0026-895X/93/030328-07$03.00/0

MOLECULAR PHARMACOLOGY, 43:328-334

Expression of Multiple Forms of Cytochrome P450 mRNAs in Primary Cultures of Rat Hepatocytes Maintained on Matrigel

THOMAS A. KOCAREK, ERIN G. SCHUETZ, and PHILIP S. GUZELIAN[1]

Division of Clinical Toxicology and Environmental Medicine, Department of Medicine (T.A.K., P.S.G.) and Department of Pathology (E.G.S.), Medical College of Virginia, Richmond, Virginia 23298

Received July 23, 1992; Accepted November 25, 1992

SUMMARY

Freshly isolated rat hepatocytes rapidly lose their cytochrome P450 (P450) proteins and mRNAs, with no evidence of subsequent restoration, after placement into traditional systems of primary culture on type I collagen. We examined the patterns of expression of 10 constitutively expressed P450 mRNAs in rat hepatocytes cultured for up to 5 days on a matrix of Matrigel, a reconstituted basement membrane that allows inducible expression of some P450s, and compared these patterns with those seen in hepatocytes cultured on type I collagen (Vitrogen). mRNA for each P450 was detected on Northern blots in samples prepared from freshly isolated male rat hepatocytes, and the amount of each mRNA decreased markedly during the first 2 days of culture in cells maintained on either matrix (decreases of 53–97% on Matrigel and 62 to >99% on Vitrogen), in contrast to the level of NADPH-P450 oxidoreductase mRNA, which increased during this interval. On subsequent days, hepatocytes cultured on Matrigel expressed the individual P450 mRNAs in one of the following four patterns. 1) P450 1A2, 2A2, 2E1, and 3A1/2 mRNAs remained low throughout the 5-day culture period. 2) 2A1 mRNA increased between days 2 and 4 but then decreased on day 5. 3) 2B1/2, 2C6, 2C7, and 4A1 mRNAs increased continually between days 2 and 5. 4) The total mRNAs detected with a 2D probe remained at constant levels between culture days 2 and 5. Our results show that rat hepatocytes cultured on Matrigel in serum-free medium spontaneously reexpress the mRNAs for several constitutive P450s in form-specific patterns, and they suggest that the Matrigel culture system will be useful for identifying the underlying regulatory mechanisms.

The P450s are a superfamily of hemoproteins, found prominently in liver, whose members catalyze the oxygenation of a variety of endogenous and xenobiotic substrates. Some of these liver enzymes are found in untreated animals (sometimes called constitutive), whereas others are expressed only in response to administration of an inducing agent. The major P450s found in the livers of untreated rats include 1A2, 2A1, 2B2, 2C6, 2C7, 2D, 2E1, 4A1, and several sex-specific forms, including 2A2, 2C11, 2C13, and 3A2 in males and 2C12 in females (for review, see Ref. 1). Many of these "constitutive" P450 forms are actually inducible, to at least some extent, by such xenobiotics as aromatic hydrocarbons (1A2 and 2A1), phenobarbital (2B2, 2C6, 2C7, and 3A2), ethanol (2E1), or peroxisome proliferators (4A1) (2–7).

Animal studies have suggested that expression of each constitutive P450 is under its own distinct set of hormonal, developmental, and tissue-specific control mechanisms (for review, see Ref. 1). However, it is difficult to examine the direct effects of drugs and hormones on expression of constitutive hepatic P450s because the inherent complexity of the whole animal includes such confounding factors as indirect effects of test agents on other organ systems, cyclical variations in endogenous hormone levels, and feedback regulatory pathways. A liver cell culture system would be ideal for this purpose, but expression of constitutive hepatic P450s is rarely, if ever, found in cell lines of hepatic origin. Furthermore, when freshly isolated hepatocytes are placed into the traditional systems for primary culture, the constitutive P450s promptly disappear (8).

We recently reported that, when rat hepatocytes are cultured on dishes coated with a thin layer of Matrigel, a reconstituted basement membrane, instead of on type I collagen, several P450s, such as 2B1/2B2, can be induced by treatments with xenobiotics in a manner qualitatively similar to that observed in living rats (9). Moreover, treatment of male rat hepatocytes maintained on Matrigel, but not on type I collagen, with growth hormone caused the appearance of the female-specific form 2C12 (10), an event consistent with findings *in vivo* (11, 12). The apparent permissive effect of Matrigel on inducible expression of some P450s might also extend to spontaneous expression of some constitutive P450 forms. We have systematically examined this possibility and now report that, although the

This work was supported in part by National Institutes of Health Grant ES04628 and by gifts from the Virginia Environmental Endowment and the General Electric Foundation. T.A.K. was supported by National Institutes of Health Postdoctoral Training Grant ES05448.

[1] Current address: University of Colorado Health Sciences Center, Denver, CO 80262.

ABBREVIATIONS: P450, cytochrome P450; P450 OR, NADPH-cytochrome P450 oxidoreductase.

amounts of mRNA for 10 constitutive P450 forms decreased markedly during the first 2 days of culture, hepatocytes maintained on Matrigel spontaneously "reexpressed" some of these P450 mRNAs.

