Biochemical Pharmacology, Vol. 45, No. 8, pp. 1583–1591, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
Pergamon Press Ltd

MAINTENANCE AND INDUCTION IN CO-CULTURED RAT HEPATOCYTES OF COMPONENTS OF THE CYTOCHROME P450-MEDIATED MONO-OXYGENASE

MAY AKRAWI, VERA ROGIERS,* YVES VANDENBERGHE,* COLIN N. A. PALMER,†‡ ANTOINE VERCRUYSSE,* ELIZABETH A. SHEPHARD§ and IAN R. PHILLIPS†

Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K.; *Department of Toxicology, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium; and †Department of Biochemistry, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, U.K.

(*Received* 2 *October* 1992; *accepted* 1 *February* 1993)

Abstract—Hepatocytes grown in culture rapidly lose many of the cytochromes P450 (CYP) responsible for metabolizing foreign compounds. Among the proteins most readily lost are members of the CYP2B subfamily. We have investigated, by RNase protection assays, the ability of rat hepatocytes, cultured conventionally or co-cultured with rat liver epithelial cells, to maintain the expression of genes encoding members of the CYP2B subfamily, and the inducibility of this expression by phenobarbital. After 4 days of conventional hepatocyte culture CYP2B mRNAs were undetectable, but remained inducible by phenobarbital. In co-cultured hepatocytes the abundance of the mRNAs remained relatively constant from 4–14 days. After 7 days of co-culture the concentration of the mRNAs was increased 12–15-fold by phenobarbital. RNase protection assays with probes capable of distinguishing between CYP2B1 and 2B2 mRNAs demonstrated that the ratios of the abundance and inducibility of the two mRNAs were the same in co-culture as *in vivo*. Co-cultured hepatocytes also maintained the expression of genes coding for two other components of the cytochrome P450-mediated mono-oxygenase, namely cytochrome P450 reductase and cytochrome b_5.

Primary cultures of adult rat hepatocytes are often used as alternatives to animals in pharmaco-toxicological studies [1–3]. However, when cultured under conventional conditions, such cells undergo a rapid decline in the activities of many of the enzymes responsible for xenobiotic metabolism [1, 4, 5], especially members of the cytochrome P450 (CYP) superfamily.‖ Consequently, results obtained from hepatocyte culture do not accurately reflect the situation *in vivo*. Recent studies have demonstrated that to maintain their differentiated state *in vitro* hepatocytes require a complex and well-defined environment. The expression of liver-specific functions is regulated not only by exogenous soluble factors, but also by cell–matrix and cell–cell interactions (reviewed in Ref. 8).

Attempts to maintain cytochrome P450-dependent functions, through modification of the culture medium by the addition of hormones [9, 10], growth factors [11], trace elements [12], special nutrients [13, 14], enzyme inducers [5, 15–17], nicotinamide [18], dimethyl sulphoxide [19] and various other substances [20], have met with mixed success. Encouraging results have recently been obtained by changing the substratum to which the cells attach. For instance, the expression and inducibility of several cytochromes P450 were found to be maintained when rat hepatocytes were cultured on a biologically derived support medium known as matrigel [21, 22], or in serum-free medium on the collagen-based substrate, vitrogen [23].

A further approach has been the modification of cell–cell interactions by co-culturing hepatocytes with non-parenchymal cells such as sinusoidal endothelial cells [24, 25], human fibroblasts [26], established fibroblastic cell lines [27–29], various epithelial and mesenchymal cell lines [30], or rat epithelial cells derived from primitive biliary duct cells [31]. Hepatocytes cultured under these conditions, especially those co-cultured with primary rat epithelial cells [1, 31], have a longer viability than hepatocytes cultured under conventional conditions, and retain to a greater extent the morphological and biochemical characteristics of adult hepatocytes *in vivo*, including cytochrome P450-mediated biotransformations. It has been claimed that as much as 100% of the cytochrome P450 content and *N*-aminopyrine demethylation activity can be maintained in co-cultured hepatocytes [31]. Some other Phase I enzymic activities also appear to be maintained, since drugs such as Ketotifen [32], oxaminozoline and clonidine [33] were able to be metabolized by several pathways. Maier [34] however has reported that aldrin epoxidase (AE¶) activity underwent a significant decrease as a function of culture time.

In our own experiments on adult rat hepatocytes

‡ Present address: Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, CA 92037, U.S.A.

§ Corresponding author. Tel. (071) 387 7050 ext. 2321; FAX (071) 380 7193.

‖ The cytochrome P450 classification and nomenclature system used is that of Nebert *et al.* [6] as modified by Nebert *et al.* [7].

