Temperature-sensitive mRNA degradation is an early event in hepatocyte de-differentiation

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The isolation and culture of metabolically active hepatocytes by proteolytic digestion of the extracellular matrix of the liver results in the transcriptional silencing of liver-specific genes encoding cytochromes *P*-450 (CYP) and albumin together with an induction of cellular RNase activity. The levels of albumin mRNA are maintained in cultured hepatocytes at similar levels to that present in the intact liver for at least 24 h, whereas the major constitutively expressed CYP2C11 mRNA is rapidly degraded. Hepatocytes heat-shocked at 40 °C during the isolation procedure (which results in an induction of heat-shock protein mRNA species) blocks the increase in RNase activity and abrogates the loss of CYP2C11 mRNA for at least 4 h. Cycloheximide-dependent inhibition of protein synthesis blocks the temperature-dependent induction of heat-shock proteins without affecting the protection afforded to CYP2C11 mRNA, indicating that CYP2C11 mRNA levels are not directly dependent on heat-

shock protein induction and suggesting that the induction of RNase activity might be responsible for the specific loss of CYP2C11 mRNA in hepatocytes isolated at 37 °C. Differential rates of degradation of CYP2C11 transcribed *in vitro* and of albumin mRNA are observed in the presence of cellular extracts from cultured hepatocytes isolated at 37 °C (which have maximally induced levels of cellular RNase activity) but not in comparable extracts from cultured hepatocytes isolated at 40 °C, supporting the hypothesis that an RNase activity is induced in culture that specifically degrades CYP2C11 mRNA but not albumin mRNA. These results suggest that an early event in hepatocyte de-differentiation involves the induction of RNase activity in addition to transcriptional silencing of liver-specific genes and that the induced RNase activity demonstrates specificity within liver-specific gene products.

INTRODUCTION

A common observation during the isolation of cells from solid tissues is their subsequent de-differentiation, which markedly limits the use of cell culture systems *in vitro* to precisely study the factors regulating organ-specific function [2,3]. This process has been examined in cultures of hepatocytes because this cell type is highly differentiated and expresses many physiologically important functions such as the genes encoding cytochromes *P*-450 (CYP) [4]. CYP has evolved into a superfamily of genes with diverse biological functions from the synthesis and degradation of steroid hormones to a role in the mechanism of odour identification [5]. CYPs expressed in the liver also have a prominent role in the metabolism and determination of xenobiotic toxicity [6]. Expression of CYP in the liver is temporarily extinguished in regenerating rat liver [7], extinguished in primary cultures of rat hepatocytes [7,8] and undetectable in many liver-derived cell lines [2].

In rat hepatocytes, the loss of CYP proteins and associated enzyme activities is due to the transcriptional silencing of liver-specific genes, which occurs during isolation and the first few hours of culture [7,9,10]. Such rapid changes in liver-specific gene expression after the isolation of hepatocytes has been termed 'culture shock' and shown in *Xenopus* hepatocytes to be associated with the induction of heat-shock proteins (HSPs) [11]. Accordingly, we have investigated whether the characteristic

alteration of CYP gene expression in rat hepatocyte culture [7,10] is also related to the induction of HSPs. In contrast with studies on *Xenopus* hepatocytes, we report here that the isolation of rat hepatocytes at the conventional temperature of 37 °C does not induce HSP gene expression and that when HSPs are induced by temperature elevation to 40 °C during isolation, this is associated with an abrogation in RNase activity induction observed when hepatocytes are isolated at 37 °C and with the protection of CYP mRNA species, apoproteins and functional activities. The results suggest that rat hepatocyte culture leads to the induction of RNase activity that results in the degradation of CYP mRNA species but not albumin mRNA. The apparent specificity in mRNA stability suggests that in addition to transcriptional silencing, the early de-differentiation process includes the degradation of specific mRNA species.

MATERIALS AND METHODS

Animals and materials

Male Sprague–Dawley CD rats purchased from Charles River Ltd. (Margate, Kent, U.K.) at a body weight of 250–300 g were used in all studies. Animals were housed for at least 1 week before use in an air-conditioned environment of 20 ± 3 °C and a humidity of 50 ± 10 % with a 12 h light/12 h dark cycle. Food (rat maintenance diet no. 1; SDS, Witham, Essex, U.K.) and water were available *ad libitum*.

Abbreviations used: CYP, the genes encoding cytochromes *P*-450; HBSS, Hanks balanced salt solution; HSP, heat-shock protein. The nomenclature for CYP genes is that recommended in [1].

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A full-length rat albumin cDNA used in these studies was cloned in this laboratory by Dr. J. Downey. A full-length CYP2C11 cDNA [12] and anti-CYP2C11 antibody [13] were provided by Dr. Zaphiropoulos (Department of Medical Nutrition, Huddinge, Sweden) and Professor C. R. Wolf (University of Dundee, Dundee, Scotland) respectively. The rat CYP2E1 1.1 kb cDNA [14] and anti-rat CYP2E1 antibody [15] were supplied by Professor M. Ingelman-Sundberg (Karolinska Institutet, Stockholm, Sweden). The rat actin 0.7 kb cDNA was provided by Dr. G. Allenby (Hofmann-La Roche, Nutley, NJ, U.S.A.). A 1.5 kb CYP3A1 cDNA [16] that hybridizes to CYP3A2 mRNA [17] was provided by Dr. F. Gonzalez (NIH, Bethesda, MD, U.S.A.). The anti-rat CYP3A antibody [18] was provided by Dr. D. Waxman (Harvard Medical School, Boston, MA, U.S.A.). Human HSP60 and HSP89 α cDNA clones, human HSP70 genomic clone and the mouse monoclonal anti-human HSP72/73 antibody were purchased from Stressgen (Vancouver, British Columbia, Canada).