Experimental Procedures

Materials. Adult male and female Sprague-Dawley rats (Dominion Labs, Dublin, VA) weighing 175–200 g were maintained in wire-bottomed cages, with free access to animal chow and water, for 2 weeks before use. Collagenase type I was purchased from Cooper Biochemical Co. (Malvern, PA) and Vitrogen was purchased from Celtrix Laboratories (Palo Alto, CA). Matrigel was prepared in this laboratory as described previously (9). Phenobarbital was from J. T. Baker, Inc. (Phillipsburg, NJ), dexamethasone and β-naphthoflavone were from Sigma (St. Louis, MO), and ciprofibrate was from Sterling-Winthrop Research Institute (Rensselaer, NY). Cloned cDNA fragments for 3A1 (pDex12) (13) and human 2E1 (HLj) (14) were isolated in this laboratory. A cDNA for P450 1A2 (p72) (15) was provided by Dr. John Fagan (Mahareshi International University, Fairfield, IA), cDNAs for 2A1 (16), 2C6 (17), 2C7 (17), 2D (18), and 4A1 (6) were supplied by Dr. Frank Gonzalez (National Cancer Institute, Bethesda, MD), a cDNA for 2B2 (pR17) (19) was provided by Dr. Milton Adesnik (New York University, New York, NY) [we did not routinely use oligonucleotide probes for 2B1 and 2B2 in these experiments, as we have in some of our studies (20, 21), because of their decreased sensitivities and increased backgrounds, relative to the cDNA probe], and a cDNA for P450 OR (pOR-7) (22) was furnished by Dr. Charles Kasper (University of Wisconsin, Madison, WI).

Hepatocyte cultures and drug treatments. Rat hepatocytes were isolated from adult male or female Sprague-Dawley rats (230–280 g) and plated onto 60-mm plastic dishes coated with 150–250 μl of Matrigel or 50 μl of Vitrogen, as described previously (9). Cultures were maintained in a humidified incubator at 35° under an atmosphere of 5% CO_2/95% air. The culture medium was a modification of Waymouth MB-752 containing insulin (1.5×10^{-7} M) as the only hormone. Media were changed and dishes were harvested at 24-hr intervals after plating of the hepatocytes. Inducers were added to 3-day-old cultures as concentrated stock solutions in water (phenobarbital) or dimethylsulfoxide (dexamethasone, β-naphthoflavone, and ciprofibrate; 0.1% of total volume).

Northern blot analysis. Total RNA was isolated from the pooled cells of three to five culture dishes as described previously (9). RNA samples (20 μg) were resolved on denaturing 1% agarose gels and capillary transferred onto reinforced nitrocellulose filters (Nitro plus; MSI, Westboro, MA). cDNA inserts were radiolabeled to $>10^8$ cpm/μg using a nick translation kit (BRL, Gaithersburg, MD) and [α-^{32}P] dCTP (3000 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA). Prehybridization and hybridization conditions have been described previously (23). Final washing conditions for the cDNAs were 0.1× standard saline citrate (20× standard saline citrate = 3 M sodium chloride, 0.3 M sodium citrate, pH 7.5), 0.1% sodium dodecyl sulfate, for 30 min at 37° (for 2E1), 50° (for 2A1, 2B2, 2C6, 2C7, 4A1, 2D, and P450 OR), or 65° (for 1A2 and 3A1). RNA bands were visualized by autoradiography and quantified by scanning densitometry.

Statistical analysis. The densitometric estimations of the amounts of each mRNA measured within a hepatocyte preparation were expressed as percentages of the amount of that mRNA detected in freshly isolated hepatocytes. Paired comparisons between percentages at different time points were made using the nonparametric sign test. Differences were considered to be statistically significant at $p < 0.05$.

Results

Hepatocytes were isolated from the livers of adult male or female rats and cultured on a thin layer of Matrigel in our standard serum-free Waymouth medium for up to 5 days. Total RNA was isolated from pooled dishes of cells harvested daily and was analyzed for levels of 1A2, 2A1/2, 2B1/2, 2C6, 2C7, 2D, 2E1, 3A1/2,[2] and 4A1[3] mRNAs, and for P450 OR mRNA, by Northern blot hybridization. Each of these mRNAs was examined in samples isolated from the hepatocytes of three to five different cell preparations, including at least one preparation from a female liver. The pattern of expression for a given mRNA was qualitatively similar among all the different cell preparations, although there was considerable variability in the mRNA level among preparations, even when the data were calculated as percentages of amounts of mRNA measured in freshly isolated hepatocytes. The results of all experiments are summarized at three time points (culture days 2, 4, and 5) in Table 1. Sex differences, where they occurred, are indicated in the text. The results shown in Fig. 1 are those obtained from a male hepatocyte preparation in which we directly compared mRNA expression in cells cultured on matrices of Matrigel versus type I collagen (Vitrogen).

TABLE 1

Summary of the time courses of P450 mRNA expression in multiple preparations of rat hepatocytes cultured on Matrigel

P450	n[a]	Relative mRNA level[b]		
		Day 2	Day 4	Day 5
		%		
1A2	3	3.37 ± 1.91	<0.1	0.853 ± 0.854
2A1/2	5	24.3 ± 5.7	61.1 ± 19.0	32.3 ± 13.3[c]
2B1/2	3	0.993 ± 0.995	21.5 ± 13.5	29.1 ± 20.7
2C6	5	11.0 ± 6.9	19.2 ± 5.3	22.9 ± 7.0[d]
2C7	5	16.3 ± 11.2	28.3 ± 10.9[d]	30.6 ± 12.2[d]
2D	3	21.5 ± 13.1	23.2 ± 13.1	21.7 ± 14.1
2E1	3	3.98 ± 1.40	2.22 ± 0.80	1.94 ± 0.43
3A1/2	3	4.74 ± 4.64	6.33 ± 5.14	3.64 ± 2.31
4A1	3	11.8 ± 4.7	33.3 ± 21.3	49.7 ± 32.8
P450 OR	3	168 ± 50	105 ± 19	118 ± 28

[a] Number of independent hepatocyte preparations.
[b] Data are expressed as percentages of the amounts of mRNA detected in freshly isolated hepatocytes.
[c] Significantly less than the day 4 value ($p < 0.05$).
[d] Significantly greater than the day 2 value ($p < 0.05$).

Hybridizable mRNA for each P450 was clearly present in freshly isolated hepatocytes, and the amount of each form decreased dramatically during the first 2 days of culture in hepatocytes maintained on either Matrigel (decreases of 53–97%; Fig. 1) or Vitrogen (decreases of 62 to >99%; Fig. 1). After this time, the hepatocytes cultured on Matrigel expressed the individual P450 isoforms in one of essentially four different patterns (summarized in Table 2).