¶ Abbreviations: AE, aldrin epoxidase; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate.

co-cultured with rat liver epithelial cells [35] it was found that a steady-state situation was obtained in which total cytochrome P450 content and 7-ethoxycoumarin *O*-deethylase and AE activities were maintained at 25, 100 and 15%, respectively, of their corresponding values in freshly isolated hepatocytes. Both the total cytochrome P450 content and 7-ethoxycoumarin *O*-deethylase activity, but not AE activity, were induced by phenobarbital treatment [35]. These results indicate a degree of selectivity in the ability of co-cultured hepatocytes to maintain the expression and inducibility of individual members of the cytochrome P450 superfamily.

For an *in vitro* system to be of any real value for pharmaco-toxicological studies, it is of the utmost importance that the content and inducibility of individual cytochromes P450 be determined, so that results obtained with the system can be interpreted correctly. Therefore, the aim of the present study was to investigate the expression, in rat hepatocytes co-cultured with rat liver epithelial cells, of genes coding for individual members of the CYP2B subfamily and their responsiveness to phenobarbital. The expression was also studied of genes coding for two other components of the cytochrome P450-mediated mono-oxygenase, namely NADPH-dependent cytochrome P450 reductase and cytochrome b_5. The results are compared with those obtained from conventionally cultured hepatocytes isolated from the same animals.

MATERIALS AND METHODS

Chemicals

Crude collagenase type I, bovine serum albumin (fraction V) and bovine insulin were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All media, foetal calf serum and trypsin-EDTA solution were from Gibco (Brussels, Belgium) and hydrocortisone hemisuccinate was from Roussel (Paris, France).

Isolation and culture conditions of rat hepatocytes

Hepatocytes were isolated, as described previously [36], from outbred male Sprague–Dawley rats (200–250 g) (Iffa-Credo, Belgium) which had been given free access to food and water. Cell integrity was tested by Trypan blue exclusion, and was between 80 and 93%. Hepatocytes were seeded at a density of 10^7 cells/175 cm^2 petri dish in 24 mL of medium. The medium consisted of 25% Medium 199 and 75% minimum essential medium, supplemented with bovine serum albumin (1 mg/mL), bovine insulin (10 μg/mL), 10% foetal calf serum and antibiotics (penicillin, 4.5 μg/mL; streptomycin sulphate, 50 μg/mL and kanamycin monosulphate, 50 μg/mL). The medium was renewed 4 hr after cell seeding, and daily thereafter. For co-cultures, hepatocytes were cultured as above for 4 hr, to allow the cells to attach, before the addition of rat liver epithelial cells [37]. Epithelial cells, prepared from the livers of 10-day-old Sprague–Dawley rats by trypsinization [38], were cultured in Williams' medium and used before they underwent spontaneous transformation.

Hydrocortisone hemisuccinate was included in the daily renewal of the hepatocyte medium at final concentrations of 7×10^{-5} and 7×10^{-6} M for conventional cultures and co-cultures, respectively, as described in Ref. 35. For the induction experiments, phenobarbital was present in the medium at a final concentration of either 3.2 or 2 mM throughout the culture period. Cultures were incubated at 37° in a humidified atmosphere of air containing 5% CO_2. Epithelial cells, cultured in the presence or absence of the appropriate concentration of phenobarbital, were included in this study as controls.

RNA isolation

Cells were harvested immediately after cell isolation and at various times during culture (4 days for conventional culture; 4, 7 and 14 days for co-culture), and washed three times in phosphate-buffered saline. The cells were sedimented by centrifugation and the pellets stored at −80°. Total RNA was isolated from frozen cell pellets and from rat liver by a guanidinium thiocyanate/LiCl method [39].

RNase protection assays

Probe preparation. CYP2B common probe: A 235 bp *Bam*HI/*Bal*I fragment of a cDNA clone encoding a rat CYP2B2 [40] was inserted between the *Bam*HI/*Eco*RI sites of a Bluescript KS plasmid (Stratagene). The resulting construct (pBS2B) was linearized by digestion with *Bam*HI, then treated with proteinase K (50 μg/mL) (Boehringer, Mannheim, Germany), 10% sodium dodecyl sulphate (SDS) for 30 min at 37°, extracted once with an equal volume of phenol/chloroform (1:1, v/v) and ethanol precipitated. A radiolabelled "antisense" transcript of the linearized DNA template (1 μg) was produced by using an *in vitro* transcription kit (Stratagene), T3 RNA polymerase and [α-^{32}P]UTP (>400 Ci/mmol, Amersham International, Amersham, U.K.). The DNA template was digested with RNase-free DNase I (Stratagene). The transcript was ethanol-precipitated, and purified by electrophoresis through an 8 M urea/6% polyacrylamide gel. After exposing the gel to an X-ray film (Fuji-RX) the portion containing full-length transcript was cut out and placed in 1 mL of 0.5 M ammonium acetate, 10 mM EDTA, 1% SDS. RNA was eluted by shaking for 2 hr at 37° in an orbital incubator and ethanol-precipitated after the addition of 30 μg of tRNA as carrier. RNA probe was resuspended to a final concentration of 10^4 cpm/μL in RNase protection hybridization buffer (80% formamide, 40 mM 1,4-piperazinediethanesulphonic acid (pH 6.4), 0.4 M NaCl, 1 mM EDTA) [41] and stored in aliquots at −20°.