Hepatocyte isolation and culture

Single step collagenase liver perfusion was performed as described [8]. In brief, rats were anaesthetized by intraperitoneal injection with 60 mg of sodium pentobarbitone (Rhone Poulenc, Dagenham, Essex, U.K.) per kg of body weight and a heparin-filled 16-gauge cannula was inserted into the portal vein. After the cannula had been secured by ligation, a cut was made in the posterior vena cava and the liver was perfused with Ca²⁺- and Mg²⁺-free Hanks balanced salt solution (HBSS) [0.14 M NaCl/5.4 mM KCl/0.34 mM Na₂HPO₄·7H₂O/0.44 mM KH₂PO₄/5.6 mM D-glucose/15.7 mM NaHCO₃]. The perfusate was gassed with O₂/CO₂ (19:1) before it was passed through the liver at a pH of 7.4 and a temperature of 37 °C, or at an elevated temperature of 40 °C. After the liver had been cleared of blood and excised from the body cavity, it was perfused with HBSS containing 0.025 % (w/v) collagenase H (Boehringer Mannheim, Lewes, East Sussex, U.K.) and recirculated for 30 min. The cells were dispersed in HBSS containing 2 % (w/v) BSA, filtered through 120 μ m nylon mesh and centrifuged at 50 g for 3 min. The cells were resuspended in culture medium (William's Medium E) (Flow Laboratories, Irvine, Scotland, U.K.) supplemented with 1 μ M insulin, 100 μ M hydrocortisone 21-hemisuccinate and 50 μ g/ml gentamycin and washed twice more in culture medium by centrifugation at 50 g for 3 min. The viability of freshly isolated hepatocytes was assessed by their ability to exclude 0.1 % (w/v) Trypan Blue in PBS [137 mM NaCl/27 mM KCl/8 mM Na₂HPO₄/1.5 mM KH₂PO₄/0.9 mM CaCl₂·2H₂O/0.5 mM MgCl₂·6H₂O (pH 7.4)]. Cells that did not exclude the dye were considered 'non-viable' and routinely comprised less than 15 % of the total cell population. Viable hepatocytes (1.8×10^7) were suspended in 20 ml of culture medium further supplemented with 1 μ g/ml fibronectin and seeded on plastic plates 150 mm in diameter in humidified air/CO₂ (19:1) at 37 °C.

Isolation of hepatocyte nuclei and nuclear run-on transcription assays

Run-on transcription assays were performed with nuclei from intact liver and cultured hepatocytes essentially as described previously [19]. The caudate liver lobe was ligated after the removal of blood during liver perfusions before the addition of collagenase to provide an intact liver preparation, and homogenized in 1 vol. of ice-cooled homogenization buffer [0.3 M sucrose/15 mM NaCl/60 mM KCl/20 mM Tris/HCl/0.5 mM EGTA/2 mM EDTA/0.5 mM spermidine/0.15 mM spermine/1

mM dithiothreitol/0.1 % Triton X-100/0.1 mM PMSF (pH 8.0)] and mixed with 3 vol. of hypertonic sucrose buffer [2 M sucrose/20 mM Tris/HCl/0.5 mM EGTA/2 mM EDTA/1 mM dithiothreitol (pH 8.0)]. The homogenate was layered over 1.3 vol. of hypertonic sucrose buffer and centrifuged at 35000 g for 2 h at 4 °C. The pellet enriched with nuclei was resuspended in nuclei storage buffer [50 mM Hepes/NaOH/5 mM MgCl₂/25 % (v/v) glycerol/0.5 mM dithiothreitol/1 μ g/ml acetylated BSA (pH 8.0)]; nuclei were counted, snap-frozen in liquid nitrogen and stored in aliquots at -70 °C. Isolated hepatocytes and cultured hepatocytes were lysed by incubating cells on ice in ice-cooled homogenization buffer and observing plasma membrane lysis visually by microscopic examination of aliquots stained with 0.1 % Trypan Blue. The lysed cellular material was aspirated and centrifuged at 900 g for 5 min at 4 °C. The pellet of intact cells and nuclei was resuspended in homogenization buffer and centrifuged at 100 g for 2 min at 4 °C. The supernatant was retained and centrifuged at 900 g to pellet nuclei, which were resuspended in nuclei storage buffer, counted and stored in aliquots at -70 °C.

