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Transfection of adult primary rat hepatocytes in culture

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Abstract

The use of adult primary hepatocytes in culture is of importance for the understanding of hepatic processes at the cellular and molecular levels, and the possibility to employ transient transfection of reporter constructs is invaluable for mechanistic studies on hepatic gene regulation. Although frequently used, there is a lack of reports addressing optimization and characterization of transfection of primary rodent hepatocytes. Here, we have shown that the efficiency of biochemical transfection reagents varies significantly and that Lipofectamine2000 was a superior transfection reagent for adult primary rat hepatocytes when using luciferase reporter vectors. The efficiency increased when the cells were allowed ample time to adapt to the in vitro milieu. Cotransfection of a second reporter gene indicated a risk for promoter competition, and we found that relating reporter activity to total cellular protein content gave consistent and reliable results. Differentiation of the cells, achieved by including biomatrix from the Engelbreth–Holm–Swarm mouse sarcoma in the culture system, was to a larger extent required for hormonal/drug responses of transfected constructs than for responses of endogenous genes and assured responses of transfected constructs. Dexamethasone (Dex) is most often included in hepatocyte culture media, but we could not demonstrate a general beneficial effect of Dex on expression of luciferease reporter contructs. Using the established protocol, we have demonstrated responses of transfected constructs to growth hormone, glucocorticoid and LXR stimuli.

Keywords: Rat liver; Primary hepatocytes; Transfection; Lipofection; Luciferase reporter; Extracellular matrix

1. Introduction

Primary culture of liver cells is a valuable system for studying the biology and pathobiology of the liver. The system allows examination of direct effects of hormones and xenobiotics as well as studies on molecular mechanisms of hepatic gene regulation. For maintenance of the adult hepatocyte phenotype in vitro, the attachment support plays a dominant role, and numerous examples in the literature corroborate the beneficial effect of adhesion of the cells to an extracellular matrix, such as the biomatrix extracted from the Engelbreth–Holm–Swarm mouse sarcoma (EHS/Matrigel®) [1]. The effect is attributed to a morphology more closely resembling that of the hepatocyte in vivo, accompanied by maintained expression of liver enriched transcription factors [2,3]. Even transformed, poorly differentiated hepatoma cells have been shown to gain some differentiated functions when cultured in the presence of EHS [2].

A common way to identify gene regulatory sequences is by transient transfection of reporter gene constructs. Liposome mediated transfection (lipofection), probably the most versatile method for introducing exogenous DNA into cells, of primary hepatocytes is, however, severely reduced when the cells are cultured on EHS [4]. This has, as shown by Shih and Towle [5], been possible to overcome by transfecting hepatocytes shortly after plating, followed by an overlay of diluted EHS. A number of studies have emerged in which primary rat hepatocytes have been transfected using different transfection reagents, methods and reporter genes. It is generally accepted that primary hepatocytes are not easy to transfect and the sensitivity of the reporter is an issue. Luciferase is a favourable reporter due to its high sensitivity and the activity is easily measured. Moreover, for cotransfection of a reporter plasmid for normalization, the use of firefly and *Renilla* luciferase reporters makes it possible to measure the respective luciferase activity in a single sample with a dual assay. Despite this, no study has, to our knowledge, addressed optimization of lipofection of primary rat hepatocytes using luciferase as reporters. This is a pertinent task in

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that the kinetics of transient expression can vary with the reporter gene used and with the type of cell transfected [6]. Furthermore, cotransfection of a second plasmid to determine transfection efficiency can affect expression of the test plasmid or vice versa [7], and we have experienced squelching of hormonal response upon cotransfection of primary hepatocytes using electroporation [8]. In an attempt to apply the method of an overlay of diluted EHS for studies on growth hormone (GH)-regulation of gene expression in primary rat hepatocytes, preliminary results indicated that a GH-response was obtained also in the absence of EHS. This prompted us to reinvestigate the prerequisite of EHS for hormonal/drug inducibility of rat hepatic genes and, following optimization of a protocol for lipofection of primary rat hepatocytes, we have investigated hormonal/drug inducibility of luciferase reporter gene activity by different response elements with and without an overlay of EHS. We have also addressed the issue of normalization for transfection efficiency.

2. Materials and methods

2.1. Primary rat hepatocyte cultures

The Stockholm South Ethical Committee of the Swedish National Board for Laboratory Animals approved all animal procedures. Hepatocytes were isolated from 7 to 8-week-old female Sprague–Dawley rats purchased from B&K Universal (Stockholm, Sweden). Hepatocytes were isolated by non-recirculating collagenase perfusion through the portal vein of isoflurane anesthetized rats as previously described [9]. The cells were filtered through a double layer of gauze, pelleted and resuspended in fresh medium four times. Cell viability was assessed by trypan blue exclusion and averaged 90–95%. For studies on induction of endogenous genes, 10×10^6 cells were plated onto 100-mm dishes uncoated or coated with EHS. For transfection experiments, 2×10^6 cells, unless otherwise stated, were plated onto uncoated 60-mm dishes. Cells plated onto uncoated dishes were plated in medium containing 2% fetal calf serum, whereas cells plated onto EHS-coated dishes were in serum free medium. Culture dishes were obtained from Nunc A/S (Roskilde, Denmark). Williams' E medium with Glutamax (Invitrogen Life Technologies, Paisley, Scotland, UK) supplemented with 10 nM insulin (Sigma–Aldrich, Stockholm, Sweden) and 100 U/ml of penicillin and 100 μg of streptomycin/ml (Invitrogen, Life Technologies) was used in all experiments except in Fig. 1B where modified Waymouth's 752 medium [10] was used. The cells were maintained at 37 °C in a humidified 5% CO_2 incubator. Cells were allowed to attach for 15–18 h, thereafter all cultures received serum free medium. EHS-matrix was prepared from Engelbreth–Holm–Swarm sarcoma propagated in C57BL/6 female mice and stored at −20 °C as previously described [1]. The EHS (10 mg/ml) was thawed on ice, and 7 μl/cm^2 was evenly spread onto the dish using a rubber policeman and allowed to form a gel