CYP2B1 and CYP2B2 specific probes: Two synthetic oligonucleotides, (a) 5′ TAATACGACTCACTATAGATCGATCAGGTGATCGGCTCACAC 3′ and (b) 5′ ATTTAGGTGACACTATAGAATCTTGGGAAGCAGGTACCCTCGGA 3′, were kindly provided by Dr David Bell of the Department of Life Sciences, University of Nottingham. Oligonucleotide (a) contained nucleotides coding for a T7 promoter followed by 26 nucleotides identical to positions 965–990 of

the coding sequence of rat CYP2B2 mRNA. Oligonucleotide (b) contained nucleotides coding for an SP6 promoter followed by 23 nucleotides complementary in sequence to positions 1145–1123 of the coding sequence of CYP2B2 mRNA. The oligonucleotides were used to prime the amplification, by a polymerase chain reaction (PCR), of a 180-bp region of CYP2B1 and 2B2 cDNAs. In this region, extending from coding nucleotides 965–1145, the two cDNAs have only 90.5% sequence similarity, but are identical in the regions complementary to the priming oligonucleotides.

PCRs were performed in a total volume of 50 μL containing 20 pmol of each oligonucleotide primer, 20 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 25 mM KCl, 0.05% Tween 20, bovine serum albumin (100 μg/mL), 100 μM of each dNTP and 100 ng of plasmid DNA containing a cDNA insert coding for either CYP2B1 (D. R. Bell, E.A.S. and I.R.P., unpublished) or CYP2B2 [40]. The reaction mixture was heated at 95° for 5 min before the addition of 2.5 units of *Taq* DNA polymerase (Promega) and then incubated for 10 cycles at 90° for 90 sec, 60° for 90 sec and 70° for 90 sec. Antisense RNA probes specific for either CYP2B1 or 2B2 mRNAs were produced from the appropriate amplified DNA templates by using SP6 RNA polymerase (Stratagene) essentially as described above for the CYP2B common probe.

Assays. Hybridizations were performed essentially as described by Myers *et al.* [41] in a total volume of 30 μL of RNase protection hybridization buffer that contained 10–20 μg of sample RNA and 0.4–1.0 × 10^5 cpm of radiolabelled antisense RNA transcript. The total amount of RNA was adjusted to 30 μg by the addition of tRNA. The hybridization mixture was heated at 80° for 10 min then incubated overnight at 45°. RNA probe that was not protected by hybridization to complementary target mRNA was digested with RNase A [41]. Hybrids were denatured in 97% formamide at 80° and electrophoresed through an 8 M urea/6% polyacrylamide gel. To detect the protected RNA species the gel was washed for 1 hr in 10% methanol, 10% acetic acid, then dried, and autoradiographed at −78° with an intensifying screen. The protected RNA probe was quantified by densitometric scanning using a VIDS V image analyser (Analytical Measuring Systems, Cambridge, U.K.). Using the method of Little and Jackson [42] the quantification of the corresponding mRNA in terms of molecules/μg of total RNA or molecules/cell was determined from a standard curve of undigested probe. For co-culture experiments, mRNA concentration is given as molecules/hepatocyte.

Northern blot hybridization

RNA samples were denatured in 60% formamide/0.66 M formaldehyde and electrophoresed through a 1.2% agarose gel containing 0.66 M formaldehyde [43]. After transfer to a nylon membrane (Hybond-N, Amersham International) the RNA was hybridized to rat cDNA clones coding for either CYP1A2 [44], cytochrome P450 reductase [45] or cytochrome b_5 [46]. Membranes were washed as described previously [47] to a final stringency of 18 mM NaCl,