Run-on transcription assays were performed with 2.5×10^6 nuclei resuspended in 50 μ l of nuclei storage buffer and added to 50 μ l of transcription buffer [50 mM Hepes/NaOH/2 mM MgCl₂/2 mM MnCl₂/300 mM NH₄Cl/1 μ g/ml acetylated BSA/80 units of placental ribonuclease inhibitor (Pharmacia)/400 μ M ATP/400 μ M GTP/400 μ M CTP]. Transcriptional elongation was initiated by the addition of 5 μ l of [α -³²P]UTP (800 Ci/mmol; Amersham, Little Chalfont, Bucks., U.K.) and incubated at 25 °C for 20 min. Linear rates of transcription with time as determined by incorporation of radiolabel into RNA was observed for up to 25 min. Transcription was halted by treatment with RNase-free DNase-I and proteinase K as outlined [19]; the radiolabelled RNA was purified by phenol/chloroform extraction, precipitation with trichloroacetic acid and washes with ethanol by using fetal calf liver tRNA as a carrier, as outlined [19]. A fixed amount of purified radiolabelled RNA from run-on transcription assays was hybridized at 45 °C to an excess (0.25 pmol) of cDNA probes immobilized on nitrocellulose membranes {after prehybridizing the membranes in 55 % [v/v] formamide/4 $\times$ SSC [20 $\times$ SSC stock: 3 M NaCl/0.3 M trisodium citrate (pH 7)]/0.1 M NaH₂PO₄/5 $\times$ Denhardt's [50 $\times$ Denhardt's stock: 1 % Ficoll type 400/1 % poly(vinylpyrrolidone)/1 % BSA]/0.1 % [w/v] SDS/100 μ g/ml salmon-sperm DNA [pH 7] for 16 h} for 48 h in 55 % (v/v) formamide/4 $\times$ SSC/0.1 M NaH₂PO₄/1 $\times$ Denhardt's/0.1 % SDS/10 μ g/ml salmon-sperm DNA (pH 7). Membranes were washed at high stringency (0.2 $\times$ SSC/0.1 % SDS at 65 °C) and the filters were exposed to autoradiography to quantify the degree of transcription.

Isolation of RNA and Northern blotting

RNA was isolated from whole liver and cultured hepatocytes by using RNazol (Biogenesis, Bournemouth, Hants., U.K.) essentially as outlined in the manufacturer's instructions. Briefly, RNA was extracted from RNazol homogenates with chloroform, precipitated with propan-2-ol and washed with 75 % (v/v) ethanol. The RNA was resuspended in water at approx. 4–8 mg/ml and aliquots were stored at -20 °C. RNA was denatured in 50 % (v/v) formamide and 6 % (w/v) formaldehyde at 65 °C for 10 min and subjected to electrophoresis on 1.5 % (w/v) agarose gel in Mops buffer [20 mM Mops/5 mM sodium acetate/1 mM EDTA (pH 7.0)] containing 1.9 % (w/v) formaldehyde. Integrity and equal loading of RNA were achieved by observing ethidium bromide-stained 28 S and 18 S mRNA bands under UV illumination. The RNA was transferred overnight

from gels to nylon membranes (Hybond-N; Amersham) by capillary action and the RNA was cross-linked to the membrane by UV irradiation (306 nm, 120 mJ). Oligonucleotides for CYP2C11 (5'-ATC CAC GTG TTT CAG CAG CAG CAG GAG TTC-3', complementary to nt 925-954) and CYP3A2 (5'-CAA ATC TTT AAC CAT GCA TCA AGA GCA GTC A-3', complementary to nt 1689-1719) were directly labelled with [γ - 32 P]ATP with a DNA 5'-end labelling kit (Promega, Southampton, Hants., U.K.) essentially as described by the manufacturer. Purified plasmid DNA was cut with the appropriate restriction endonuclease(s) to give 1 μ g of insert DNA and the digested insert was purified by electrophoresis on 0.7-2.5% low-melting-point agarose in TAE buffer [4 mM Tris/1 mM EDTA/0.0114% (v/v) acetic acid (pH 7.6)] containing 1 μ g/ml ethidium bromide. The purified insert was excised from the gel and 25 ng of cDNA was denatured by heating to 100 °C for 2 min and cooling rapidly on ice; the cDNA was radiolabelled with [α - 32 P]dCTP by random priming with a kit supplied by Promega. Unincorporated label from oligonucleotide and cDNA labelling assays was removed by gel filtration (NucTrap[®] push columns; Stratagene, Cambridge, U.K.). Hybridizations with oligonucleotides and cDNA probes were performed with QuikHyb (Stratagene) as in the manufacturer's instructions. Briefly, oligonucleotide hybridization temperatures were performed at 5-10 °C below the calculated melting temperature (T_m) and for cDNA probes at 65 °C. Hybridization was performed for 1-2 h. Membranes were washed at room temperature twice with $2 \times$ SSC/0.1% SDS for 30 min followed by a wash in $0.1 \times$ SSC/0.1% SDS for 30 min. A high-stringency wash (5-10 min) was performed in $0.1 \times$ SSC/0.1% SDS at 5-10 °C below T_m (for oligonucleotides) or between 40 and 55 °C (for cDNA species) before the membranes were wrapped in Saran wrap and subjected to autoradiography at -70 °C.