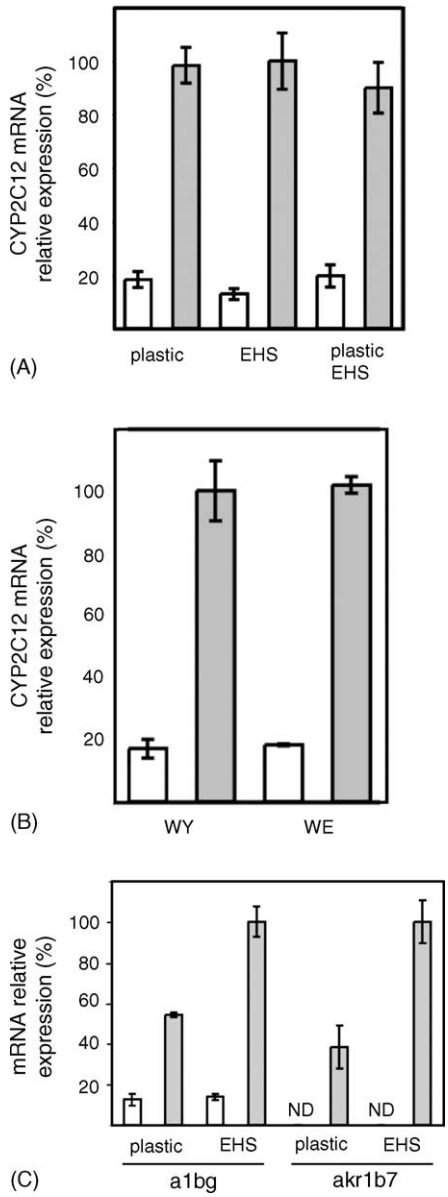

Fig. 1. Effect of different culture conditions on GH-induced gene expression in female derived primary rat hepatocytes. (A) Cells were cultured in modified Williams' E medium on uncoated dishes (plastic), on EHS-coated dishes (EHS) or on uncoated dishes with an overlay with EHS at the time of GH treatment (plastic/EHS). At 42 h of culture age, cells were treated (grey bars) or not (open bars) with 4.5 nM GH and harvested 20 h later. Prepared tNA samples were analyzed for CYP2C12 mRNA contents. The results are expressed in percent of the level in GH treated cells cultured on EHS-coated dishes. (B) Cells were cultured on uncoated dishes in modified Waymouth 752 (WY) or Williams' E (WE) medium, treated with GH and analyzed as in (A), the level of CYP2C12 mRNA in GH treated cells (grey bars) cultured in WY was set to 100%. (C) Expression of a1bg and akr1b7 mRNAs in response to GH treatment (grey bars) in cells cultured on uncoated (plastic) or EHS-coated (EHS) dishes. The levels of expression in cells cultured on EHS with GH treatment were set to 100%. Values shown are the mean ± S.D. from three independent dishes. ND, not detectable.

at room temperature prior to cell isolation. For cells receiving an overlay of EHS, the EHS was diluted 20-fold in ice-cold medium so that following medium change the amount of EHS equalled 7 μl/cm^2.

2.2. Solution hybridization assay

Cells were harvested in SET buffer (1% SDS, 10 mM EDTA, 20 mM Tris–HCl pH 7.5) and total nucleic acids (tNA) were prepared as previously described [11]. The concentration of nucleic acids in tNA samples was measured spectrophotometrically and the DNA concentration was quantified using a fluorimetric assay [12]. The mRNA levels of CYP2C12, a1bg and akr1b7 were analyzed using [^{35}S]UTP-labeled cRNA probes as previously described [13–15]. Results are expressed as attomoles mRNA/μg DNA.