The first pattern ("crash") was a continued decrease in the amount of mRNA during days 3–5 in culture, with little or no mRNA being detectable during this time in hepatocytes cultured on either matrix. This pattern was displayed by four P450s, i.e., 1A2, 2A2, 2E1, and 3A1/2. P450 1A2 is expressed constitutively in rat liver and is induced by aromatic hydrocarbons and isosafrole (2). 1A2 mRNA disappeared almost completely by day 2 in the cultured hepatocytes and was essentially undetectable henceforth in cells cultured on either matrix (Fig. 1; Table 1). 2A2 is expressed constitutively only in the livers of male rats (3), although, unlike two other male-specific P450s,

[2] Because the Dex12 cDNA hybridizes to the mRNAs for both 3A1 and 3A2, we use 3A1/2 to describe our Northern blot data.
[3] The 4A1 cDNA probe may also hybridize to the 4A2 and 4A3 mRNAs.

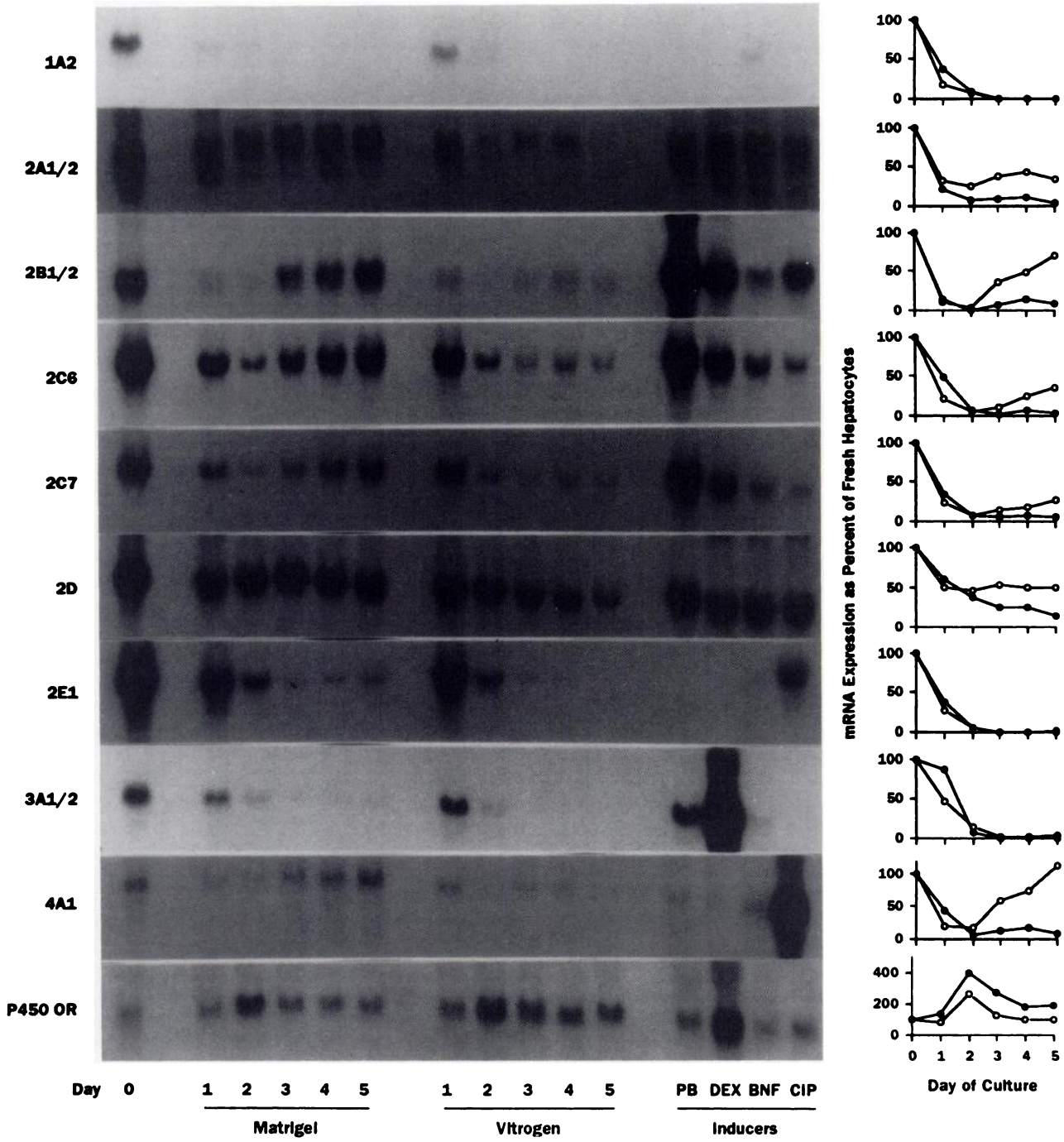

Fig. 1. Time course for expression of constitutive P450 mRNAs in male rat hepatocytes cultured on Matrigel or Vitrogen. Freshly isolated hepatocytes prepared from the liver of a single male rat were placed into primary culture on dishes coated with either Matrigel or Vitrogen and were maintained in standard, serum-free medium for up to 5 days. Hepatocytes were harvested daily, and total RNA was analyzed by Northern blot hybridization, as described in Experimental Procedures. Autoradiographs show the amounts of each mRNA over the 5-day culture period in hepatocytes maintained on Matrigel or Vitrogen and, for comparison, in hepatocytes that were cultured on Matrigel and treated for 2 days (days 3–5 in culture) with one of four prototypical inducers, phenobarbital (*PB*) (10^{-4} M), dexamethasone (*DEX*) (10^{-5} M), β-naphthoflavone (*BNF*) (10^{-5} M), or ciprofibrate (*CIP*) (10^{-4} M). A graphic representation of the densitometrically quantified data, expressed as percentages of the amount of mRNA detected in fresh hepatocytes (day 0), is shown for each time course, to the *right* of the respective autoradiograph (○, Matrigel; ●, Vitrogen).

