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VIRAL TRANSFER TECHNOLOGY ***RESEARCH ARTICLE***

Baculovirus vectors repress phenobarbital-mediated gene induction and stimulate cytokine expression in primary cultures of rat hepatocytes

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Baculovirus transfection strategies have proven successful at transferring foreign DNA into hepatoma cells and primary hepatocytes. When testing the utility of these methodologies in cultured hepatocytes, we discovered that the presence of baculovirus disrupts the phenobarbital (PB) gene induction process, a potent transcriptional activation event characteristic of highly differentiated hepatocytes, and repressed expression of the albumin gene. In concert with previous reports from our laboratory demonstrating that increased cAMP levels can completely repress the induction of specific cytochrome P450 (CYP) genes, cAMP concentrations and PKA activities were measured in the primary hepatocytes subsequent to baculovirus exposure. However, neither parameter was affected by the presence of the virus. To evaluate whether immune response modulation was triggered by baculovirus exposure, RNase protection assays were performed and demonstrated that baculovirus infection activates TNF-α, IL-1α and IL-1β expression in the primary hepatocyte cultures. Immunocytochemical experiments indicated that the production of cytokines was likely due to the presence of small numbers of Kupffer cells present in the culture populations. Exogenously added TNF-α was also effective in repressing PB induction, consistent with other reports indicating that inflammatory cytokines are capable of suppressing expression of biotransformation enzyme systems. Comparative studies demonstrated the specificity of these effects since exposures of hepatocytes to adenoviral vectors did not result in down-regulation of hepatic gene responsiveness. These results indicate that baculovirus vectors enhance the expression of inflammatory cytokines in primary hepatocyte cultures, raising concerns as to whether these properties will compromise the use of baculovirus vectors for study of cytochrome P450 gene regulation, as well as for liver-directed gene therapy in humans. Gene Therapy (2000) **7,** 1274–1283.

Keywords: *baculovirus; transfection; hepatocyte; cytochrome P450; cytokines; TNF-α*

Introduction

As the liver is the predominant organ responsible for the metabolism of most endogenous and exogenous compounds, understanding the factors governing liver-specific gene regulation is an important issue. Recent advances in primary hepatocyte culture techniques using defined preparations of serum-free media coupled with extracellular matrices now allow for the *in vitro* preservation of differentiated hepatocyte function.[1–3] One parameter that has been especially difficult to model in primary hepatocytes, or in established hepatoma cell lines, is the phenobarbital (PB) gene induction response, a process that is transcriptionally regulated and results in dramatically enhanced expression of a battery of genes, including several involved in drug biotransformation.[4]

A continued difficulty with current primary hepatocyte culture protocols is the inability to perform efficient DNA transfections, a procedure commonly used for investigating gene regulatory processes. Previous studies have reported that baculovirus (*Autographa californica* nuclear polyhedrosis virus) vectors can be used to shuttle foreign genes into mammalian cells.[5–8] It is noteworthy that the uptake of the baculovirus was reported to be predominantly specific for hepatic cell types, with associated transfection efficiencies ranging greater than 70%.[6]

In this investigation, we explored the utility of the baculovirus system as a potential means to increase transfection efficiencies in primary hepatocyte cultures, thereby enabling molecular approaches into the mechanisms that control the PB-mediated induction of the rat CYP2B gene subfamily. Although baculovirus was determined to be highly efficient at delivering foreign genes into the primary cells, the presence of the virus triggered a repression of PB-mediated gene responsiveness, together with a reduction in levels of albumin mRNA expression. Since enhanced cAMP production and/or PKA stimulation were reported to repress similarly PB inducibility in primary hepatocytes,[9] the effects of baculovirus on these pathways were tested but viral treatments did not result in cAMP or PKA modulation. However, infection with virus markedly stimulated the production of several cytokines in the primary cell cultures. These results raise several issues regarding the suitability of baculovirus vectors for studies of CYP gene regulation as well as for liver-directed gene therapy in humans.

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Results

Transfection determinations

We tested baculovirus-mediated strategies in an effort to improve DNA transfection efficiencies in hepatoma cells and in primary hepatocyte cultures. As demonstrated in Figure 1, when virus carrying TK-Luciferase DNA was used to transfect human hepatoma HuH-7 cells, a high level of luciferase expression was detected with laser cytometry using an anti-luciferase antibody. Increasing the titer (MOI) of virus applied to the cells resulted in a corresponding increase in luciferase expression. Both luciferase fluorescence intensity levels (pixels per cell) and luciferase activity from harvested cells increased concordantly with MOI of the baculovirus (Table 1). This trend was consistent between multiple preparations of virus and similar results were obtained in transfection studies with multiple preparations of primary rat hepatocytes (Table 1). It was estimated that approximately 65% of the hepatoma cells were transfected with baculovirus MOIs of 200.