2.3. SDS–PAGE and Western blot

Cells used for Western blot experiments were treated with 10 μM β-naphtoflavone (BNF) (Aldrich Chemical Co., Milwuakee, WI), 100 μM phenobarbital (PB) (Apoteksbolaget, Sweden), 4.5 nM bovine GH (GH) (American Cynamide, Wayne, NJ), 10 μM dexamethasone (Dex) (Sigma–Aldrich) or 10 μM Wy14.643 (ChemSyn Science Laboratories, Lenexa, KS) at 45 h of culture age and harvested 24 h later. The cells were washed in phosphate buffered saline (PBS), harvested in PBS containing 5 mM EDTA and incubated for 1 h on ice to dissolve the EHS. Cells cultured in the absence of EHS were treated in the same way for comparative purposes. After centrifugation, the cells were resuspended in HEPES buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1% Triton X-100, 100 μM Na_3VO_4, 10 mM NaF and one tablet Complete Mini protease inhibitor cocktail per 10 ml (Roche Diagnostics, Bromma, Sweden)). Complete lysis was ensured by aspiration of the cells 10 times through a 27G-needle. The cell lysate was cleared by centrifugation and the supernatant was kept as cellular extract at −80 °C. Proteins were separated on a 9% SDS–PAGE gel and transferred to PVDF-Plus membrane (Osmonics Inc. Westborough, MA) by semi-dry blotting. Membranes were blocked for 1 h in PBS containing 0.1% Tween 20 and 10% fat-free milk protein and incubated with primary antibodies over night at +4 °C. The membranes were washed and incubated for 1 h with horseradish peroxidase (HRP) conjugated secondary antibody. Antibody signals were visualized on X-ray film using the ECL blotting analysis system (Amersham Biosciences, Uppsala, Sweden). The antibodies used were: anti-CYP2C12 [16], anti-CYP2B (Xenotech, Lenexa, KS), anti-CYP3A (Human Biologics, Phoenix, AZ) and anti-CYP4A provided by Dr. G. Gibson, University of Surrey, Surrey, UK. HRP-conjugated secondary antibodies were obtained from Dako A/S, Denmark.

2.4. Transfection and reporter gene assays

Cells were transfected after 40–45 h in culture unless otherwise indicated. The transfection reagents, Lipofectamine2000 (Invitrogen), GeneJammer (Stratagene, AH Diagnostics, Sweden), FuGene6 (Roche Diagnostics), Escort III (Sigma–Aldrich) and DreamFect (Ozbiosciences, Marseille, France) were used according to the manufacturers' recommendations and as described in Section 3. Transfections were terminated by medium change, and cells received medium containing diluted EHS or not and when appropriate, hormones/ligands or vehicle as described in figure legends. The cells were harvested in 1 ml ice-cold phosphate buffered saline (PBS) containing 5 mM EDTA and incubated on ice for 1 h to dissolve the EHS gel. Cells cultured without an overlay of EHS were treated identically. After centrifugation, the cells were resuspended in 100 μl Passive Lysis Buffer (Promega, SDS Biosciences, Sweden) and frozen at −70 °C. Following thawing, the cell debris was pelleted and the supernatant assayed for luciferase activity using the GenGlow 1000 Kit (Thermo Labsystems, Dalarö, Sweden) or the Dual Luciferase assay system (Promega). Protein concentration was determined using the Bradford-Coomassie assay (Pierce Chemical Co., Boule Nordic, Sweden).

2.5. Plasmids

The pGL3-control plasmid encoding the firefly luciferase gene driven by the SV40 promoter (#1741), the pRLtk vector encoding the *Renilla* luciferase gene driven by the thymidine kinase promoter (#2241), the pGL3-promoter plasmid harbouring the SV40 promoter (#1761) and the pGL3-basic vector (#1751) were from Promega. The *Hin*dIII fragment containing two glucocorticoid response elements (GRE), aagcttcGTTACAAACTGTTCTgatcccgggctagacctagctaggggatcAGAACAGTTTGTAACcaagctt [17], was subcloned into the *Sma*I site of the pGL3-promoter plasmid to create $(GRE)_2$pGL3p. Two liver X receptor response elements (LXRE), AGGTCAtttcAGGTCA spaced by 64 bases were cloned into the *Mlu*I and *Bgl*II sites in pGL3-promoter plasmid to create $(LXRE)_2$pGL3p. The *a1bg*-pGL3b plasmid was obtained by cloning of 189 bases upstream of the ATG (−160/+29) of the rat *a1bg* gene into the *Bgl*II and *Mlu*I sites in the polylinker of the pGL3-basic vector.

2.6. Statistical analysis

Data shown are results from pooled experiments or representative data from multiple experiments performed independently. Values are expressed as the mean ± S.D. Comparisons between groups were made using Student's *t*-test or one-way ANOVA followed by Tukey's or Neuman–Keul's test. Samples were considered significantly different at $P < 0.05$.

3. Results

3.1. Expression of endogenous CYP genes at different culture conditions

The *CYP2C12* gene constitutes the prototypical hepatic gene induced by GH in female rats [18]. GH induction of CYP2C12 mRNA was investigated in female derived primary rat hepatocytes cultured in Williams' E medium on EHS-coated or uncoated dishes with or without an overlay of diluted EHS. Unexpectedly, the *CYP2C12* gene was equally induced at all three culture conditions (Fig. 1A). Previous studies, demonstrating the requirement of EHS for GH induction of the *CYP2C12* gene in cultured primary hepatocytes, were carried out using a modified Waymouth 752 medium [10]. Therefore, we compared the GH-induction of the *CYP2C12* gene in hepatocytes cultured without EHS in modified Waymouth 752 or the standard Williams' E medium. No difference in CYP2C12 mRNA expression was observed (Fig. 1B). We could also show that two other GH-regulated genes, *a1bg* [14] and *akr1b7* [15], were induced by GH in cells cultured in the absence of EHS albeit at lower magnitude than in the presence of EHS (Fig. 1C). Cells cultured on dishes from four different manufacturers without EHS responded equally well to GH in terms of *CYP2C12* gene induction (data not shown). The results encouraged us to investigate the induction of *CYP* genes of other subfamilies than the *CYP2C* family by their prototypic inducers in cells cultured in Williams' E medium on uncoated or EHS-coated dishes. Western blot experiments showed that in cells cultured without EHS-coating CYP1A, CYP2C12, CYP3A and to a minor extent CYP4A were induced by BNF, GH, Dex and Wy-14.643, respectively (Fig. 2). However, with the exception of CYP2C12, the induction was lower in cells cultured without than with EHS-coating, and CYP2B was induced by PB only in cells cultured on EHS-coated dishes, which is consistent with other studies [3]. The multiple bands seen with the antibodies directed against CYP2B or CYP4A most likely reflects detection of different isoforms, and the CYP2B antibody obviously also recognizes CYP2B unrelated proteins.