Preparation of microsomes

Tissue/hepatocytes were washed with ice-cooled PBS and homogenized in TKMSG buffer (50 mM Tris/HCl, pH 7.5, containing 25 mM KCl, 5 mM MgCl₂, 0.25 M sucrose and 5 mM reduced glutathione) with an Ultra-Turrax blender type T25 (Janke and Kunkel, Stauten, Germany) and the resultant pellet was centrifuged at 12000 *g* for 20 min at 4 °C. The supernatant was retained and centrifuged at 100000 *g* for 1 h at 4 °C. The soluble (cytosolic) fraction was aspirated and the microsomal pellet was resuspended in the same buffer with a Potter-Elvehjem homogenizer (Jencons Scientific, Leighton Buzzard, Beds., U.K.) and re-centrifuged at 100000 *g* for 1 h at 4 °C. Microsomal pellets were finally resuspended in microsomal buffer [10 mM potassium phosphate buffer, pH 7.25, containing 1 mM EDTA and 20% (v/v) glycerol] and stored in liquid nitrogen at a protein concentration of approx. 20 mg/ml. The protein concentration was determined by the method of Lowry et al. [20] with BSA as standard.

Western blotting

Microsomal protein was separated by a discontinuous SDS/PAGE system with a 9% (w/v) separating gel and a 4% (w/v) stacking gel. Protein samples were denatured under reducing conditions by addition to loading buffer [62.5 mM Tris buffer, pH 6.8, containing 10% (v/v) glycerol, 2% (w/v) SDS, 100 mM dithiothreitol and 0.002% Bromophenol Blue] and heating by placing in a boiling-water bath for 3 min. After separation the proteins were transferred to nitrocellulose (4.2 μ m nitrocellulose sheets; Schleicher and Schuell, Anderman and Co., Kingston, Surrey, U.K.) in 25 mM Tris/192 mM glycine/20% (v/v)

methanol (Merck Ltd, AnalaR grade) (pH 8.3). Nitrocellulose blots were washed in TBS [0.2 M NaCl/20 mM Tris/HCl (pH 7.4)] and non-specific binding sites were blocked overnight at 4 °C in TBS containing 3% (w/v) BSA and 0.3% (v/v) Tween-20. After blocking, the membrane was washed in TBS-T [TBS supplemented with 0.05% (w/v) Tween-20] and then incubated with the appropriate antisera diluted with incubating buffer [TBS-T supplemented with 0.3% (w/v) BSA] for up to 1 h. The blots were washed before incubation for 1 h with the appropriate species-specific horseradish peroxidase-conjugated anti-(anti-IgG) (Bio-Rad). The membrane was thoroughly washed before detection by exposing X-ray film to chemiluminescence generated by a reaction mediated by the antibody-conjugated horseradish peroxidase with an enhanced chemiluminescence kit (Amersham).

CYP spectrophotometric and enzyme assays

Total CYP concentrations in microsomal extracts were determined in samples after dilution in Renex Buffer [50 mM Tris/HCl, pH 7.5, containing 0.2% (v/v) Renex 690, 20% glycerol and 50 μ M EDTA] by measuring the reduced carbon monoxide bound compared with reduced difference spectra between 500 and 400 nm. The difference in absorbance at 490 and 450 nm was used to calculate the concentration of the cytochrome by using an absorption coefficient of 91000 M⁻¹·cm⁻¹ [21]. Microsomal steroid hydroxylase activities were determined as described previously [19] by using [4-¹⁴C]androstenedione (Amersham). After incubation, the metabolites were separated by TLC and located by autoradiography. Metabolites were identified by co-migration of authentic standards (Steraloids). Formation of metabolites was quantified by scintillation counting after they had been scraped from the plate.

RNase assays

Extracts for assay of RNase activity were prepared by homogenizing intact liver or cultured hepatocytes in 20 mM Tris buffer, pH 8.0, supplemented with 15 mM NaCl, 135 mM KCl and 2 mM EDTA and snap-freezing aliquots in liquid nitrogen for storage at -70 °C. Extracts were defrosted and adjusted to 5 mg/ml protein containing 0.5% (w/v) sodium deoxycholate and incubated at room temperature for 30 min to solubilize the protein. Solubilized extract (0.1 ml) was added to 0.35 ml of 180 mM Tris/6 mM EDTA (pH 8.0) and warmed to 37 °C before the addition of 0.25 ml of 1% (w/v) calf liver RNA. Samples were incubated for 15 min before the addition of 0.7 ml of ice-cooled 0.75% (w/v) lead acetate in 25% (w/v) HClO₄. Samples were vortex-mixed and left on ice for 15 min before centrifugation at 12500 *g* for 15 min at 0 °C to precipitate protein and long-chain RNA. The absorbance of the supernatant at 260 nm was used to determine the concentration of nucleotides by using an absorption coefficient of 40 M⁻¹·cm⁻¹. The absorbance produced by isolated hepatocytes was typically 0.20, whereas blanks produced by incubation of cell extracts without calf liver RNA substrate typically gave an absorbance of 0.035, which was subtracted from test values. Incubation of RNA substrate without cell extract resulted in an absorbance of 0.002.