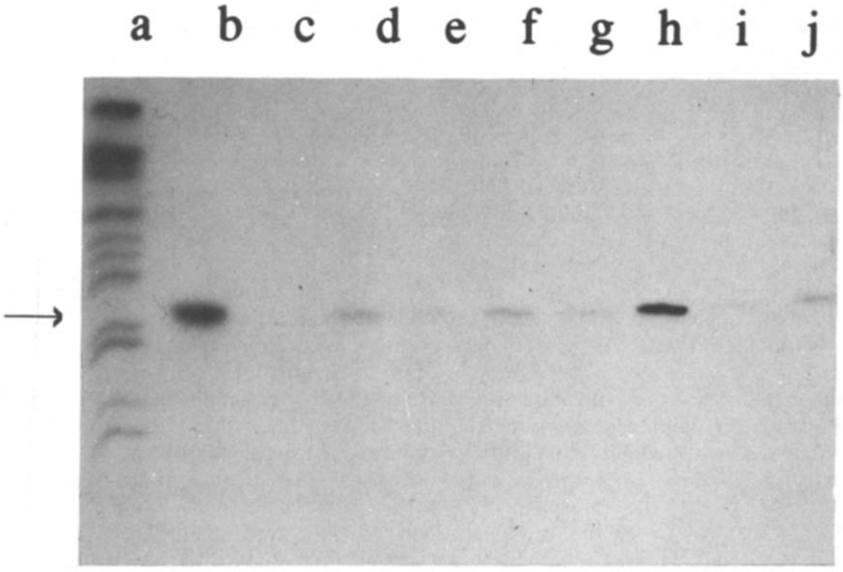

Fig. 1. RNase protection assays of CYP2B1/2B2 mRNAs. Total RNA was extracted from freshly isolated hepatocytes (b), hepatocytes cultured under conventional conditions for 4 days (c, d) and hepatocytes co-cultured for 4 days (e, f), 7 days (g, h) and 14 days (i, j). RNA samples in tracks d, f, h and j were isolated from cultures treated with 3.2 mM sodium phenobarbital. Each hybridization reaction contained 10 μg of total RNA and 1 × 10^4 cpm of radiolabelled antisense RNA complementary to both CYP2B1 and 2B2 mRNAs. Track a shows molecular mass standards (1 kb ladder, Bethesda Research Laboratories). The arrow indicates the fully protected 235 nucleotide fragment.

1 mM sodium phosphate (pH 7.7), 0.1 mM EDTA, 0.1% SDS at 50° and autoradiographed at −78° with an intensifying screen.

Western blot analysis

Frozen cell pellets were prepared as described above for RNA isolation. Prior to electrophoresis cell pellets were homogenized in a solution containing 10 mM potassium phosphate (pH 7.25), 1 mM EDTA, 20% (v/v) glycerol and 2 mM phenylmethanesulphonylfluoride. Proteins were separated by electrophoresis through a 7.5% acrylamide gel prior to electro-blotting onto a nitrocellulose filter (Hybond C-extra, Amersham International). CYP2B protein was detected using an anti-CYP2B serum and a goat anti-rabbit immunoglobulin G horseradish peroxidase kit (Bio-Rad Laboratories Ltd, Watford, U.K.).

RESULTS

Expression of CYP2B mRNAs

The expression and induction of members of the CYP2B gene subfamily in rat hepatocytes cultured conventionally, or in the presence of rat liver epithelial cells, were investigated through the use of a quantitative RNase protection assay. The antisense RNA probe used in these experiments was derived from a region of a CYP2B2 cDNA clone, the sequence of which is identical with those of all published CYP2B1 and 2B2 variants but is distinct from that of CYP2B3 [48]. Thus, RNase protection assays using this probe would quantify all known CYP2B1 and 2B2 mRNAs, but not CYP2B3 mRNA.

Initially, we investigated a time course of expression and induction of CYP2B1/2B2 genes in hepatocytes co-cultured with rat liver epithelial cells. In untreated cultures, the concentration of CYP2B1/2B2 mRNAs remained remarkably constant (3–3.5 molecules/cell) between 4 and 14 days of co-culture (Fig. 1, tracks e, g and i). When hepatocytes were incubated in the presence of phenobarbital (3.2 mM) the concentration of CYP2B1/2B2 mRNAs (molecules/cell) increased to 4.5 after 4 days (Fig. 1, track f), 10.5 after 7 days (Fig. 1, track h) and 5.25 after 14 days (Fig. 1, track j). CYP2B1/2B2 mRNAs were undetectable in epithelial cells cultured in either the absence or presence of phenobarbital (data not shown), strongly suggesting that the hybridization signals obtained from RNA derived from co-cultures were due to mRNAs present in hepatocytes.