3.2. Optimization of transfection

These findings made us think that gene regulatory studies in primary hepatocytes using reporter gene transfections could be done without the use of EHS and we set out to establish a lipofection protocol. First, we compared five different transfection reagents for transfection of primary rat hepatocytes; GeneJammer, EscortIII, FuGene6, DreamFect and Lipofectamine2000. The plasmid pGL3-control harbouring an SV40 promoter fused to the firefly luciferase gene was used. In our initial experiments, we did not employ cotransfection of a second plasmid, instead firefly luciferase activity was related to cellular

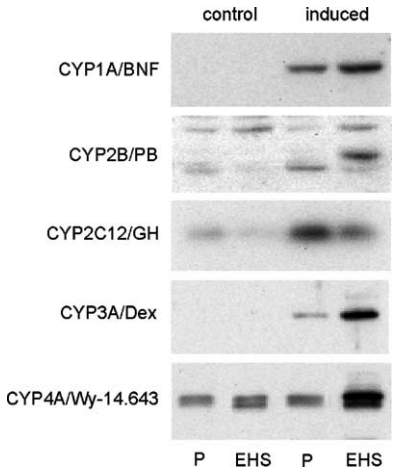

Fig. 2. Western blot analysis of CYP induction in primary rat hepatocytes cultured in Williams' E medium on uncoated (P) or EHS-coated (EHS) dishes. At 42 h of culture age, cells were treated for 20 h with 10 μM β-naphtoflavone (BNF), 100 μM Phenobarbital (PB), 4.5 nM GH, 10 μM dexamethasone (Dex) or 10 μM Wy-14.643 and analyzed for content of CYP1A, CYP2B, CYP2C12, CYP3A and CYP4A, respectively. Control cells received vehicle only.

protein content. As shown in Fig. 3, Lipofectamine2000, used at the DNA/Lipofectamine2000 ratio 1:2, was by far the most potent reagent, giving luciferase activities 10 times higher than the second best reagent, EscortIII. Increased luciferase activity correlated to a higher number of cells being transfected as judged by the number of blue cells quantified following transfection of a RSV-βgalactosidase reporter gene vector and X-gal incubation (data not shown). The ratio DNA/transfection reagent employed and the length of transfection were according to the manufacturers' recommendations, and we made no attempt to optimize these conditions and can therefore not exclude that GeneJammer, EscortIII, FuGene6 or DreamFect would give better results if other conditions were employed. On the other hand, varying the ratios of DNA/Lipofectamine2000 had little impact on the effi-

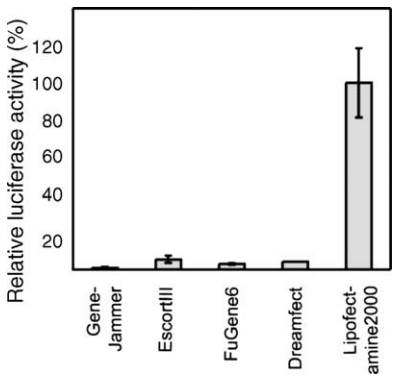

Fig. 3. Relative efficiency of different transfection reagents. Primary hepatocytes were cultured on uncoated dishes and transfected with 2 μg pGL3-control at 40 h of culture age, medium was changed after 6 h of transfection and the cells were harvested 20 h later. Results were expressed as luciferase units/μg protein. Data are given as relative luciferase activity and are the mean ± S.D.; $n = 3$.

ciency (data not shown), and we concluded that of the tested reagents Lipofectamine2000 was the most effective reagent for transfection of primary rat hepatocytes. We did not perform any viability test, but examination of the cell cultures did not reveal any visible differences in cell appearance after exposure to the different reagents. We also compared the transfection activity at different cell densities, 1, 2 or 3.5×10^6 cells per 60-mm dish. There was an inverse relation between cell density and luciferase activity upon lipofection of the same amount of plasmid when the luciferase activity was expressed per μg protein (data not shown). Because luciferase readings in samples from 2 or 3.5×10^6 cells per 60-mm dish were more robust than in samples from 1×10^6 cells, 2×10^6 cells were chosen as the standard number of cells.

Isolated primary hepatocytes cultured on EHS have been shown to require some time in culture to adapt to the in vitro milieu and to regain differentiated functions [10], and this is likely to be the case also for cells cultured without EHS. Therefore, we compared two different time points for transfection, 16 and 40 h after seeding of the cells, and a time-course measurement of the reporter gene activity was carried out. Higher luciferase activities were detected at all time points when the cells were transfected at 40 h than at 16 h of culture age (Fig. 4). Maximal luciferase activity was achieved 24–31 h after start of transfection, thereafter the activity declined. According to the manufacturer's instruction, maximal transfection efficiency is obtained upon 3–8 h of cellular exposure to the plasmid/Lipofectamin2000 mixture, we did not observe any difference in activity between 3 and 24 h (data not shown) and for practical reasons, 5–6 h of transfection was chosen as standard time.