The CYP2C11 cDNA was subcloned into pGEM 3Z at the *Eco*R1 site and the T7 promoter was used to drive the transcription of CYP2C11 mRNA *in vitro*. The T3 promoter was used to drive the transcription *in vitro* of the albumin cDNA cloned into pBluescript SK + / - at the *Eco*R1/*Xho*I sites. Briefly, 40 μ g of plasmid DNA was added to transcription mix with a final volume of 100 μ l containing 80 mM Hepes/KOH, 32 mM MgCl₂, 2 mM spermidine, 10 mM dithiothreitol, 2.5 units of

Rnasin, 6 mM GTP, 6 mM CTP, 6 mM ATP and 100 μ Ci of [α - 32 P]UTP (800 Ci/mmol). Transcription was initiated by the addition of 100 units of the appropriate polymerase and the reactions were incubated for 3.5 h at 37 °C. The RNA was purified by gel filtration on Nucrap push columns followed by extraction with phenol/chloroform; the integrity and size were determined by electrophoresis. Albumin mRNA (1956 nt) can be predicted from the plasmid map to contain the entire coding sequence, the 3' untranslated region but no poly(A) tail. CYP2C11 mRNA (1865 nt) can be predicted from the plasmid map to contain 5 nt of 5' untranslated region, the full coding sequence, the 3' untranslated region and a 27-mer poly(A) tail. For the assay of mRNA degradation, 100 μ g of radiolabelled mRNA was incubated with 0.1 ml of solubilized cell extract incubated as for calf liver RNA. The lead acetate/HClO₄ supernatants were analysed for nucleotide concentration by liquid scintillation.

RESULTS

Expression of liver-specific and housekeeping genes after isolation and culture of hepatocytes

The results presented in Figure 1 demonstrate that the procedure to isolate rat hepatocytes by collagenase perfusion extinguishes the transcription of albumin but not that of actin, CYP2E1 or of the liver-specific CYP2C11. At 24 h of culture, rat hepatocytes have also extinguished CYP2C11 and CYP2E1 expression. Analysis of the size range of the transcripts produced by the nuclear run-on assay demonstrate that during hepatocyte isolation and the first 4 h of culture there is a decrease in the mean RNA transcript size compared with the intact liver (Figure 2). After 24 h of culture the mean transcript size of RNA produced is similar to that produced by nuclei from the intact liver. The results presented in Figure 3 show that despite the silencing of albumin transcription, the abundance of albumin mRNA with respect to total cellular ribosomal RNA remains at levels comparable with those in the intact liver.

Figure 1 Nuclear run-on transcription of albumin, actin, CYP2E1 and CYP2C11 genes in intact liver, isolated hepatocytes and hepatocytes cultured for 24 h

Run-on transcription assays from intact liver (lane A), isolated hepatocytes (lane B) and hepatocytes cultured for 24 h (lane C). Radiolabelled RNA (10⁷ c.p.m.) from each preparation of nuclei was hybridized to 0.25 pmol of the cDNA probes and pBR322 plasmid as outlined in the Materials and methods section. Results shown are typical of two separate experiments.

Figure 2 Electrophoresis of run-on transcribed RNA from nuclei prepared from intact liver (IL), isolated hepatocytes (IH), hepatocytes cultured for 4 h (4hr) and 24 h (24hr)

Purified run-on radiolabelled RNA (1.1 $\times$ 10⁵ c.p.m.) from each assay was subjected to electrophoresis under denaturing conditions on a 1% (w/v) agarose gel. After separation, the gel was dried and exposed to X-ray film overnight. Results shown are typical of three separate experiments.

Figure 3 Albumin mRNA abundance in the intact liver and in hepatocytes cultured for up to 24 h

Total RNA (20 μ g per lane) was subjected to Northern blotting: lane 1, intact liver (IL); lane 2, isolated hepatocytes (IH); lanes 3–6, hepatocytes cultured for the indicated times. (A) RNA hybridized with 32 P-labelled rat albumin cDNA. (B) the same blot stained with ethidium bromide, demonstrating equal loading of total RNA. Results shown are typical of three separate experiments.

Figure 4 Effect of isolation temperature on the abundance of CYP mRNA species in intact liver and cultured hepatocytes

Total RNA (20 μ g per lane) was subjected to Northern blotting: lane 1, intact liver (IL); lane 2, isolated hepatocytes (IH); lanes 3–5, hepatocytes cultured for the indicated times. For each probe, the upper blot is RNA from hepatocytes isolated at 37 °C and the lower blot is RNA from hepatocytes isolated at 40 °C. (A) RNA hybridized with 32 P-labelled CYP2C11 oligonucleotide; (B) RNA hybridized with 32 P-labelled CYP2E1 cDNA; (C) RNA hybridized with 32 P-labelled CYP3A2 oligonucleotide. Results shown are typical of at least three separate experiments.