We also examined the expression and induction of CYP2B1/2B2 genes in hepatocytes cultured conventionally. After 4 days of conventional culture the mRNAs were undetectable (<0.5 molecules/cell) (Fig. 1, track c). In the presence of phenobarbital, the concentration of the mRNAs increased to 4.75 molecules/cell (Fig. 1, track d). As hepatocytes cultured under these conditions began to deteriorate after 4 days it was not possible to continue a time course for these cells.

Having established that the induction of CYP2B1/2B2 mRNAs in co-cultured hepatocytes appears to be maximal at 7 days this time point was chosen for subsequent experiments. We next examined the effect on induction of a lower concentration of phenobarbital, namely 2 mM. These conditions resulted in concentrations of CYP2B1/2B2 mRNAs of up to 50 molecules/cell (Fig. 2). Results obtained from replicate culture plates of a single hepatocyte preparation differed by less than 10%. Although different preparations of hepatocytes were found to differ in their concentration of CYP2B1/2B2 mRNAs, the differences applied both to untreated and phenobarbital-treated cells and consequently the inducibility of the mRNAs in different hepatocyte preparations was very similar (between 12–15-fold).

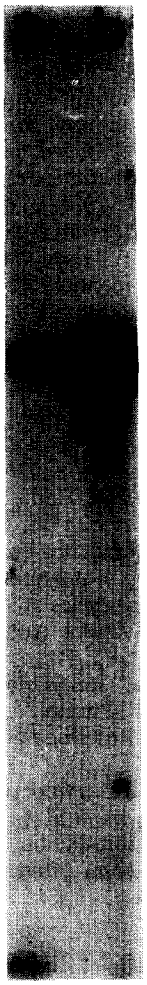

Fig. 2. Induction of CYP2B1/2B2 mRNAs in co-cultured hepatocytes. Total RNA was isolated from hepatocytes co-cultured for 7 days in the presence (b) or absence (a) of 2 mM sodium phenobarbital. CYP2B1/2B2 mRNAs were assayed by RNase protection with a CYP2B1/2B2 common probe as described in Materials and Methods and the legend of Fig. 1.

Having established that, in rat hepatocytes co-cultured for 7 days, CYP2B mRNAs were substantially inducible by phenobarbital we wished to determine the concentrations and inducibility of mRNAs encoding individual members of the CYP2B subfamily, namely CYP2B1 and CYP2B2, at this

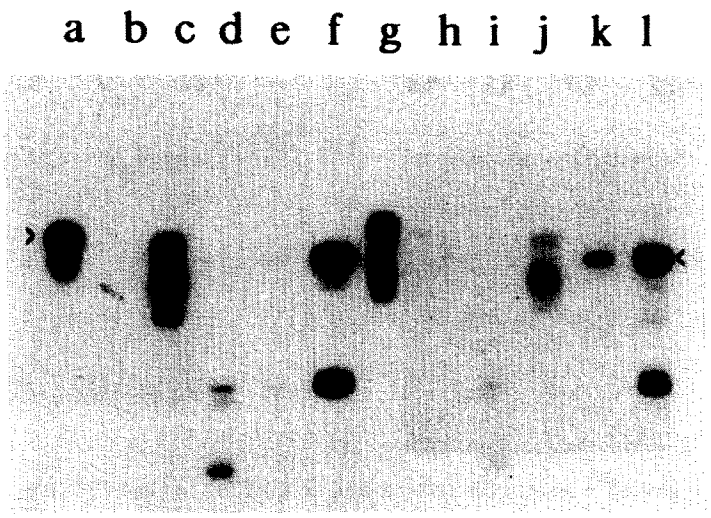

Fig. 3. RNase protection assays using probes specific for mRNAs coding for either CYP2B1 or 2B2. Antisense RNA probes specific for either CYP2B1 (a–f) or CYP2B2 (g–l) mRNAs were hybridized to 20 μg of tRNA (tracks b and h), 100 ng of DNA template coding for CYP2B1 (c and i) or CYP2B2 (d and j), or 10 μg of total RNA from the livers of untreated rats (e and k) or phenobarbital-treated rats (f and l). Tracks a and g contain undigested CYP2B1 and 2B2 probes, respectively. The positions of undigested probes (220 nucleotides) and fully protected fragments (180 nucleotides) are indicated by arrowheads.

stage of co-culture. Our approach was to use the RNase protection technique with antisense RNA probes designed specifically to distinguish between CYP2B1 and 2B2 mRNAs. To establish the specificity of the assays, each probe was hybridized to DNA molecules, coding for either CYP2B1 or 2B2, derived by PCR amplification as described in Materials and Methods. After treatment with RNase A, fully protected fragments (180 nucleotides long) were obtained when either probe was hybridized to its homologous DNA (Fig. 3, tracks c and j), but not when they were hybridized to the heterologous DNAs (Fig. 3, tracks d and i). The smaller protected fragments produced in the latter case are the result of RNase cleavage at positions of mismatches within the hybrid molecules. Thus, the two antisense probes permit complete discrimination between nucleotide sequences encoding CYP2B1 and 2B2.