2C11 and 2C13, its expression is reported not to be dependent on the male pulsatile pattern of growth hormone secretion (24). The 2A1 cDNA used for our Northern blot analyses detects three clearly resolved bands, the upper and lower reportedly corresponding to 2A1 and the middle corresponding to 2A2 (3, 16). Although we were unable to quantify the middle band separately, examination of the autoradiograph in Fig. 1 demonstrates that this band, corresponding to 2A2, was strongly expressed in the fresh hepatocytes, disappeared by 2 days in culture, and was not detected subsequently. 2A2 mRNA was not detected in fresh hepatocytes prepared from female rats, nor was it detected at any time in culture (data not shown). 3A1 is the major P450 induced by glucocorticoids in rat liver (see effect of dexamethasone treatment in Fig. 1) (25, 26). 3A2,

TABLE 2

Summary of expression patterns exhibited by P450 mRNAs in primary cultured rat hepatocytes on Matrigel

Name	Common names	Pattern	Inducers[a]
1A2	d	Crash	BNF
2A1	a	Bounce	
2A2		Crash	
2B2	e	Crash-rise	PB, DEX, CIP
2C6	k,PB1	Crash-rise	PB, DEX
2C7	f	Crash-rise	PB, DEX
2D1,2,3,5	db1,2,3,5	Coast	
2E1	j	Crash	CIP
3A1;3A2	p,pcn1; pcn2	Crash	DEX, PB
4A1	LAω	Crash-rise	CIP

[a] PB, phenobarbital; DEX, dexamethasone; BNF, β-naphthoflavone; CIP, ciprofibrate.

a related form sharing 90% nucleotide sequence similarity with 3A1, is expressed constitutively only in the livers of male rats (5, 24). 3A1/2 mRNA disappeared almost completely during the first 2 days in culture, with only small amounts being detectable (<4% of the level of expression in fresh hepatocytes) by day 5 in the hepatocytes maintained on Matrigel (Table 1). Consistent with previous findings (27), 3A1/2 mRNA was not detected in freshly isolated hepatocytes prepared from female rats or in untreated cultures of these hepatocytes (data not shown). 2E1 is the P450 form induced by ethanol, acetone, isoniazid, and pyrazole and during diabetic ketosis (7). 2E1 mRNA disappeared almost completely by day 2 in culture and was only barely detectable on day 5 in the cells cultured on Matrigel (<2% of the level detected in fresh hepatocytes).

A second pattern of expression ("bounce") was observed for 2A1 mRNA, which corresponded to the upper and lower bands detected on Northern blots with the 2A1 cDNA probe (3, 16). Amounts of this form decreased during the first 2 days of culture, increased strongly between days 3 and 4, and then decreased again on day 5 (Fig. 1; Table 1). Because the 2A2 mRNA had disappeared from the hepatocyte cultures by day 2, quantification of the 2A mRNA levels present in the cultures from days 3 through 5 reflected only 2A1 mRNA. The 2A1 mRNA attained a level on day 4 that was an average of 61% of the total amount of 2A seen in freshly isolated hepatocytes (Table 1). Hepatocytes isolated from male or female rats displayed similar relative increases in 2A1 mRNA during this time, suggesting that higher absolute levels of 2A1 mRNA were actually attained in the female-derived cultures, because 2A1 expression is known to be higher in adult female than in adult male rat liver (3, 16). The decrease in 2A1 mRNA that occurred between days 4 and 5 was statistically significant (Table 1). Although the patterns of 2A1 mRNA expression were qualitatively similar in hepatocytes cultured on the two matrices, the amounts of mRNA were at all times much greater in the cells maintained on Matrigel (Fig. 1).

A third pattern ("crash-rise") was displayed by the mRNAs for P450s 2B1/2, 2C6, 2C7, and 4A1, in which expression steadily increased between days 2 and 5 of culture. 2B1 and 2B2 are two remarkably similar P450s, that share 97% amino acid sequence identity (19, 28, 29) and are the major forms induced by phenobarbital in rat liver (see effect of phenobarbital treatment in Fig. 1). Northern blot hybridization with a cDNA recognizing both 2B1 and 2B2 revealed that, although 2B1/2 mRNA disappeared almost completely in hepatocytes during the first 2 days of culture, the cells cultured on Matrigel displayed a definite trend toward an increase in 2B1/2 mRNA during the next 3 days, attaining a level on day 5 that was as high as 70% (Fig. 1) and an average of 29% (Table 1) of that detected in fresh hepatocytes. Results obtained using synthetic oligonucleotide probes that discriminate between the 2B1 and 2B2 mRNAs indicated that 2B2 was the major form that was reexpressed (data not shown), as is found in untreated rat liver (30).

We have not reported in our previous studies of 2B1/2 induction the presence of detectable amounts of 2B1/2 mRNA in 5-day-old control hepatocyte cultures on Matrigel (9, 20, 21). As shown in Fig. 1, the phenobarbital-induced 2B1/2 mRNA levels were much greater than the spontaneously expressed 2B1/2 mRNA levels. These low levels of spontaneously expressed 2B1/2 mRNA would probably have gone unnoticed under our standard assay conditions. In this study, however, we compensated for the expected low mRNA levels in untreated hepatocytes by using increased amounts of total RNA (to 20 μg) in our Northern blot analyses and longer film exposure times than we had previously used for detecting inducible 2B1/2 mRNA expression.