Heat shock during isolation retards the decline of CYP mRNA species and results in the maintenance of CYP protein and activity

Figure 4 indicates that, in contrast with albumin mRNA levels, CYP2C11, CYP2E1 and CYP3A2 mRNA species decline in cultured hepatocytes isolated at 37 °C. However, isolating hepatocytes at 40 °C prevents the loss of these CYP transcripts for at least 4 h when cultured at 37 °C (Figure 4). Isolation at 40 °C was without effect on albumin mRNA abundance during the subsequent culture of hepatocytes at 37 °C (results not shown). Figure 5 indicates that the isolation of hepatocytes at 40 °C

Figure 5 Effect of isolation temperature on the abundance of HSP mRNA species in intact liver and cultured hepatocytes

Total RNA (20 μ g per lane) was subjected to Northern blotting: lane 1, intact liver (IL); lane 2, isolated hepatocytes (IH); lanes 3–5, hepatocytes cultured for the indicated times. For each probe, the upper blot is RNA from hepatocytes isolated at 37 °C and the lower blot is RNA from hepatocytes isolated at 40 °C. (A) RNA hybridized with 32 P-labelled human HSP60 cDNA, which also hybridizes to rodent homologues; (B) RNA hybridized with 32 P-labelled fragment of human HSP70B genomic sequence, which also hybridizes with rodent homologues; (C) RNA hybridized with 32 P-labelled human HSP89 α cDNA, which also hybridizes with rodent homologues but does not hybridize with human HSP89 β mRNA. Results shown are typical of at least three separate experiments.

results in the induction of mRNA species encoding HSP60, HSP70 and HSP90, which were not detectable to any appreciable extent in the intact liver or in hepatocyte cultures prepared at 37 °C. Isolation of hepatocytes at 37 °C followed by culture at 40 °C did not retard the decline of CYP2C11 (results not shown),

Figure 6 Effect of isolation temperature on the abundance of CYP protein in intact liver and hepatocytes cultured for 24 h

Microsomal protein (1.2 μ g per lane) was subjected to Western blotting: lane 1, intact liver (IL); lane 2, hepatocytes cultured for 24 h. For each probe, the upper blot is protein from hepatocytes isolated at 37 $^{\circ}$ C and the lower blot is protein from hepatocytes isolated at 40 $^{\circ}$ C. (A) Probed with anti-CYP2C11 antibody; (B) probed with anti-CYP2E1 antibody; (C) probed with anti-CYP3A antibody. Results shown are typical of three separate experiments.

whereas hepatocytes isolated and cultured at 40 $^{\circ}$ C were dead after 4 h of culture. The effect of isolation at 40 $^{\circ}$ C on the maintenance of CYP mRNA species is sufficient to result in a maintenance of the individual CYP2C11, CYP2E1 and CYP3A2 apoproteins because the levels of immunoreactive protein found in microsomes from hepatocytes cultured for 24 h were similar to those in the donor liver, in contrast with microsomes from hepatocytes isolated at 37 $^{\circ}$ C (Figure 6). The CYP proteins in hepatocytes isolated at 40 $^{\circ}$ C are also catalytically active because there is a maintenance of spectrophotometrically detectable CYP after 24 h of culture, indicative

Table 1 Effect of heat shock during hepatocyte isolation on the total microsomal CYP and microsomal androstenedione hydroxylase activities in hepatocytes cultured for 24 h

Results are means $\pm$ S.D. for three separate experiments at each isolation temperature. Abbreviations: IH, isolated hepatocytes; 7 α -OH, 7 α -hydroxyandrostenedione; 6 β -OH, 6 β -hydroxyandrostenedione; 16 α -OH, 16 α -hydroxyandrostenedione. Levels in microsomes from isolated hepatocytes, 0.60 $\pm$ 0.17 nmol/mg of protein (IH), 80 $\pm$ 14 pmol/min per mg (7 α -OH), 1.1 $\pm$ 0.18 nmol/min per mg (6 β -OH) and 2.1 $\pm$ 0.44 nmol/min per g (16 α -OH). *Significantly different levels compared with microsomes from hepatocytes isolated at 37 $^{\circ}$ C (P < 0.05) with Student's t test (two-tailed).

Isolation temperature ($^{\circ}$ C)	Total CYP at 24 h (% versus IH)	Androstenedione hydroxylase activities at 24 h (% versus IH)		
		7 α -OH	6 β -OH	16 α -OH
37	34 $\pm$ 6	60 $\pm$ 8	44 $\pm$ 16	49 $\pm$ 8
40	104 $\pm$ 11*	85 $\pm$ 9*	80 $\pm$ 10*	84 $\pm$ 7*

Figure 7 Stabilization of CYP2C11 mRNA in cultured hepatocytes isolated at 40 $^{\circ}$ C is unaffected by inhibition of protein synthesis

Hepatocytes isolated at 40 $^{\circ}$ C were seeded with (+) or without (ethanol vehicle only) (–) 3 μ M cycloheximide. (A) RNA subjected to Northern blotting and probed with 32 P-labelled CYP2C11 oligonucleotide. Lane 1, isolated hepatocytes (IH); lanes 2–7, hepatocytes cultured for the indicated periods. (B) Western blot of total cell protein (10 μ g per lane) probed with anti-human HSP72/73 antibody, which cross-reacts with rodent HSP70. Lane 1, intact liver (IL); lane 2, isolated hepatocytes (IH); lanes 3 and 4, hepatocytes cultured for 2 h (2hr). Results shown are typical of three separate experiments.

of haem insertion into the CYP apoprotein. Furthermore microsomes prepared from cultures of hepatocytes isolated at 40 $^{\circ}$ C perform the CYP2C11-mediated 16 α -hydroxylation, the CYP2A1-mediated 7 α -hydroxylation and the CYP3A2-mediated 6 β -hydroxylation [14,18] of androstenedione at rates comparable with those of the donor liver, whereas hepatocyte cultures prepared at 37 $^{\circ}$ C retain approximately half of these activities (Table 1).