CYP induction in baculovirus transfected primary hepatocytes

Figure 2 presents the PB-mediated induction responses for CYP2B1 and CYP3A1, and βNF-mediated CYP1A1 induction. Increasing titers of baculovirus added to the cultures resulted in the repression of CYP2B1 (panel a) and CYP3A1 mRNA induction (panel b). However, CYP1A1 induction responses were not affected by the presence of virus (panel c). As can be visualized in each panel, increasing the MOI of baculovirus resulted in a corresponding decrease in albumin mRNA levels in the primary cells, although the steady-state 18S ribosomal RNA levels remain constant with these same treatments.

Effects of baculovirus cAMP and PKA pathways

Previous results reported by our laboratory demonstrated that cAMP analogs and adenylate cyclase activators can completely repress the PB-mediated CYP2B induction event.[9] To examine whether the repressive effect of baculovirus on PB induction was related to these parameters, we investigated the effects of the baculovirus on cAMP levels and subsequent PKA activity in the primary hepatocyte cultures. Figure 3 shows the results of treating the rat hepatocytes with TE pH 8 (negative control), baculovirus (MOI 100), or forskolin (positive control). Forskolin treatments resulted in a 90-fold increase in intracellular cAMP levels within 5 min of treatment. Despite this capacity for response, baculovirus exposures exerted no observable effects on cAMP levels within the hepatocytes. Similar results were obtained when treatment periods were extended to 24 h. Higher MOIs of baculovirus were also without effect on cAMP levels in the cells (data not shown).

In addition, we used a synthetic Kemptide substrate to assess PKA activities following treatments with either TE pH 8, baculovirus (MOI 100), or forskolin. As demonstrated in Figure 4, only the forskolin treatment produced an increase in PKA activation in the primary hepatocytes. Baculovirus exposures exhibited no effect on PKA activity. The results presented are representative of data obtained from three separate hepatocyte preparations.