2C6 and 2C7 are related constitutive P450s that are expressed at very high levels in untreated rats and that have been reported to be moderately induced by phenobarbital or kepone treatments, respectively (17, 31, 32). The mRNAs detected with the 2C6 and 2C7 cDNAs showed very similar patterns of reexpression (Fig. 1), with levels of each increasing significantly between 2 and 5 days in hepatocytes cultured on Matrigel (Table 1), although they did not approach the large amounts detected in fresh hepatocytes (23% and 31% of the fresh hepatocyte levels for 2C6 and 2C7, respectively; Table 1). Because of the similar patterns displayed by the 2C6 and 2C7 mRNAs and the possibility for cross-reactivity of the cDNA probes with the related class 2C mRNA species, these forms are referred to as 2C6/2C7 throughout the remainder of the manuscript.

The mRNA for P450 4A1, the principal peroxisome proliferator-inducible form (see effect of ciprofibrate treatment in Fig. 1), exhibited a pattern that was very much like that seen for 2B1/2 mRNA, in that 4A1 mRNA was present at a relatively low level in fresh hepatocytes but showed a definite trend toward an increase between days 2 and 5 of culture in cells maintained on Matrigel, to a level that was as high as 114% (Fig. 1) and an average of 50% (Table 1) of the amount present in fresh hepatocytes. A common finding for the P450s displaying the crash-rise pattern was that the mRNAs were reexpressed to much lower levels in hepatocytes maintained on Vitrogen than in those cultured on Matrigel (Fig. 1).

The fourth pattern ("coast"), exhibited by the 2D subfamily P450 mRNAs, involved a less dramatic decrease during the first 2 days in culture, followed by maintenance over the next 3 days of relatively constant amounts in the cells on Matrigel (approximately 22% of the fresh hepatocyte level; Table 1) that were at all times greater than the amounts of 2D mRNA seen in the cells cultured on Vitrogen, which continued to decline gradually (Fig. 1). It must be emphasized, however, that the 2D cDNA used in this study hybridizes to at least four separate 2D subfamily members that are expressed in the livers of Sprague-Dawley rats (i.e., 2D1, 2D2, 2D3, and 2D5) and are unresolved on Northern blots (18, 33). Therefore, the apparently stable pattern of expression observed here may actually represent the summation of four more dynamic profiles.

For comparison with the P450 mRNAs, we examined the expression in culture of P450 OR mRNA and found that, in marked contrast to the pattern observed for all the P450s, the amount of P450 OR mRNA did not decrease during the first 2 days in culture but, if anything, increased (Fig. 1; Table 1). The level of P450 OR mRNA remained relatively constant for the rest of the culture period in hepatocytes maintained on either matrix (Fig. 1; Table 1).

We included treatments with four prototypical P450 inducers in each of our experiments to ensure that the cultured hepatocytes gave normal responses and for comparison with the spontaneous levels of expression. We found that the inducers produced the expected effects (summarized in Table 2); phenobarbital induced 2B1/2, as well as 2C6/2C7 and 3A1/2, dexamethasone strongly induced 3A1/2 and, to a lesser extent, 2B1/2, β-naphthoflavone produced only a small induction of 1A2, consistent with the relative intractability of this form to induction by polycyclic aromatic hydrocarbons in our standard culture system (9), and ciprofibrate strongly induced 4A1 mRNA. Ciprofibrate treatment also produced a smaller increase in 2B1/2 mRNA levels, consistent with previous reports that treatments with clofibric acid, a related peroxisome proliferator, induced 2B1/2 protein and activity in rats and rat hepatocyte cultures (34, 35). Ciprofibrate treatment additionally produced a distinct increase in the amount of 2E1 mRNA, consistent with the level of increase of immunoreactive 2E1 protein previously reported in clofibric acid-treated rats (35). To our knowledge, this finding marks the first demonstration of 2E1 mRNA induction in cultured hepatocytes.

Discussion

Hepatocytes undergo numerous phenotypic changes after their introduction into primary culture, including loss of P450 content (8). We previously reported that the amounts of total spectral P450 detected in rat hepatocytes cultured on type I collagen in serum-free medium declined 68% during the first 3 days in culture but that the sum of the amounts of immunoreactive protein for seven different P450 forms declined only 24% during this period (36). These results suggested that loss of heme, rather than protein, accounted for much of the observed P450 loss in the cultured hepatocytes. Subsequent studies have revealed that amounts of P450 mRNAs also decline dramatically during the first days in culture (9, 20), and the current results extend this finding to include the mRNAs for 10 constitutively expressed P450 forms. However, loss of monooxygenase-associated mRNAs during the first 2 days in culture is not universal, as demonstrated by the pattern exhibited by P450 OR mRNA. Transcription of the liver-specific genes albumin and α_1-antitrypsin has been reported to be decreased in cultured rat hepatocytes (37). Moreover, we have previously demonstrated that cultured hepatocytes are refractory to P450 induction between 1 and 2 days in culture (25, 38), consistent with impaired transcriptional activation. It therefore seems likely that an inability to transcribe the P450 genes, coupled with a failure to stabilize the preexisting P450 mRNAs, combine to account for the loss of P450 that occurs early in culture.