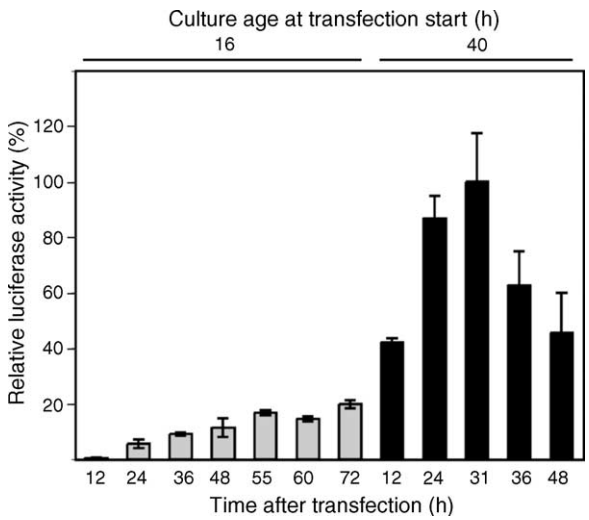

Fig. 4. Effect of culture age on transfection efficiency. Primary hepatocytes were cultured on uncoated dishes and transfected with 2 μg pGL3-control at 16 h (grey bars) or 40 h (black bars) of culture age using Lipofectamine2000. Cells were harvested at different time points and analyzed for luciferase expression. Results were expressed as luciferase units/μg protein. Data are given as relative luciferase activity and are the mean ± S.D.; $n = 3$.

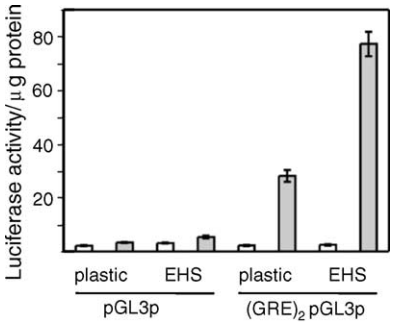

Fig. 5. Dexamethasone activation of $(GRE)_2$pGL3p transcription in primary hepatocytes cultured with or without EHS overlay. Cells were transfected with 2 μg plasmid at 40 h of culture age; after 6 h, the medium was changed to medium containing vehicle (open bars), 1 μM Dex (grey bars) and diluted EHS (EHS) or not (plastic). Cells were harvested 20 h later. Data are expressed as luciferase activity/μg protein and are the mean ± S.D.; $n = 3$.

3.3. EHS-mediated cellular differentiation assures induction of reporter gene constructs

Using the conditions found to be most efficient for lipofection of primary hepatocytes, we set out to examine hormonal/drug induction of reporter gene constructs with or without an overlay of EHS. First, we examined the effect of the synthetic glucocorticoid Dex on a luciferase reporter gene construct harbouring two glucocorticoid response elements in front of the SV40 promoter in the pGL3-promoter vector, $(GRE)_2$pGL3p. As shown in Fig. 5, induction of luciferase activity in response to Dex occurred both with and without EHS-overlay. This is in line with the Dex induction of the endogenous *CYP3A* gene (cf. Fig. 2) obtained both in the absence and presence of EHS. The fold-induction was, however, higher in the presence than in the absence of EHS, on average 30-fold versus 15-fold. The pGL3p plasmid not harbouring the GREs was stimulated 1.6-fold by the treatment with 1 μM Dex.

Induction by the synthetic liver X receptor (LXR) ligand T0901317 [19] of luciferase activity from the same plas-

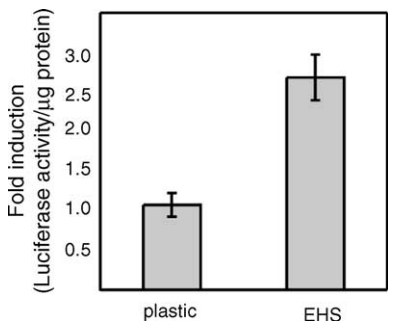

Fig. 6. T0901317 activation of $(LXRE)_3$pGL3p transcription in primary hepatocytes cultured with or without EHS overlay. Cells were transfected with 2 μg plasmid at 40 h of culture age; after 6 h, the medium was changed to medium containing vehicle or 1 μM T0901317 and diluted EHS. Cells were harvested 20 h later. Data are expressed as fold induction of luciferase activity/μg protein in stimulated vs. unstimulated cells and are the mean ± S.D.; $n = 3$.

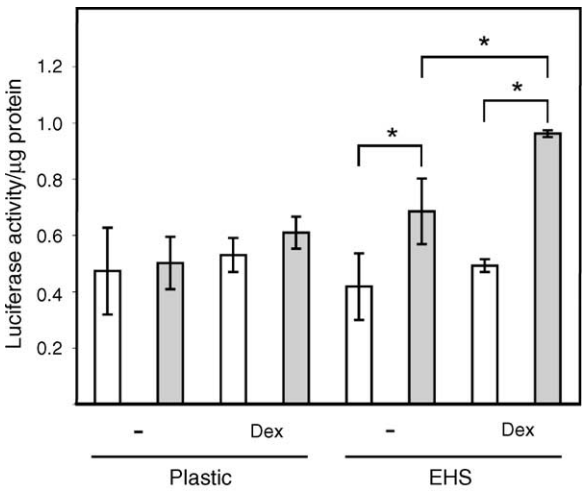

Fig. 7. GH activation of *a1bg*-pGL3b transcription in primary hepatocytes cultured with or without EHS overlay and with or without the addition of low concentration of dexamethasone. Cells were transfected with 2 μg plasmid at 40 h of culture age; after 6 h, the medium was changed to medium containing 4.5 nM GH (grey bars) or not (open bars), 10 nM Dex (Dex) or not (−), and diluted EHS (EHS) or not (plastic). Cells were harvested 24 h later. Data are expressed as luciferase activity/μg protein and are the mean ± S.D.; $n = 3$. (*) Significant difference between groups, $P < 0.05$.