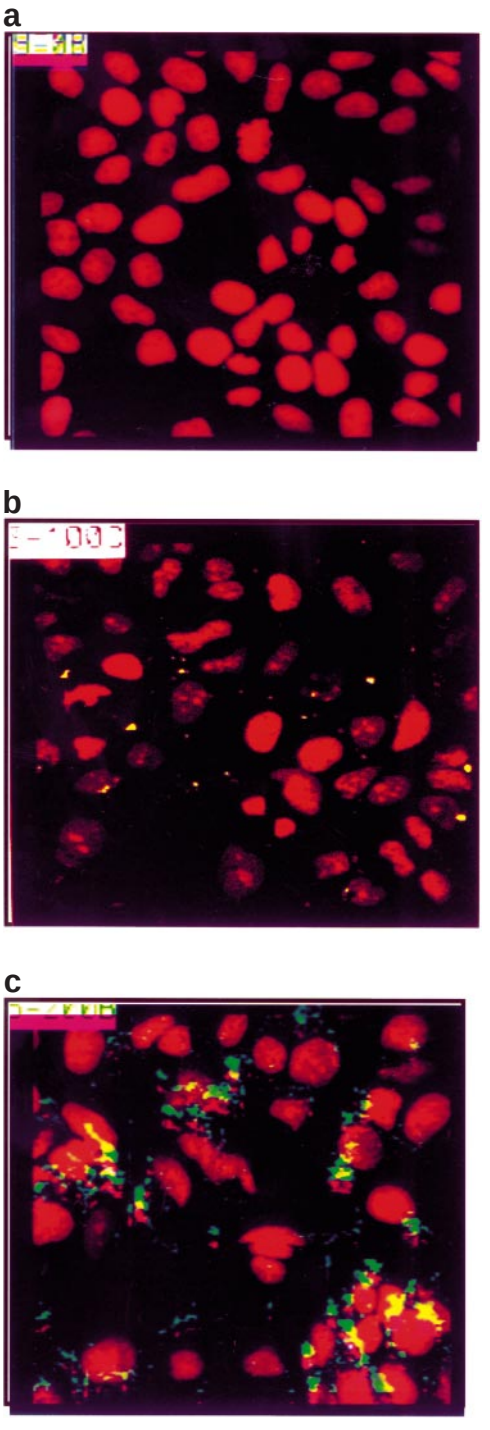

Figure 1 *Baculovirus-mediated transfections in HuH-7 cells. HuH-7 cells were exposed to TK-Luc virus and examined 24 h later for luciferase expression using laser cytometry. Propidium iodide was used for nuclei detection (red signal). FITC-conjugated luciferase antibody allows detection of luciferase expression (green/yellow signal). (a) No virus; (b) TK-Luc virus MOI 100; (c) TK-Luc virus MOI 200. We estimated that the transfection efficiency in HuH-7 cells was >60% with the baculovirus vector at a MOI 200. The use of a matrigel overlay in the primary hepatocyte culture system quenched detection of the fluorescence signals in parallel experiments (data not shown).*

Table 1 Luciferase expression in cultured liver cells transfected with varying MOI of baculovirus

Baculovirus MOI	*Fluorescence pixels/cell*[a,c]	*Luciferase activity (RLU)*[a,d]	*Luciferase activity (RLU)*[b,d]	*Luciferase activity (RLU)*[b,d]	*Luciferase activity (RLU)*[b,d]
0	5	8	25	9	14
100	12	2165	596	968	688
200	64	10381	1204	1962	1817

[a]Values obtained from transfection of HuH7 human hepatoma cells.
[b]Values obtained from transfection of primary rat hepatocytes in culture, shown for three independent experiments.
[c]Fluorescence (pixels per cell) was measured using the ACAS laser cytometer (see Methods). The FITC (luciferase) expression was a measure of total expression within the field, divided by No. of nuclei present as determined from propidium iodide staining.
[d]Luciferase activity (RLU, relative light units) was measured using a luminometer and normalized to protein concentrations of each cell extract. Values shown are means of duplicate determinations.

Effects of baculovirus on cytokine expression

To investigate further the effects of the baculovirus on primary hepatocytes, we examined cytokine expression levels as a function of virus exposure. The use of a multiprobe RNase protection analysis enabled screening for multiple cytokine messages with the same RNA sample. Figure 5 illustrates a representative assay result. In control or PB-treated cell preparations there was no detectable cytokine expression (lanes 1 and 6). However, in the presence of virus, TNF-α production and low levels of IL-1β were detected. In some hepatocyte preparations, virus treatment produced detectable levels of both IL-1α and IL-1β, although this expression was variable between hepatocyte preparations (data not shown). We were unable to detect TNF-β, IL-3, IL-4, IL-5, IL-6, IL-2, or INF-γ in any of the baculovirus-exposed samples. In all lanes there is detectable L32 and GADPH, confirming that an equal level of mRNA was present within each gel register.

To determine whether potential endotoxin contamination was occurring in our baculovirus preparations, we conducted limulus amebocyte lysate (LAL) testing and determined that the endotoxin levels in the baculovirus samples were extremely low (<19 ng/ml). In addition, we tested the effects of a 100-fold higher concentration of purified lipopolysaccharide (LPS) in the primary hepatocytes, and at these levels no repression of CYP induction was detected (data not shown).

To investigate further the effects of cytokines on CYP2B expression, we examined the effects of TNF-α in more detail. As predicted from previous reports, additions of TNF-α to the hepatocyte cultures resulted in decreased CYP2B expression (Figure 6), although this effect was not as pronounced as the repression demonstrated upon addition of baculovirus (Figure 2).

Kupffer cell detection

To identify the potential origins of cytokine production, we assessed hepatocyte cultures using the KU-1 antibody directed against Kupffer cells. As seen in Figure 7, although their abundance was low, Kupffer cells were present in the cell population. The level of Kupffer cells varied from none to eight cells within fields of approximately 100 hepatocytes.

Viral specificity and Kupffer cell disruption

We examined whether the effects noted for baculovirus on hepatic gene expression endpoints were specific for this vector. Primary hepatocytes were similarly treated with an MOI of 100 of purified adenovirus particles and monitored for PB induction responsiveness and albumin mRNA expression levels. As demonstrated in the data presented in Figure 8, addition of adenovirus was without any compromising effect on either expression parameter. Cells treated with adenovirus remained fully PB responsive and maintained levels of albumin mRNA production equaling the untreated cells. Both higher and lower MOIs of adenovirus were similarly tested and were without apparent adverse effect on the treated cells (data not shown). In these experiments, baculovirus-treated cells again demonstrated a concentration-dependent repression of PB induction and down-regulated albumin levels (Figure 8), indicating that the baculovirus-mediated effects on hepatocyte gene expression were specific to the latter vector.