In our earlier study (36), the rates of decline for the individual P450 proteins varied greatly, but it did not appear that once the amounts of the various proteins had decreased, their levels were later increased, suggesting that the cultured hepatocytes did not subsequently restore the conditions necessary for constitutive P450 expression. Possible explanations for this finding are that 1) the culture conditions did not support expression of a differentiated enough phenotype by the hepatocyte, 2) the damage exacted during the initial 2 days of culture was irreversible, 3) extrahepatic factors are necessary for constitutive P450 expression, or 4) the cell culture system contained inhibitory factors preventing constitutive P450 expression. The recent discovery that a variety of cell types cultured on a matrix of Matrigel, an extract of the Engelbreth-Holm-Swarm sarcoma, exhibit a generally more differentiated phenotype than occurs on other matrices (39–41), including inducible expression of several P450s in cultured rat hepatocytes (9, 10), provided us with an opportunity to examine the first possibility. Our results revealed that the mRNAs for several constitutive P450s were spontaneously reexpressed, after their initial decays, to much higher levels in hepatocytes cultured on Matrigel than in hepatocytes cultured on type I collagen. We observed four distinct patterns of expression over 5 days in culture for 10 constitutive P450 mRNAs, summarized in Table 2 as 1) crash, i.e., little or no reexpression at any time of culture (1A2, 2A2, 2E1, and 3A1/2), 2) bounce, i.e., strong reexpression between 2 and 4 days, followed by a statistically significant decrease on day 5 (2A1), 3) crash-rise, i.e., strong reexpression between 2 and 5 days (2B2, 2C6/2C7, and 4A1), and 4) coast, i.e., maintained mRNA levels between 2 and 5 days (2D). We emphasize that these changes occurred in the presence of a culture medium that is completely devoid of serum growth factors or hormones, except insulin, which is necessary for maintenance of hepatocyte viability. Although insulin likely affects constitutive P450 expression, because all mRNAs were measured in the same RNA preparations, differential exposures of the hepatocytes to insulin cannot account for the different patterns of mRNA expression that were observed.

The four distinct patterns of P450 mRNA expression suggest that some constitutive forms may share control mechanisms governing their spontaneous expression in culture. For example, the 2B1/2, 2C6/2C7, and 4A1 mRNAs displayed very similar patterns of reexpression in the cultured hepatocytes. In contrast, the bounce pattern depicted by 2A1 suggests a possible feedback control mechanism that permits this form to rapidly overcome the suppressive conditions early in culture but later become once again suppressed. The crash pattern displayed by 1A2, 2A2, 2E1, and 3A1/2 may suggest that extrahepatic factors are absolute requirements for constitutive expression of these forms or, alternatively, that the culture environment contains factors that inhibit expression of these particular cytochromes. A requirement for extrahepatic factors also may explain why some mRNAs that were spontaneously reexpressed, such as those for 2C6/2C7, did not approach the levels detected in fresh hepatocytes. Recent reports suggesting that growth hormone is required for maximal 2C7 expression in rats support this idea (42, 43). Another possible explanation for the different patterns exhibited by the P450 mRNAs may relate to the levels of expression of the hepatic transcription factors. For example, recent evidence that HNF-1 (hepatocyte nuclear factor 1) and DPB (D site binding protein) regulate expression of 2E1 and 2C6, respectively (44, 45), may suggest that DPB, but not HNF-1, is active in the hepatocyte cultures.

It was recently reported that a unique form of P450, belonging to the 2C subfamily, was expressed only in cultured rat hepatocytes and not in rat liver and, moreover, that the level

of expression of this form was higher in cells cultured on type I collagen than it was in cells cultured on Matrigel (46). Each P450 form examined in our study is expressed constitutively in rats and was expressed at a higher level in hepatocytes cultured on Matrigel than in cells cultured on Vitrogen, as would be expected if Matrigel produces the more differentiated environment. These data indicate that our Northern blot analyses most probably detected the authentic P450 forms themselves, rather than some unusual culture-derived relatives, and, taken together, our data strongly suggest that the different patterns of reexpression observed in the hepatocytes cultured on Matrigel resulted from reestablishment of the normal intracellular control mechanisms governing constitutive P450 gene expression. If this interpretation is correct, then use of Matrigel as the hepatocyte substratum should prove as useful for defining the factors affecting constitutive P450 gene expression as it has been for studying P450 induction.

Acknowledgments

We thank Donna Li for her excellent technical assistance in the preparation of the hepatocyte cultures.