mid as above but with two LXR response elements (LXRE) instead of the GREs, $(LXRE)_2$pGL3p, was in contrast to induction of $(GRE)_2$pGL3p by Dex, dependent on the presence of EHS (Fig. 6). T0901317 did not affect expression of the pGL3p plasmid lacking the LXREs (data not shown).

As shown in Fig. 1C, GH induced the endogenous *a1bg* gene in primary hepatocytes both in the absence and presence of EHS. This gene harbours known GH responsive elements, Stat5 and HNF-6 [20,21], in the proximal promoter. The 160 bp promoter fused to the firefly luciferase gene (*a1bg*-pGL3b) was lipofected into primary hepatocytes and GH responsiveness was investigated in cultures with or without an overlay of EHS. Since low concentrations of Dex together with EHS have been suggested to be crucial for preservation of hepatocyte morphology associated with a differentiated phenotype in vitro, and for maintained expression of liver characteristic transcription factors [3], we also investigated expression of luciferase activity from the *a1bg*-pGL3b construct in the absence or presence of 10 nM Dex. In the absence of EHS, GH failed to stimulate *a1bg*-pGL3b expression, whereas in the presence of EHS, there was a significant GH stimulation of expressed luciferase activity (Fig. 7). The results also indicated that co-treatment with Dex augmented luciferase readings and potentiated the GH effect, this was, however, not a consistent finding in repeated experiments. We also investigated whether co-treatment with 10 nM Dex affected the T0901317-stimulated luciferase expression from $(LXRE)_2$pGL3p in cells overlaid with EHS. Dex had no effect on the T0901317-stimulated expression (data not shown).

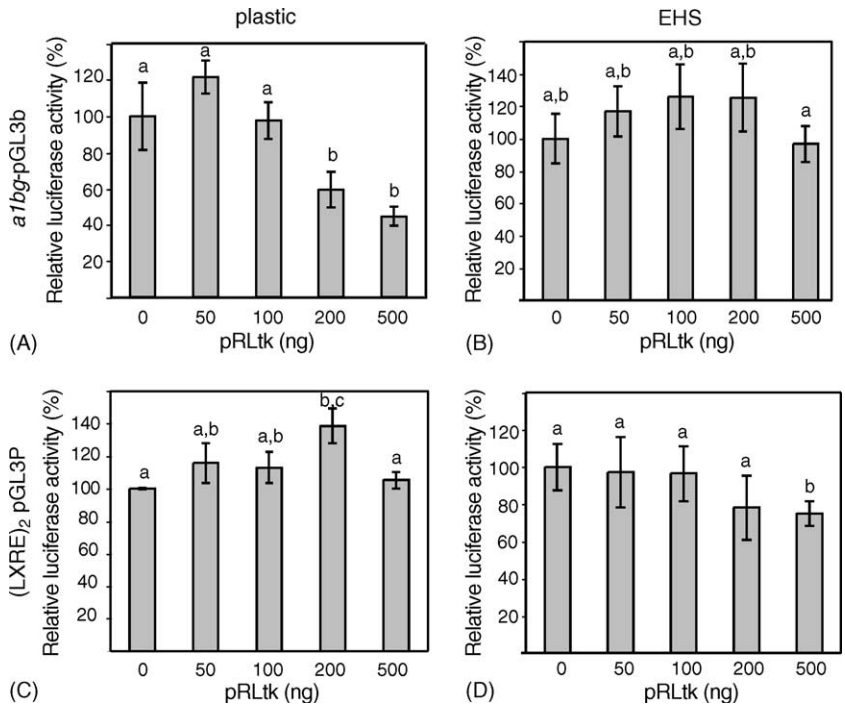

Fig. 8. Effect of cotransfection with pRLtk on *a1bg*-pGL3b and $(LXRE)_2$pGL3p expression in primary hepatocytes cultured with or without EHS overlay. Cells were transfected at 40 h of culture age with 2 μg *a1bg*-pGL3b (A, B) or $(LXRE)_2$pGL3p (C, D) and varying amounts of pRLtk and pGL3-basic so that all cells received the same amount of plasmid. After 6 h, the medium was changed to medium without EHS (A, C) or to medium containing EHS (B, D). Cells were harvested 20 h later. Results were expressed as firefly luciferase units/μg protein. Data are given as relative firefly luciferase activity with the level of expression in cells not given pRLtk set to 100% and are the mean ± S.D.; $n = 3$–6. Bars with different superscripts are significantly different, $P < 0.05$.