We also attempted to abrogate the Kupffer cell influence in the culture system by treating the cells with gadolinium chloride, a standard Kupffer cell poison. However, at concentrations required for Kupffer cell ablation, this agent proved deleterious to the hepatocyte phenotypic responses as well, such that the gadolinium chloride treatments also repressed PB-mediated induction of the CYP2B1 gene and suppressed albumin expression in the cells (Figure 8). These endpoints are highly sensitive indicators of the highly differentiated hepatocyte phenotype.[1–3] Attempts to rescue the baculovirus-mediated repression of PB induction or albumin expression with gadolinium chloride were without effect. Adding gadolinium chloride to adenovirus-exposed cells also resulted in negative modulation of these endpoints (Figure 8).

Discussion

The PB induction response is largely liver specific, and requires a highly differentiated hepatocyte phenotype since transformed hepatoma lines such as Fao, HepG2 and HuH-7, are largely refractive to PB induction.[1] Modeling this response *in vitro* therefore requires a well-defined system exhibiting concordant expression of a battery of other liver-specific markers and signaling pathways.[1,2,4] To assist with the study of molecular mechanisms involved in the PB induction response and expression mechanisms of other liver-specific genes, we have tested a diverse array of DNA transfection techniques and reagents in cultured primary hepatocytes. Our best results were obtained using lipofectin as a DNA delivery lipid, however, β-galactosidase staining experi-

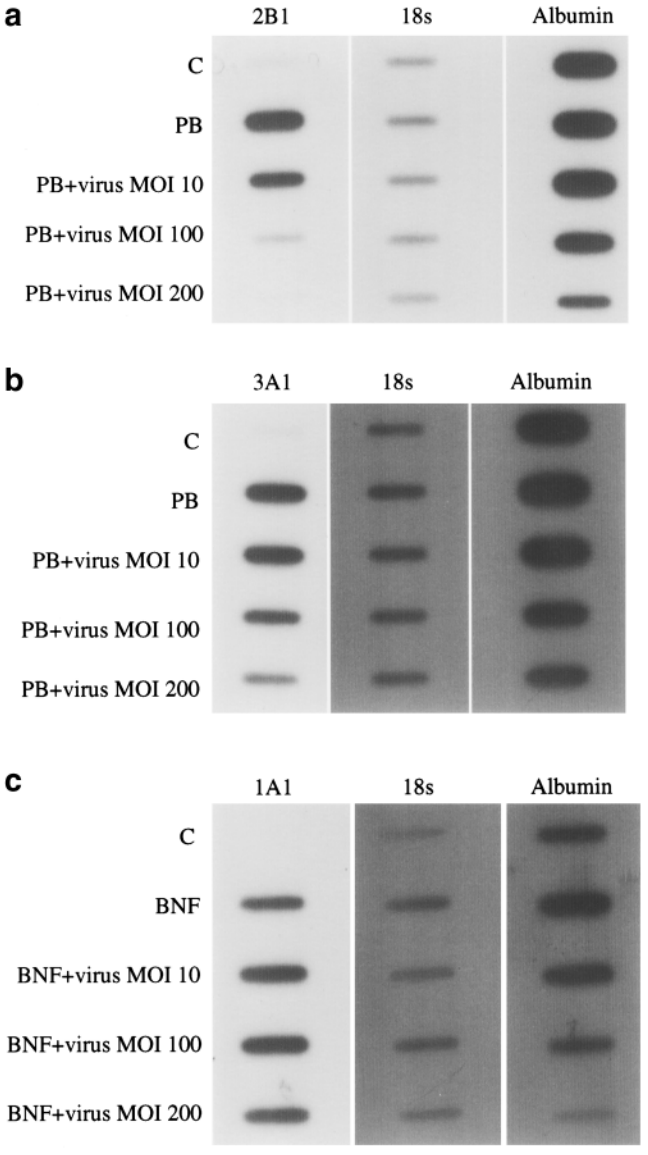

Figure 2 *CYP mRNA expression in baculovirus-transfected primary hepatocytes. Primary hepatocytes were either untreated (C) or exposed to PB (500 μM) or BNF (22 μM) alone or in the presence of TK-Luc virus, MOI 10–200 as stated. (a) CYP2B1 mRNA expression; (b) CYP3A1 mRNA expression; (c) CYP1A1 mRNA expression. Corresponding 18S ribosomal RNA levels and albumin levels are shown for each sample. The blots shown are representative of data obtained from at least three independent hepatocyte preparations; each treatment was performed in duplicate.*