References

1. Gonzalez, F. J. The molecular biology of cytochrome P450s. *Pharmacol. Rev.* **40:**243–288 (1989).
2. Thomas, P. E., S. Bandiera, L. M. Reik, S. L. Maines, D. E. Ryan, and W. Levin. Polyclonal and monoclonal antibodies as probes of rat hepatic cytochrome P-450 isozymes. *Fed. Proc.* **46:** 2563–2566 (1987).
3. Matsunaga, T., K. Nagata, E. J. Holsztynska, D. P. Lapenson, A. Smith, R. Kato, H. V. Gelboin, D. J. Waxman, and F. J. Gonzalez. Gene conversion and differential regulation in the rat P-450IIA gene subfamily: purification, catalytic activity, cDNA and deduced amino acid sequence, and regulation of an adult male-specific hepatic testosterone 15α-hydroxylase. *J. Biol. Chem.* **263:**17995–18002 (1988).
4. Friedberg, T., D. J. Waxman, M. Atchison, A. Kumar, T. Haaparanta, C. Raphael, and M. Adesnik. Isolation and characterization of cDNA clones for cytochromes P-450 immunochemically related to rat hepatic P-450 form PB-1. *Biochemistry* **25:**7975–7983 (1986).
5. Gonzalez, F. J., B. J. Song, and J. P. Hardwick. Pregnenolone 16α-carbonitrile-inducible P-450 gene: gene conversion and differential regulation. *Mol. Cell. Biol.* **6:**2969–2976 (1986).
6. Hardwick, J. P., B.-J. Song, E. Huberman, and F. J. Gonzalez. Isolation, complementary DNA sequence, and regulation of rat hepatic lauric acid ω-hydroxylase (cytochrome P-450LAω): identification of new cytochrome P-450 gene family. *J. Biol. Chem.* **262:**801–810 (1987).
7. Yang, C. S., J.-S. H. Yoo, H. Ishizaki, and J. Hong. Cytochrome P450IIE1: roles in nitrosamine metabolism and mechanisms of regulation. *Drug Metab. Rev.* **22:**147–159 (1990).
8. Bissell, D. M., and P. S. Guzelian. Phenotypic stability of adult rat hepatocytes in primary monolayer culture. *Ann. N. Y. Acad. Sci.* **349:**85–98 (1980).
9. Schuetz, E. G., D. Li, C. J. Omiecinski, U. Muller-Eberhard, H. K. Kleinman, B. Elswick, and P. S. Guzelian. Regulation of gene expression in adult rat hepatocytes cultured on a basement membrane matrix. *J. Cell Physiol.* **134:**309–323 (1988).
10. Guzelian, P. S., D. Li, E. G. Schuetz, P. Thomas, W. Levin, A. Mode, and J. A. Gustafsson. Sex change in cytochrome P-450 phenotype by growth hormone treatment of adult rat hepatocytes maintained in a culture system on Matrigel. *Proc. Natl. Acad. Sci. USA* **85:**9783–9787 (1988).
11. Mode, A., G. Norstedt, B. Simic, P. Eneroth, and J. A. Gustafsson. Continuous infusion of growth hormone feminizes hepatic steroid metabolism in the rat. *Endocrinology* **108:**2103–2108 (1981).
12. Morgan, E. T., C. MacGeoch, and J.-A. Gustafsson. Hormonal and developmental regulation of expression of the hepatic microsomal steroid 16α-hydroxylase cytochrome P-450 apoprotein in the rat. *J. Biol. Chem.* **260:**11895–11898 (1985).
13. Wrighton, S. A., E. G. Schuetz, P. B. Watkins, P. Maurel, J. Barwick, B. S. Bailey, H. T. Hartle, B. Young, and P. Guzelian. Demonstration in multiple species of inducible hepatic cytochromes P-450 and their mRNAs related to the glucocorticoid-inducible cytochrome P-450 of the rat. *Mol. Pharmacol.* **28:**312–321 (1985).
14. Wrighton, S. A., P. E. Thomas, D. T. Molowa, M. Haniu, J. E. Shively, S. L. Maines, P. B. Watkins, G. Parker, G. Mendez-Picon, W. Levin, and P. S. Guzelian. Characterization of ethanol-inducible human liver *N*-nitrosodimethylamine demethylase. *Biochemistry* **25:**6731–6735 (1986).
15. Fagan, J. B., J. V. Pastewka, S. C. Chalberg, E. Gozukara, F. P. Guengerich, and H. V. Gelboin. Noncoordinate regulation of the mRNAs encoding cytochromes P-450BNF/MC-B and P-450ISF/BNF-G. *Arch. Biochem. Biophys.* **244:**261–272 (1986).
16. Nagata, K., T. Matsunaga, J. Gillette, H. V. Gelboin, and F. J. Gonzalez. Rat testosterone 7α-hyroxylase: isolation, sequence, and expression of cDNA and its developmental regulation and induction by 3-methylcholanthrene. *J. Biol. Chem.* **262:**2787–2793 (1987).
17. Gonzalez, F. J., S. Kimura, B. J. Song, J. Pastewka, H. V. Gelboin, and J. P. Hardwick. Sequence of two related P-450 mRNAs transcriptionally increased during rat development: an R.dre.1 sequence occupies the complete 3′ untranslated region of a liver mRNA. *J. Biol. Chem.* **261:**10667–10672 (1986).
18. Gonzalez, F. J., T. Matsunaga, K. Nagata, U. A. Meyer, D. W. Nebert, J. Pastewka, C. A. Kozak, J. Gillette, H. V. Gelboin, and J. P. Hardwick. Debrisoquine 4-hydroxylase: characterization of a new P450 gene subfamily, regulation, chromosomal mapping, and molecular analysis of the DA rat polymorphism. *DNA* **6:**149–161 (1987).
19. Kumar, A., C. Raphael, and M. Adesnik. Cloned cytochrome P-450 cDNA: nucleotide sequence and homology to multiple phenobarbital-induced mRNA species. *J. Biol. Chem.* **258:**11280–11284 (1983).
20. Kocarek, T. A., E. G. Schuetz, and P. S. Guzelian. Differentiated induction of cytochrome P450b/e and P450p mRNAs by dose of phenobarbital in primary cultures of adult rat hepatocytes. *Mol. Pharmacol.* **38:**440–444 (1990).
21. Kocarek, T. A., E. G. Schuetz, and P. S. Guzelian. Selective induction of cytochrome P450e by kepone (chlordecone) in primary cultures of adult rat hepatocytes. *Mol. Pharmacol.* **40:**203–210 (1991).
22. Gonzalez, F. J., and C. B. Kasper. Cloning of DNA complementary to rat liver NADPH-cytochrome *c* (P-450) oxidoreductase and cytochrome P-450b mRNAs: evidence that phenobarbital augments transcription of specific genes. *J. Biol. Chem.* **257:** 5962–5968 (1982).
23. Schuetz, E. G., S. A. Wrighton, S. H. Safe, and P. S. Guzelian. Regulation of cytochrome P-450p by phenobarbital and phenobarbital-like inducers in adult rat hepatocytes in primary monolayer culture and *in vivo*. *Biochemistry* **25:**1124–1133 (1986).
24. Waxman, D. J., G. A. LeBlanc, J. J. Morrissey, J. Staunton, and D. P. Lapenson. Adult male-specific and neonatally programmed rat hepatic P-450 forms RLM2 and 2a are not dependent on pulsatile plasma growth hormone for expression. *J. Biol. Chem.* **263:**11396–11406 (1988).
25. Schuetz, E. G., S. A. Wrighton, J. L. Barwick, and P. S. Guzelian. Induction of cytochrome P-450 by glucocorticoids in rat liver. I. Evidence that glucocorticoids and pregnenolone 16α-carbonitrile regulate *de novo* synthesis of a common form of cytochrome P-450 in cultures of adult rat hepatocytes and in the liver *in vivo*. *J. Biol. Chem.* **259:**1999–2006 (1984).
26. Schuetz, E. G., and P. S. Guzelian. Induction of cytochrome P-450 by glucocorticoids in rat liver. II. Evidence that glucocorticoids regulate induction of cytochrome P-450 by a nonclassical receptor mechanism. *J. Biol. Chem.* **259:**2007–2012 (1984).
27. Burger, H.-J., E. G. Schuetz, J. D. Schuetz, and P. S. Guzelian. Divergent effects of cycloheximide on the induction of class II and class III cytochrome P450 mRNAs in cultures of adult rat hepatocytes. *Arch. Biochem. Biophys.* **281:**204–211 (1990).
28. Fujii-Kuriyama, Y., Y. Mizukami, K. Kawajini, K. Sogawa, and M. Muramatsu. Primary structure of a cytochrome P-450: coding nucleotide sequence of phenobarbital-inducible cytochrome P-450 cDNA from rat liver. *Proc. Natl. Acad. Sci. USA* **79:**2793–2797 (1982).
29. Yuan, P. M., D. E. Ryan, W. Levin, and J. E. Shively. Identification and localization of amino acid substitutions between two phenobarbital-inducible rat hepatic microsomal cytochromes P-450 by micro sequence analyses. *Proc. Natl. Acad. Sci. USA* **80:** 1169–1173 (1983).
30. Omiecinski, C. J. Tissue-specific expression of rat mRNAs homologous to cytochromes P-450b and P-450e. *Nucleic Acids Res.* **14:**1525–1539 (1986).
31. Waxman, D. J., and C. Walsh. Cytochrome P-450 isozyme 1 from phenobarbital-induced rat liver: purification, characterization, and interactions with metyrapone and cytochrome b_5. *Biochemistry* **22:**4846–4855 (1983).
32. Bandiera, S., D. E. Ryan, W. Levin, and P. E. Thomas. Age- and sex-related expression of cytochromes P450f and P450g in rat liver. *Arch. Biochem. Biophys.* **248:**658–676 (1986).
33. Matsunaga, E., U. M. Zanger, J. P. Hardwick, H. V. Gelboin, U. A. Meyer, and F. J. Gonzalez. The *CYP2D* gene subfamily: analysis of the molecular basis of the debrisoquine 4-hydroxylase deficiency in DA rats. *Biochemistry* **28:**7349–7355 (1989).
34. Bars, R. G., A. M. Mitchell, C. R. Wolf, and C. R. Elcomb. Induction of cytochrome P-450 in cultured rat hepatocytes: the heterogeneous localization of specific isoenzymes using immunocytochemistry. *Biochem. J.* **262:**151–158 (1989).
35. Wortelboer, H. M., C. A. de Kruif, A. A. J. van Iersel, H. E. Falke, J. Noordhoek, and B. J. Blaauboer. Comparison of cytochrome P450 isoenzyme profiles in rat liver and hepatocyte cultures: the effects of model inducers on apoproteins and biotransformation activities. *Biochem. Pharmacol.* **42:**381–390 (1991).
36. Steward, A. R., G. A. Dannan, P. S. Guzelian, and F. P. Guengerich. Changes in the concentration of seven forms of cytochrome P-450 in primary cultures of adult rat hepatocytes. *Mol. Pharmacol.* **27:**125–132 (1985).
37. Jefferson, D. M., D. F. Clayton, J. E. Darnell, Jr., and L. M. Reid. Posttranscriptional modulation of gene expression in cultured hepatocytes. *Mol. Cell. Biol.* **4:**1929–1934 (1984).
38. Sinclair, P. R., W. J. Bement, S. A. Haugen, J. F. Sinclair, and P. S. Guzelian.