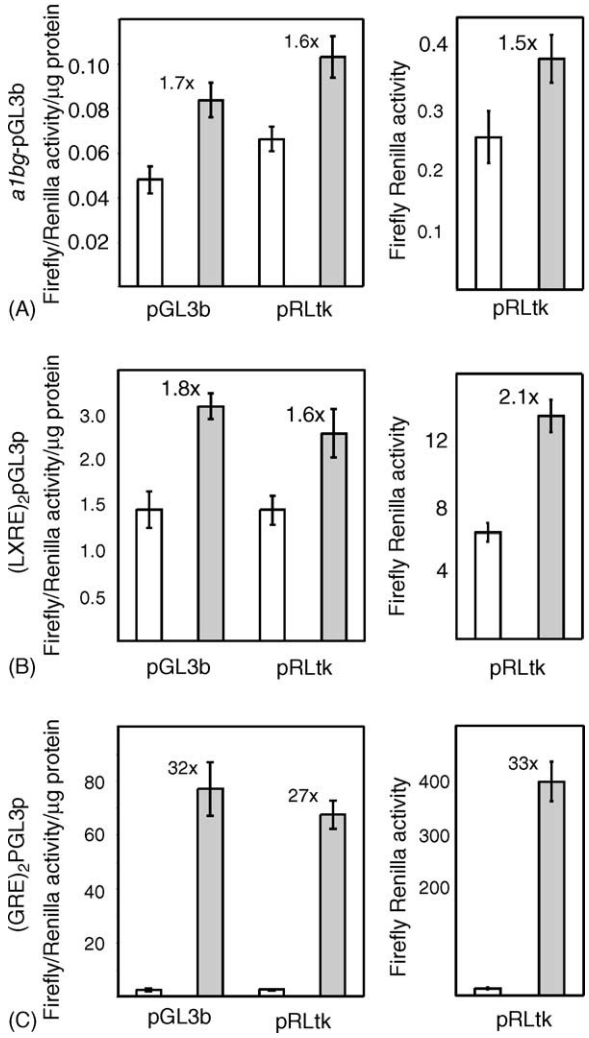

Fig. 9. Effect of cotransfection with pRLtk on hormone/drug induced reporter gene expression. Cells were transfected with 2 μg *a1bg*-pGL3b (A), 2 μg $(LXRE)_2$pGL3p (B) or 2 μg $(GRE)_2$pGL3p together with 100 ng pGL3-basic or 100 ng pRLtk at 40 h of culture age; after 6 h, the medium was changed to medium containing EHS and ligand ((A), GH 4.5 nM; (B) T0901317 1 μM; (C) Dex 1 μM) (grey bars) or vehicle (open bars). Cells were harvested 20 h (B, C) or 24 h (A) later. Data are expressed as firefly luciferase activity/μg protein and as firefly versus *Renilla* activity when cotransfected with pRLtk. The values are the mean ± S.D.; $n = 5$. The induction in hormone/drug treated cells vs. vehicle treated cells was statistically significant in all cases, $P < 0.05$.

3.4. Normalization of transfection efficiencies and promoter competition

To assess the utility of cotransfection of a second plasmid for normalization purposes, varying amounts of a *Renilla* luciferase vector driven by the thymidine kinase promoter (pRLtk) were cotransfected with a constant amount of the firefly luciferase reporter construct *a1bg*-pGL3b or $(LXRE)_2$pGL3p. As shown in Fig. 8, expression from the *a1bg*-promoter, *a1bg*-pGL3b, or from the SV40 promoter, $(LXRE)_2$pGL3p, was not significantly affected by cotransfection with 50 or 100 ng of pRLtk, neither in the absence nor in the presence of EHS. However, at higher amounts of pRLtk, luciferase activity generated from *a1bg*-pGL3b was reduced in cells cultured without an overlay of EHS indicating that there were limiting amounts of components necessary for transcription of the two plasmids. This was also supported by consistently lower *Renilla* luciferase readings in these samples (data not shown). Taken together, the data indicate that a high degree of hepatocyte differentiation, i.e. cells cultured in the presence of EHS, diminish the risk for promoter competition in transient transfection experiments.

3.5. Inducibility of reporter constructs upon cotransfection with pRLtk for normalization purposes

Finally, we examined hormonal/drug responses of reporter constructs with or without cotransfection with pRLtk. The effect of GH, T0901317 and Dex on their cognate reporter plasmids in cells cotransfected or not with pRLtk was compared. As shown in Fig. 9, the induction of the respective reporter construct was not affected by cotransfection with 100 ng pRLtk, neither did the fold induction differ when the firefly luciferase activities were related to protein amounts or *Renilla* luciferase activities.

4. Discussion

The aim of this study was to establish a protocol for the use of luciferase reporter gene constructs in transient transfection experiments in primary rat hepatocytes. A number of vectors and techniques for transfection of cells have been developed over the last decades, each having advantages and disadvantages in their applicability on cultured primary hepatocytes. Primary hepatocytes are efficiently transfected by electroporation [22], however, large amounts of plasmid are needed and many cells die. Furthermore, with luciferase as the reporter of choice, having a relatively narrow expression window [6], electroporation is less suitable since the cells need some time after isolation to adapt to the in vitro milieu and to regain responsiveness to external stimuli. Viral systems are generally highly efficient, but are relatively cumbersome to prepare and their use for transfection of non-dividing cells is limited. For examining gene regulation in transient transfection experiments, non-viral vectors and synthetic transfection reagents are convenient tools to use. High transfection activity is obtained in commonly used cell lines with essentially all commercial transfection reagents, whereas primary cells in general have proven more difficult to transfect. Here, we have shown that Lipofectamine2000 is superior for transient transfection of primary rat hepatocytes. For obvious reasons, the formulas of the commercial reagents are not fully disclosed. However, the description of the tested reagents placed in the order of their effectiveness in this study is: cationic liposome,

liposomal formulation, lipopolyamine, non-liposomal blend of lipids and formulation of polyamines. This indicates that for transfection of primary rat hepatocytes liposomes are advantageous as gene delivery vehicles. It has previously been shown that for transfection of rat hepatocytes, the lipid reagent requirements vary with the age of the culture [4]. Our comparisons of transfection activities were performed with cells at 40 h of culture age, and it cannot be excluded that the various transfection reagents are differently effective at different time points.