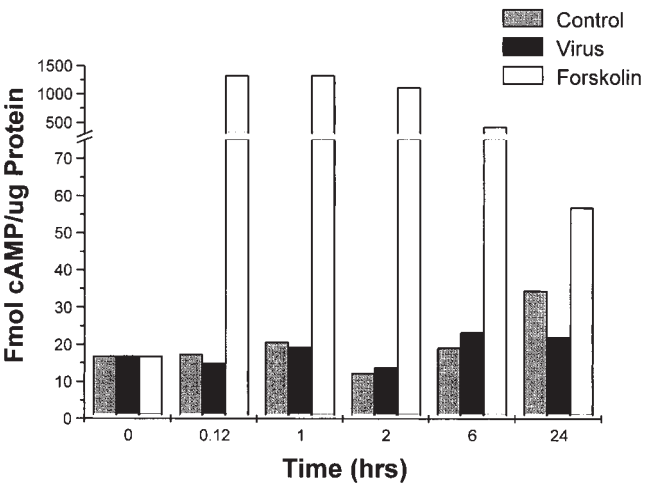

Figure 3 *Effects of baculovirus on intracellular cAMP levels in primary hepatocytes. Primary rat hepatocytes were plated 24 h before initiation of treatments. The effects of TE pH 8 (Control), baculovirus, MOI 100, and Forskolin (100 μM) on intracellular cAMP levels are shown. Forskolin treatments were included as a positive control. cAMP values represent mean data from experiments conducted in duplicate using pooled samples derived from three separate hepatocyte and virus preparations.*

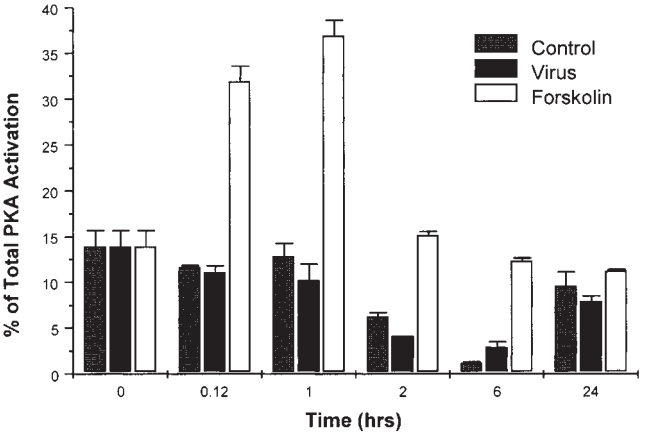

Figure 4 *Effects of baculovirus on PKA levels in primary hepatocytes. Primary rat hepatocytes were plated 24 h before treatments were initiated. The effects of TE pH 8 (Control), baculovirus, MOI 100, and Forskolin (100 μM) on PKA activity levels are shown. Forskolin treatment was included as a positive control. Values represent data obtained from three individual virus preparations and hepatocye isolations. % of total PKA activation is the amount of endogenous PKA activity/the amount of total available PKA as determined by the addition of exogenous cAMP (5 μM) to the sample. Means of PKA activity levels, determined in the absence of exogenously added cAMP, are indicated together with calculated standard deviations.*

ments indicated that even this reagent yielded <0.1% transfection efficiency in the primary cells (data not shown). Similar extremely low levels of transfection efficiencies of primary hepatocytes have been reported by other investigators.[10] It has been suggested that cell cycle division is a driving factor for lipofection-mediated gene delivery. Since hepatocytes are in a quiescent G0 state, this may explain their low transfectability.[10] Typically, transfection efficiencies in hepatoma cell lines are also relatively low, ranging from 3% to 8%.[10] When conducting studies to examine gene regulation, it is important that the transfected cells are representative of the total cell population under study.[11] Due to the phenotypic heterogeneity of hepatocyte populations, with very low transfection efficiencies it is difficult to determine whether any observed responses are truly reflective of the population at large.