CYP2B1 mRNA was undetectable in hepatocytes co-cultured for 7 days (Fig. 4A, track c) but was highly inducible by phenobarbital (>19-fold) (Fig. 4A, track d). CYP2B2 mRNA however was present at very low concentrations in the co-cultured hepatocytes (Fig. 4B, track c). This mRNA was also inducible by phenobarbital, but to a lesser extent (3-fold) than the CYP2B1 mRNA (Fig. 4B, track d). Neither of the mRNAs was present nor inducible in biliary epithelial cells (Fig. 4A and B, tracks a and b). The ratio of the abundance and inducibility of CYP2B1 and 2B2 mRNAs in co-cultured hepatocytes (Fig. 4) was similar to those found *in vivo* (Fig. 3, tracks e, f, k and l).

Expression of CYP2B proteins

To determine whether the observed increases in CYP2B mRNAs were reflected in corresponding increases in CYP2B protein we examined the expression and induction of these proteins in both conventionally and co-cultured hepatocytes (Fig. 5). The expression of CYP2B protein decreases in hepatocytes cultured either conventionally or co-cultured with epithelial cells. However, hepatocytes grown under either culture conditions maintain the ability to respond to phenobarbital (Fig. 5, tracks c, e, g and i). The maximum induction (17–18-fold) of CYP2B protein by phenobarbital was observed in hepatocytes after 7 days of co-culture (Fig. 5, track g). Thus, the maximal induction pattern of CYP2B protein by phenobarbital mirrored that observed for CYP2B mRNAs. CYP2B protein was not detectable in the epithelial cells used in the co-culture system nor was the protein inducible by phenobarbital in these cells (Fig. 5, tracks j and k).

Expression of mRNAs encoding cytochrome P450 reductase and cytochrome b_5

We also investigated the expression in conventionally cultured and co-cultured hepatocytes of genes coding for other components of the cytochrome P450-mediated mono-oxygenase. Northern blot hybridization demonstrated that hepatocytes cultured for 4 days either conventionally or in co-cultures contained similar concentrations of cytochrome b_5 mRNA (Fig. 6A, tracks d and f). The concentration of the mRNA was maintained in 7 day co-cultures (Fig. 6A, track h) but was beginning to decline after 14 days of culture (data not shown). In all cases the mRNA was inducible between 2- and 4-fold by phenobarbital (Fig. 6, tracks e, g, i, and data not shown). In epithelial cells cytochrome

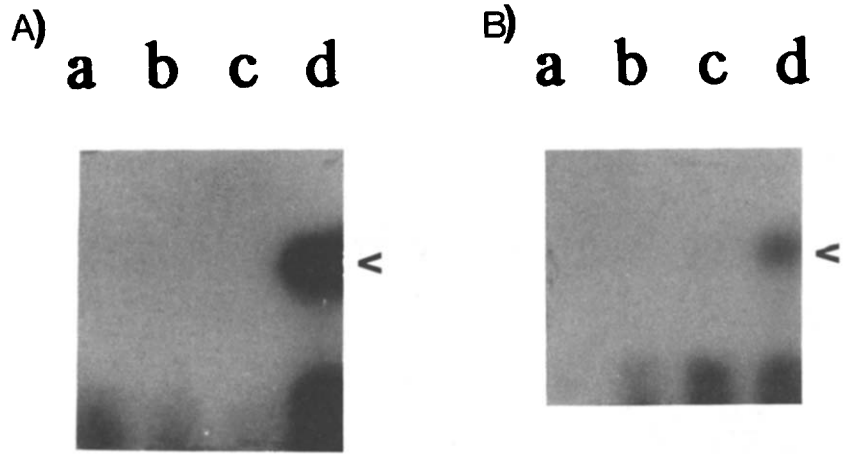

Fig. 4. RNase protection assays of mRNAs coding for CYP2B1 (A) and 2B2 (B). Total RNA was isolated from liver epithelial cells (a, b) and hepatocytes co-cultured for 7 days (c, d). RNA samples in tracks b and d were isolated from cultures treated with 2 mM phenobarbital. Each hybridization reaction contained 10 μg of total RNA and 1×10^4 cpm of radiolabelled antisense probe complementary to either CYP2B1 (A) or CYP2B2 (B) mRNAs. The arrowheads indicate the fully protected 180 nucleotide fragments.

