specific probes for HLp protein and HLp mRNA coupled with an immortalized human liver cell culture that exhibits inducible expression of these molecules should greatly facilitate future investigation of the mechanisms controlling expression of this gene family and its relevance to humans.

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