Table 2 Effect of hepatocyte isolation temperature on cellular RNase activity in cultured hepatocytes

Results are means $\pm$ S.D. for three separate experiments at each isolation temperature. Cellular RNase activity was determined with calf liver RNA as substrate and is expressed as μ g of nucleotides released/min per mg of cell protein. Abbreviation: IH, isolated hepatocytes before culture. *Significantly different levels compared with extracts from hepatocytes isolated at 40 °C ($P < 0.05$) with Student's *t* test (two-tailed).

Isolation temperature (°C)	Cellular RNase activity during culture (μ g/min per mg)			
	IH	2 h culture	4 h culture	24 h culture
37	23 $\pm$ 2	49 $\pm$ 6*	79 $\pm$ 2*	29 $\pm$ 8
40	18 $\pm$ 12	23 $\pm$ 9	32 $\pm$ 8	29 $\pm$ 4

Table 3 Degradation of CYP2C11 and albumin mRNA species transcribed *in vitro* in hepatocyte extracts from isolated hepatocytes and hepatocytes cultured for 2 h after isolation at 37 or 40 °C

Results are means $\pm$ S.D. for three separate experiments at each isolation temperature, and are presented as nucleotides released/min per mg of cell protein. Albumin mRNA (1956 nt) can be predicted from the plasmid map to contain the full coding sequence, the 3' untranslated region but no poly(A) tail; CYP2C11 mRNA (1865 nt) can be predicted to contain 5 nt of the 5' untranslated region, the full coding sequence, the 3' untranslated region and a 27-mer poly(A) tail. Abbreviations: IH, isolated hepatocytes; n.d., not detectable. *Significantly different levels compared with cell extracts from hepatocytes isolated at 40 °C ($P < 0.05$) with Student's *t* test (two-tailed).

Isolation temperature (°C)	Nucleotide release from mRNA (nucleotides/min per mg)			
	IH		2 h culture	
	CYP2C11	Albumin	CYP2C11	Albumin
37	760 $\pm$ 45*	63 $\pm$ 40*	3200 $\pm$ 1050*	n.d.
40	240 $\pm$ 13	n.d.	240 $\pm$ 21	n.d.

Inhibition of temperature-dependent HSP induction by cycloheximide does not reverse the effects of heat shock on the protection of CYP mRNA

HSPs 60 and 90 are expressed constitutively [22] and hence were found to be so abundant that it was not possible to determine the effect of isolation at 40 °C on their expression. Therefore the effect of inhibiting the synthesis of HSPs *de novo* on CYP2C11 mRNA abundance was monitored via HSP70 protein induction after treating hepatocytes isolated at 40 °C with 3 μ M cycloheximide, which from previous studies is known to block hepatocyte protein synthesis by more than 90% [23]. Figure 7 demonstrates that cycloheximide blocked the induction of HSP70 and that this was without effect on the maintenance of CYP2C11 transcripts in hepatocytes prepared at 40 °C over a 6 h time frame.

Isolation of hepatocytes at 40 °C inhibits the induction of cellular RNase activity in cultured hepatocytes

Table 2 shows that the isolation of hepatocytes at 37 °C results in an induction of cellular ribonuclease activity that does not occur in hepatocytes isolated at 40 °C. Table 3 indicates that cellular extracts from hepatocytes cultured for 2 h, when isolated at 37 or 40 °C, express induced levels of RNase activity, which preferentially degrades CYP2C11 mRNA over albumin mRNA when incubated with each radiolabelled mRNA prepared by

transcription *in vitro* from plasmids containing the full-length cDNA inserts. The addition of RNase A degrades albumin mRNA when added to extracts from hepatocytes isolated at either temperature, suggesting that the albumin mRNA is not protected by factors within the extract.

DISCUSSION

Results in this report indicate that liver-specific gene expression is extinguished at the transcriptional level in rat hepatocytes and that the maintenance of some liver-specific genes (such as albumin) in hepatocytes cultured in serum-free medium is due to mRNA stabilization, in agreement with previous studies with mouse hepatocytes [9,24,25]. A transient decrease in the mean transcript size of RNA synthesized in nuclear run-on assays prepared from hepatocytes cultured for a few hours might be associated with decreased RNA synthesis due, for example, to limited elongation of RNA transcripts. However, the decrease in mean transcript size is correlated with the transient increase in cellular RNase activity, suggesting that the induction of an RNase with exoribonuclease activity might be responsible for the transient decrease in transcript length. In this respect, a transient decrease in poly(A)⁺ RNA between 4 and 6 h of culture is observed in cultured hepatocytes isolated at 37 °C that is correlated with the induction of cellular RNase activity (C. P. Hodgkinson, X.-J. Wang and A. J. Paine, unpublished work). In contrast with albumin mRNA, CYP2C11, CYP2E1 and CYP3A2 mRNA species decline rapidly in cultured hepatocytes isolated at 37 °C, indicating that there is differential stability between liver-specific mRNA species. The rapid degradation of CYP2C11 mRNA transcribed *in vitro* but not of albumin mRNA in the presence of extracts from cultured hepatocytes expressing maximal induction of RNase activity supports the suggestion that an RNase is responsible for the decrease in the mean transcript length in run-on transcription assays from nuclei isolated from hepatocytes at this time and that this RNase is specific between liver-specific transcripts. An additional mechanism(s) could involve the loss of proteins during hepatocyte isolation that protect specific mRNAs from degradation.