Induction of cytochrome P-450 and 5-aminolevulinate synthase activities in cultured rat hepatocytes. *Cancer Res.* **50**:5219–5224 (1990).
39. Hadley, M. A., S. W. Byers, C. A. Suarez-Quian, H. K. Kleinman, and M. Dym. Extracellular matrix regulates Sertoli cell differentiation, testicular cord formation, and germ cell development *in vitro*. *J. Cell Biol.* **101**:1511–1522 (1985).
40. Bendayan, M., M. A. Duhr, and D. Gingras. Studies on pancreatic acinar cells in tissue culture: basal lamina (basement membrane matrix) promotes three-dimensional reorganization. *Eur. J. Cell Biol.* **42**:60–67 (1986).
41. Li, M. L., J. Aggeler, D. A. Farson, C. Hatier, J. Hassell, and M. J. Bissell. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. *Proc. Natl. Acad. Sci. USA* **84**:136–140 (1987).
42. Sasamura, H., K. Nagata, Y. Yamazoe, M. Shimada, T. Saruta, and R. Kato. Effect of growth hormone on rat hepatic cytochrome P-450f mRNA: a new mode of regulation. *Mol. Cell. Endocrinol.* **68**:53–60 (1990).
43. Westin, S., A. Strom, J.-A. Gustafsson, and P. G. Zaphiropoulos. 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. *Mol. Pharmacol.* **38**:192–197 (1990).
44. Ueno, T., and F. J. Gonzalez. Transcriptional control of the rat hepatic *CYP2E1* gene. *Mol. Cell. Biol.* **10**:4495–4505 (1990).
45. Yano, M., E. Falvey, and F. J. Gonzalez. Role of the liver-enriched transcription factor DBP in expression of the cytochrome P450 *CYP2C6* gene. *Mol. Cell. Biol.* **12**:2847–2854 (1992).
46. Emi, Y., C. Chijiiwa, and T. Omura. A different cytochrome P450 form is induced in primary cultures of rat hepatocytes. *Proc. Natl. Acad. Sci. USA* **87**:9746–9750 (1990).

Send reprint requests to: Thomas A. Kocarek, Institute of Chemical Toxicology, 2727 Second Avenue, Room 4000, Detroit, MI 48201.