The literature provides numerous examples of the beneficial effect of EHS for the expression of liver specific/characteristic genes in primary rat hepatocytes in response to prototypical inducers, and we have previously found that EHS is a prerequisite for the GH-induction of the *CYP2C12* gene [10] and unpublished observations. Therefore, the similarity in GH-response of CYP2C12 mRNA expression without and with EHS substratum found in this study was unexpected. We cannot exclude that different concentrations of GH and/or time of treatment could lay behind this. However, another possible explanation for the discrepancy is the sex of the animal from which the cells were derived. As indicated in our previous studies [9,15] and convincingly demonstrated by Thangavel et al. [23], female derived hepatocytes, in contrast to male derived hepatocytes, have intrinsic, apparently irreversible, high capacity to express GH-regulated and female-dependent liver genes. Almost all studies using cultured primary rodent hepatocytes are carried out with cells obtained from male animals. Although the induction of *CYP* genes other than *CYP2C12* to a much greater extent required the presence of EHS, it cannot be excluded that the use of female derived cells could be advantageous also for in vitro studies on other hepatic genes.

The presence of EHS was not a prerequisite for GH-induction of the endogenous target genes *CYP2C12*, *a1bg* and *akr1b7*, but EHS was required for the GH effect on the lipofected promoter construct, *a1bg*-pGL3b. Additional GH response elements not contained within the construct may be responsible for this difference, but it is also possible that limited expression of transcription factors or cofactors in cells cultured in the absence of EHS could be the explanation. Interestingly, the two constructs $(GRE)_2$pGL3p and $(LXRE)_2$pGL3p induced by Dex and T0901317, respectively, differed with regard to their need for EHS to be induced. Since the constructs differ only with regard to the cloned response elements, it is conceivable that the expression of LXR and/or necessary cofactors is dependent on a higher degree of cellular differentiation than expression of factors necessary for the Dex response. Glucocorticoids, often Dex, are commonly included in hepatocyte culture media and have together with EHS been suggested to have critical and cooperative roles for the degree of differentiation of primary rat hepatocytes [3]. We could not demonstrate a general beneficial effect of Dex on expression of luciferase reporter constructs, but our observation that Dex in some experiments augmented the GH induction of *a1bg*-pGL3b could indicate that glucocorticoids could have a differentiating role in relation to subtle variations in quality of the cells between experiments. Furthermore, a differentiating role of Dex could lay behind the induction of luciferase activity from $(GRE)_2$pGL3p in cells cultured without an EHS-overlay. Even though glucocorticoids have a general differentiating effect on expression of liver characteristic transcription factors [3], Dex clearly impairs GH-induction of endogenous *CYP2C12* and *akr1b7* gene expression in primary rat hepatocytes [15,23]. Thus, gene specific effects of Dex may hamper its general utility as a differentiating agent for cultured hepatocytes. From our results, it is evident that for cells cultured in the presence EHS, the commercially available standard medium Williams' E without any other addition than insulin and antibiotics is sufficient for maintenance of a differentiated hepatocyte phenotype that allows investigations of the expression of hepatic genes in response to various inducers.

In transient transfection experiments where several constructs are introduced into cells, there is a risk for promoter competition. This was indicated when hepatocytes, cultured in the absence of EHS, were transfected with the *a1bg*-pGL3b construct together with increasing amounts of pRLtk. However, when using hepatocytes in a more differentiated state, i.e. cultured in the presence of EHS, this effect was abolished. Thus, the degree of differentiation of the cells is an important factor as is titration of the amounts of cotransfected plasmids. The latter is also illustrated by the fact that the firefly activity generated from the $(LXRE)_2$pGL3p construct was reduced upon cotransfection with 500 ng pRLtk, but not with lower amounts of pRLtk, in cells cultured in the presence of EHS. The risk for promoter competition and misleading results might be particularly pronounced when promoters of different strength are used [24] and balancing the amount expression plasmids with promoter-less plasmids would not necessarily be an adequate control. We conclude that when these issues are carefully addressed, the firefly/*Renilla* system is convenient and useful for normalization purposes in transfection experiments also in primary hepatocytes. To our surprise, relating firefly luciferase activities to *Renilla* luciferase activities did not give rise to lower experimental variances than when firefly activities were related to cellular protein content and suggests that using protein content for normalization purposes is as useful as the much more costly firefly/*Renilla* system.

In conclusion, we have shown that primary rat hepatocytes can be efficiently transfected using Lipofectamine2000 and by using an EHS overlay hormonal/drug responses of transfected constructs can be assured. Dex is most often included in hepatocyte culture media, but we could not demonstrate a general beneficial effect of Dex on expression of luciferease reporter contructs. For normalization purposes, relating reporter gene activities to cellular protein content appears as useful as relating it to the reporter activity of a cotransfected vector. This also alle-

viates the risk of one reporter gene expression affecting the other.

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