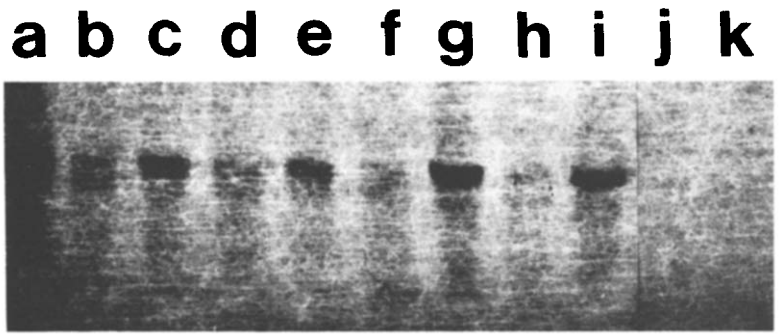

Fig. 5. Western blot analysis of total cell homogenates isolated from hepatocytes cultured under conventional conditions for 4 days (b, c) or co-cultured for 4 (d, e), 7 (f, g) or 14 (h, i) days or from liver epithelial cells (j, k). Proteins in track a were from freshly isolated hepatocytes prior to plating. Protein samples in tracks c, e, g, i and k were isolated from cultures treated with 2 mM phenobarbital. Tracks a–i contained 75 μg protein, and tracks j and k 50 μg of protein. CYP2B protein was detected by western blotting using an anti-CYP2B serum.

b_5 mRNA was present in very low amounts and was not inducible by phenobarbital (Fig. 6A, tracks a and b). A very similar pattern of expression and induction in hepatocytes and epithelial cells was obtained for the mRNA coding for cytochrome P450 reductase (Fig. 6B). We also examined the expression and induction of cytochrome b_5 and cytochrome P450 reductase using western blotting and antibodies specific to these proteins. As was the case for CYP2B protein the pattern of expression and induction of cytochrome b_5 and cytochrome P450 reductase mirrored that found for the mRNAs encoding these proteins (data not shown).

To demonstrate that treatment of the co-cultured cells with phenobarbital does not cause an increase in the concentrations of all cellular mRNAs we examined the expression of the mRNA encoding CYP1A2. This mRNA is known not to be inducible by phenobarbital *in vivo*. Northern blot hybridization, using a 3′ non-coding CYP1A2 cDNA probe which does not cross-hybridize with CYP1A1 mRNA, showed that the abundance of the CYP1A2 mRNA remained relatively constant from 4 to 14 days of co-culture and was not affected by phenobarbital (data not shown).

DISCUSSION

We have used a sensitive method, based on RNase protection, to measure the expression and inducibility in rat hepatocyte cultures of genes coding for members of the CYP2B subfamily. Although

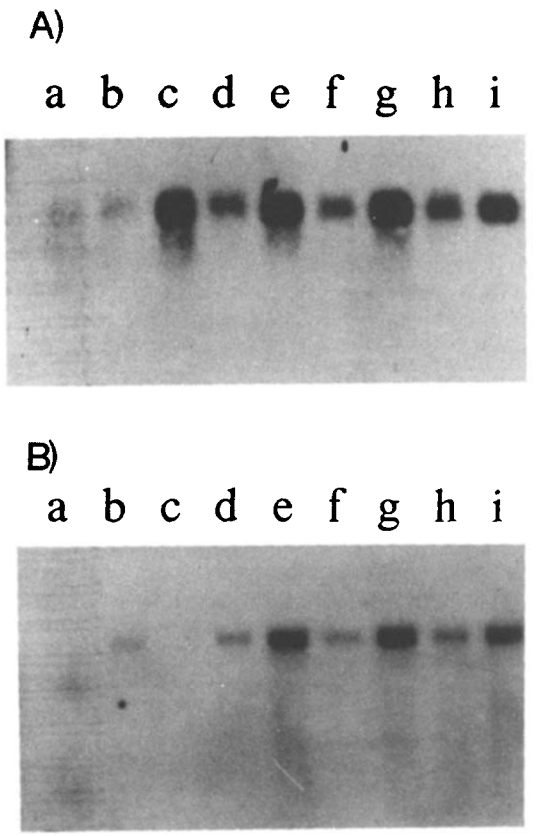

Fig. 6. Northern blot hybridization of mRNAs encoding cytochrome b_5 (A) and cytochrome P450 reductase (B). Total RNA was isolated from liver epithelial cells (a, b), freshly isolated hepatocytes (c), hepatocytes cultured under conventional conditions for 4 days (d, e), and hepatocytes co-cultured for 4 days (f, g) or 7 days (h, i). RNA samples in tracks b, e, g and i were isolated from cultures treated with 3.2 mM phenobarbital. Each track contained 12 μg of RNA. Filters were hybridized with rat cDNA clones encoding cytochrome b_5 (A) or cytochrome P450 reductase (B).