The application of viral gene delivery strategies, including retrovirus and adenovirus vectors, has had a marked impact in the gene therapy arena. As retrovirus vectors require cellular proliferation to achieve high transfection efficiencies,[12] their use for hepatic gene transfer is limited. Adenovirus does not require cellular division for infection, but these vectors do not specifically

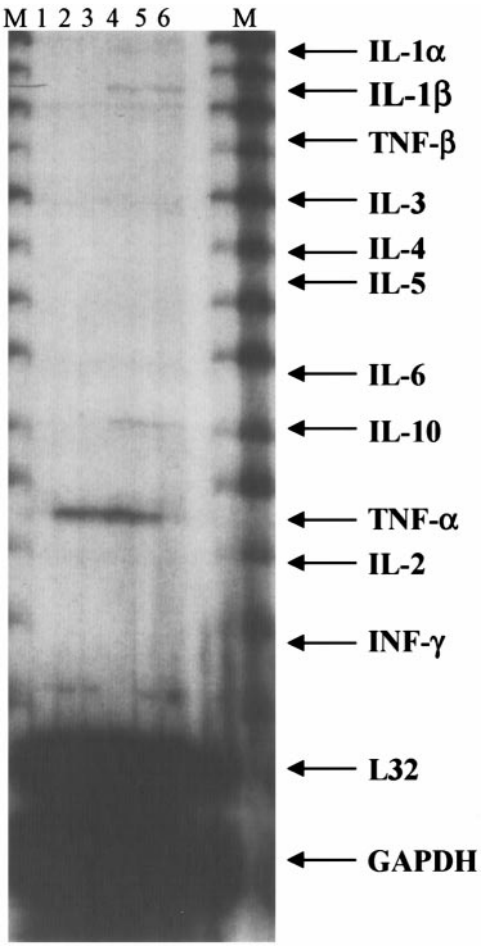

Figure 5 *Effects of baculovirus on cytokine expression. Multi-probe RNase protection assays were conducted to examine the effects of the baculovirus and heat-inactivated baculovirus on cytokine expression in primary rat hepatocytes. Hepatocytes were transfected with TK-Luc baculovirus 24 h after plating. Cells were harvested and RNA was isolated 24 h after transfections. All PB treatments were 500 μM. Lane 1, Control TE pH 8 (C); lanes 2 and 4, C + virus MOI 100; lanes 3 and 5, PB + virus MOI 100; lane 6, PB. The arrows shown depict the sizes of the expected protected probes for the individual cytokines. M represents the ladder of unprotected probe fragments, each of which has a known length and can serve as a size marker to which we can compare the protected probe sizes. An earlier exposure of the films was conducted to determine that all RNA levels were equivalent by examining L32 and GAPDH levels. The data shown are representative of results obtained from three individual hepatocyte and virus preparations.*

target the liver and cell toxicity has been documented.[13,14] Recent improvements in adenoviral vectors have limited the toxicity[15] and the advent of liver-specific promoters should help target these vectors to the liver.

Baculovirus has historically been used as a vector for protein overexpression in insect cells.[16,17] The risk of infection to humans is minimal as the baculovirus apparently replicates only in invertebrate cells; baculovirus expression in cells of mammalian or plant origin has never been reported.[17] Demonstrations of highly efficient gene delivery into hepatocytes and liver cell lines, presumably by an endosomal uptake pathway, has made baculovirus-mediated transfection appealing for the study of liver-specific gene regulation.[5,6,8] Indeed, in our experiments we achieved a high level of transfection efficiency with the use of this vector in hepatocytes and

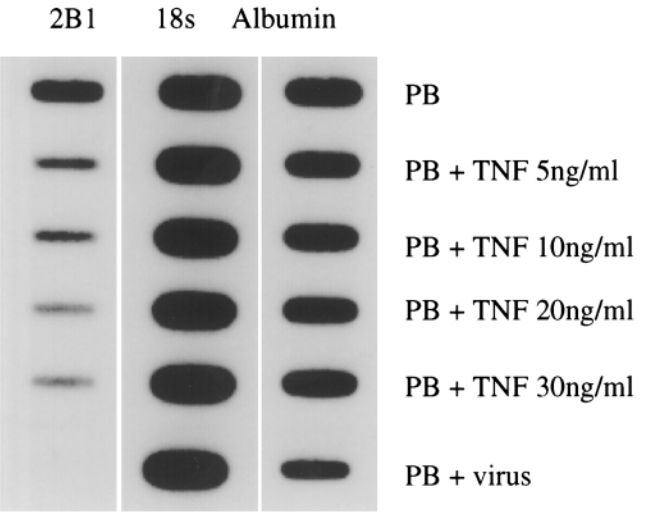

Figure 6 *Effects of TNF-α on CYP2B1 expression. CYP2B1 and corresponding 18S ribosomal RNA and albumin levels were measured in RNA extracted from primary hepatocytes treated with PB (500 μM) and differing levels of TNF-α as stated. Baculovirus was used at a MOI of 100. Cells were harvested for RNA 24 h after treatment.*