The abrogation of CYP mRNA loss effected by isolating hepatocytes at a temperature sufficient to activate HSP gene transcription indicates that the effect of heat-shocking the cells during isolation is not associated with a generalized inhibition of gene transcription. The induction of HSP expression is believed to serve as an adaptive or survival function involving a transient reprogramming of cell metabolism to protect the cell from irreversible damage [22]. In addition to heat, other stresses such as disease states, cell cycle progression and treatment of cells with amino acid analogues or heavy metals might result in the induction of HSP expression [22]. However, the results in this paper suggest that the abrogation of CYP mRNA degradation by heat shock is not associated with HSPs because inhibition of HSP synthesis by cycloheximide-dependent protein synthesis inhibition does not reverse the effects of isolation at 40 °C on CYP2C11 mRNA stability. Heat shock might therefore have the specific effect of inhibiting the induction of RNase activity.

These results suggest that a component of the de-differentiation process in rat hepatocytes involves the active expression of an RNase with differential specificity for different liver-specific mRNA species. The active removal of mRNA species that code for factors promoting the expression of tissue-specific proteins might limit the ability of the cells to re-differentiate fully. The role and mechanism of this process might have implications in cellular de-differentiation *in vivo* such as in tissue regeneration and in neoplasia, and warrants further investigation.

This research was supported by a grant (AP9237/45/29) from the Animal (Scientific Procedures) Committee of the U.K. Home Office and by the Joint Research Board of St. Bartholomew's Hospital (grant reference XMNM).

REFERENCES

- 1 Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W. et al. (1996) *Pharmacogenetics* **6**, 1–42
- 2 Paine, A. J. (1990) *Chem-Biol. Interact.* **74**, 1–31
- 3 Cereghini, S. (1996) *FASEB J.* **10**, 267–282
- 4 Paine, A. J. (1991) *Int. J. Exp. Pathol.* **72**, 349–363
- 5 Gonzalez, F. J. (1990) *Pharmacol. Therap.* **45**, 1–38
- 6 Paine, A. J. (1995) *Hum. Exp. Toxicol.* **14**, 1–7
- 7 Padgham, C. R. W., Boyle, C. C., Wang, X.-J., Raleigh, S. M., Wright, M. C. and Paine, A. J. (1993) *Biochem. Biophys. Res. Commun.* **197**, 599–605
- 8 Wright, M. C. and Paine, A. J. (1992) *Biochem. Pharmacol.* **43**, 237–243
- 9 Clayton, D. F. and Darnell, J. E. (1983) *Mol. Cell. Biol.* **3**, 1552–1561
- 10 Padgham, C. R. W. and Paine, A. J. (1993) *Biochem. J.* **289**, 621–624
- 11 Wolffe, A. P., Glover, J. F. and Tata, J. R. (1984) *Exp. Cell Res.* **154**, 581–590
- 12 Zaphiropoulos, P. G., Mode, A., Strom, A., Husman, B., Andersson, G. and Gustafsson, J.-A. (1988) *Acta Med. Scand. Suppl.* **723**, 161–167
- 13 Borlakoglu, J. T., Scott, A., Henderson, C. J. and Wolf, C. W. (1993) *Int. J. Biochem.* **25**, 1659–1668
- 14 Song, B. J., Gelbion, H. V., Park, S. S., Young, S. C. and Gonzalez, F. J. (1986) *J. Biol. Chem.* **261**, 16689–16697
- 15 Johansson, I., Ekstrom, G., Scholte, B., Puzycki, D., Jornvall, H. and Ingelman-Sundberg, M. (1988) *Biochemistry* **27**, 1925–1934
- 16 Gonzalez, F. J., Nebert, D. W., Hardwick, J. P. and Kasper, C. B. (1985) *J. Biol. Chem.* **260**, 7435–7441
- 17 Gonzalez, F. J., Song, B. J. and Hardwick, J. P. (1986) *Mol. Cell. Biol.* **6**, 2969–2976
- 18 Waxman, D. J., Dannan, G. A. and Guengerich, F. P. (1985) *Biochemistry* **24**, 4409–4417
- 19 Wright, M. C., Wang, X.-J., Pimenta, M., Ribeiro, V., Paine, A. J. and Lechner, M. C. (1996) *Mol. Pharmacol.* **50**, 856–863
- 20 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 21 Omura, T. and Sato, R. (1964) *J. Biol. Chem.* **239**, 2379–2385
- 22 Burdon, R. H. (1986) *Biochem. J.* **240**, 313–324
- 23 Blaauboer, B. J. and Paine, A. J. (1979) *Biochem. Biophys. Res. Commun.* **90**, 368–374
- 24 Jefferson, D. M., Clayton, D. F., Darnell, J. E. and Reid, L. M. (1984) *Mol. Cell. Biol.* **4**, 1929–1934
- 25 Clayton, D. F., Harrelson, A. L. and Darnell, J. E. (1985) *Mol. Cell. Biol.* **5**, 2623–2632

Received 3 July 1997/22 August 1997; accepted 9 September 1997