hepatocytes did not express CYP2B genes after 4 days of conventional culture, they retained the ability to induce the expression of these genes in response to treatment with phenobarbital. The expression of CYP2B genes and its induction were retained for up to 14 days in co-cultured hepatocytes. Thus, the presence of the epithelial cells (which themselves do not express CYP2B genes) not only maintains the viability of hepatocytes but also their ability to express CYP2B genes. As the induction was greater at 7 days than at 4 days of co-culture, the increase cannot entirely be accounted for by phenobarbital merely decreasing the rate of decline of the mRNA concentration, but must be due, at least in part, to a more positive effect either at the level of gene transcription, or RNA processing or stability. Although the precise mechanism of induction of CYP2B gene expression in co-cultures is not known, it is interesting to note that the ratio of CYP2B1 and 2B2 mRNAs in untreated cells, and the relative inducibility of each of the mRNAs by phenobarbital, reflect closely the situation *in vivo*. However, as treated cells were continuously exposed to phenobarbital, we cannot exclude the possibility that the induction may be due entirely to an increase in the stability of CYP2B mRNAs.

Our results on the expression of CYP2B mRNAs and protein in conventional hepatocyte culture are in broad agreement with those of several groups on the expression of the corresponding proteins [5, 14, 17, 49]. To date, no information has been published concerning the quantification of cytochrome P450 mRNAs in co-cultured rat hepatocytes. Schuetz *et al.* [22], working with rat hepatocytes co-cultured with a rat liver epithelial cell line (ARL15) on vitrogen-coated dishes, observed no immunoreactive CYP2B1 and 2B2 proteins following treatment with phenobarbital. This lack of induction may be explained by the fact that many factors play a role in the development of a functionally intact co-culture system, including the nature of the helper cells, the matrix and the culture medium [8]. For instance, corticosteroids (which are required to obtain the "co-culture effect" [37]) were not present in the system described in [22]. In addition, these workers used an established cell line rather than primary epithelial cells, vitrogen-coated culture dishes and a different culture medium.

Considerable success in the maintenance and induction of specific cytochromes P450 has also been achieved through the use of various biologically derived support matrices such as matrigel [22] and vitrogen [23]. The latter group found that foetal calf serum inhibited the induction of CYP2B proteins by more than 90%. Despite the fact that our culture medium contained 10% foetal calf serum we were able to obtain significant levels of CYP2B1 and 2B2 mRNA expression and induction in co-cultured hepatocytes. However, it may be possible to achieve even greater inducibility in the absence of foetal calf serum.

Co-cultured hepatocytes also maintained mRNAs encoding two important components of the cytochrome P450-mediated mono-oxygenase system: NADPH-dependent cytochrome P450 reductase, which is essential for all reactions catalysed by cytochromes P450 of the endoplasmic reticulum, and cytochrome b_5, which is required as a second electron donor for many cytochrome P450-catalysed reactions. However, in contrast to the CYP2B mRNAs, the mRNAs coding for cytochrome P450 reductase and cytochrome b_5 were equally well maintained (at least for 4 days) in conventional hepatocyte culture. The induction by phenobarbital of cytochrome P450 reductase and cytochrome b_5 mRNAs in both conventionally cultured and co-cultured hepatocytes is similar to that found *in vivo* ([50]; E.A.S. and I.R.P., unpublished). An induction of cytochrome P450 reductase activity by phenobarbital *in vitro* has been reported previously for hepatocytes cultured on vitrogen [23].

Our co-culture system also expressed low levels of CYPIA2 mRNA, an mRNA which several groups have been unable to detect in hepatocytes cultured conventionally in the absence of an appropriate inducer [49, 51, 52].

In conclusion, hepatocytes co-cultured with rat liver epithelial cells maintain, for up to 14 days, significant levels of expression and inducibility of genes coding for specific cytochromes P450 and for two other major components of the cytochrome P450-mediated mono-oxygenase system, namely cytochrome P450 reductase and cytochrome b_5. In this respect, co-cultured hepatocytes more accurately reflect the situation found *in vivo* than do cells cultured conventionally, and thus offer a better *in vitro* system for the investigation of the metabolism of foreign compounds.

Acknowledgements—We thank Dr David Bell for the synthetic oligonucleotides. This work was supported by grants from the Cancer Research Campaign and the City and Hackney Health Authority in the United Kingdom and by the National Fund for Scientific Research in Belgium.

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