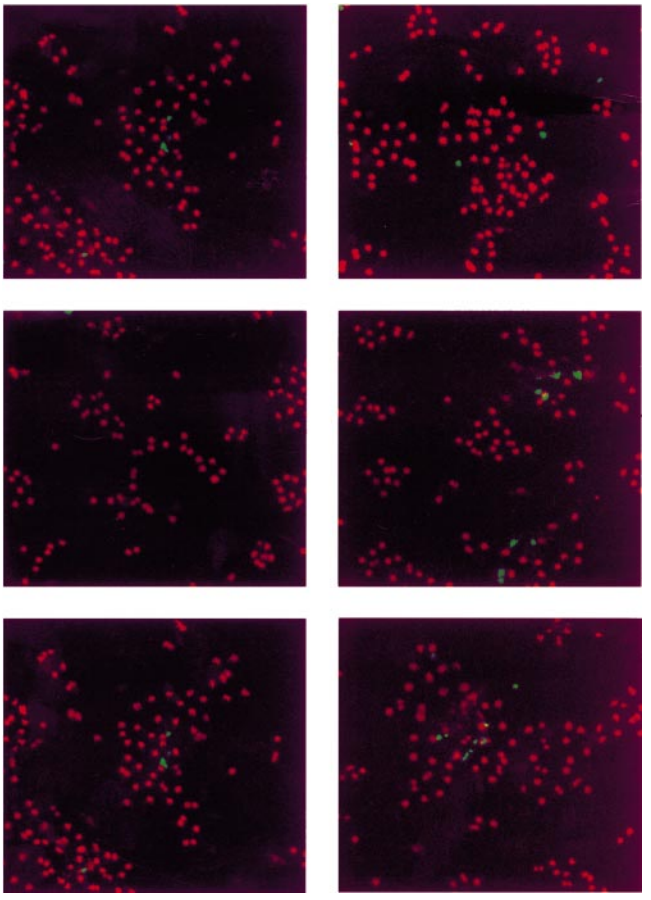

Figure 7 *Kupffer cell detection in primary hepatocytes. After 72 h in culture, primary hepatocytes were fixed and incubated with KU-1 antibody. Expression levels were detected using a FITC-conjugated secondary antibody. Using laser cytometry, the Kupffer cells are recognized by their green staining and small size. Red pseudocolor represents hepatocyte nuclei as detected using Hoechst dye.*

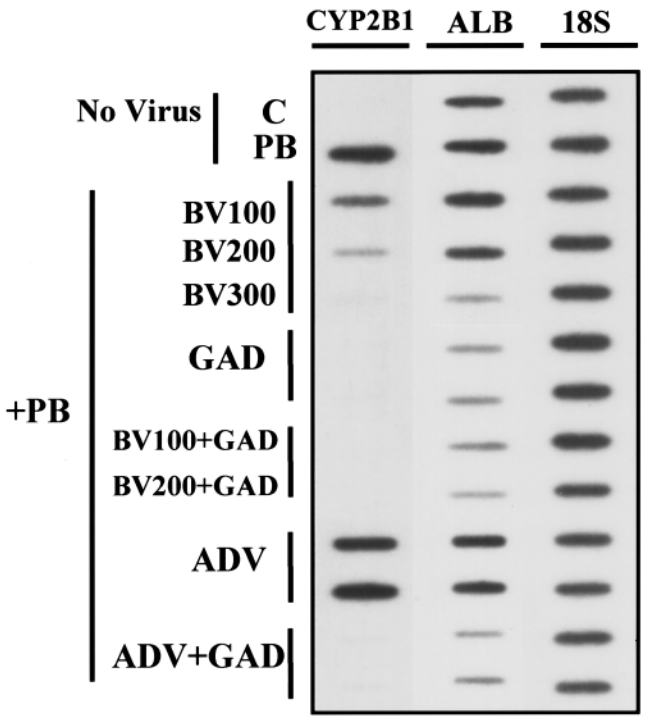

Figure 8 *Effects of adenovirus and gadolinium chloride on PB induction and albumin mRNA expression in primary hepatocytes. Cells were exposed to combinations of PB; baculovirus (BV) at MOIs of 100, 200 or 300; gadolinium chloride (GAD) at 100 μg/ml; and/or adenovirus (ADV) at a MOI of 100. Levels of CYP2B1 mRNA, albumin mRNA (ALB) and 18S rRNA expression in primary cultures of hepatocytes were determined using specific ^{32}P-oligomer probes in slot blot hybridization experiments as described in Methods.*

hepatoma lines (Figure 1, Table 1), especially when compared with lipofection or calcium phosphate-mediated transfection methods. Since luciferase reporter expression is cytoplasmic, it was difficult to quantify baculovirus transfection efficiencies on a per cell basis, but we estimate >60% of the cells were transfected with viral titers equivalent to an MOI of 200. Although no overt toxicity was noted in the presence of the baculovirus, we detected a dose-dependent decrease in albumin mRNA expression levels in treated cells (Figures 2 and 8). At low infectivity levels, ie MOI 10, no decreases in albumin expression were detected. However, even these lower levels of baculovirus exposure repressed PB-inducible CYP2B1 gene expression. This repression increased in magnitude with increasing MOI (Figures 2 and 8). Similar repression of CYP3A1 induction was also noted whereas βNF-inducible CYP1A1 expression was not affected by the presence of baculovirus (Figure 2). Thus, the response we observed appeared specific to the PB induction pathway.

To investigate the potential mechanism of the virus-mediated repression of CYP2B1 and CYP3A1 induction, we examined known effector pathways of these genes. Previous results from our laboratory demonstrated that cAMP analogs and activators of cAMP inhibit PB-mediated CYP2B and CYP3A1 gene expression.[9] However, the baculovirus vectors themselves did not affect cAMP expression levels or associated PKA activity levels (Figures 3 and 4), thus it is not likely that the baculovirus-mediated repression of the CYP gene induction is occurring through the cAMP signaling pathway.

In vivo attempts of gene transfer using baculovirus vectors have been largely unsuccessful in mice and rats either with systemic, intraportal, or direct injection into the liver parenchyma.[18] It was suggested that the baculovirus was being inactivated by a classic complement cascade, similar to the pathway that inactivates retrovirus gene transfer and long-term expression. Further research has confirmed that the complement system is responsible for baculovirus inactivation in human serum.[19] Using antibodies directed against specific complement components, baculovirus survival in the presence of human serum was subsequently demonstrated. Although this finding allows for a possible solution to one associated aspect of baculovirus-mediated human gene transfer, other issues also must be considered.

The effects of baculovirus vectors on cytokine expression in hepatocyte preparations have not been examined previously. It is well established that certain cytokines act directly on human and rat hepatocytes and *in vivo* in rat liver to depress inducible CYP gene expression.[20–22] For example, CYP2B expression and activity is repressed by several cytokines, including IL-6, IL-1α, IL-1β, TNF-α and INF-γ.[21,23–25] We therefore examined the specific effects of baculovirus on cytokine expression. Although we were unable to detect any of the examined cytokines in our control or PB-treated hepatocyte preparations, exposure of the cells to baculovirus resulted in activation of TNF-α, IL-1α, and IL-1β mRNA levels (Figure 5). The presence of TNF-α and IL-1 in our baculovirus-treated hepatocytes may explain the observed decreases in albumin expression since both of these cytokines have been shown previously to depress albumin expression.[26–28]

Although purified TNF-α additions repressed the PB induction response in the hepatocyte cultures, the magnitude of repression noted was not as marked as that caused by the baculovirus vectors. It is possible that IL-1α and IL-1β or other cytokines, may exert additive influence in repression of PB-inducible CYP gene expression. In a study of two different hepatocyte cell lines and human hepatocytes, all expressed numerous cytokines after stimulation with chemokines; although IL-1α was not among the detected cytokines.[29] There are few data demonstrating the ability of hepatocytes to express cytokines; most studies have focused on the Kupffer cell. Representing the largest population of tissue macrophages, the Kupffer cells of the liver take on many important functions including the presentation of antigen, the clearance of endotoxin and the production and release of many cytokines and eicosanoids. A rat liver contains approximately 12 Kupffer cells for every 100 hepatocytes.[30] Although our hepatocyte isolation procedure is optimized through the use of differential centrifugation and subsequent culturing conditions to yield cultures that contain ~95% hepatocytes, the potential for Kupffer cell contamination does exist. Using a monoclonal antibody, KU-1, we did in fact determine that Kupffer cells were present at very low abundance in the primary hepatocyte cultures, from none to eight cells per 100 hepatocytes (Figure 7). As the Kupffer cell is strongly responsive to an inflammatory challenge, it is probable that the cytokine expression we detected upon exposure to baculovirus is emanating from these cells. Milosevic *et al*[31] recently reported on the use of a co-culture system to further substantiate a pivotal role for Kupffer cells in

mediating inflammatory stimuli and communicating these effects to primary hepatocytes. Even small differences in the levels of Kupffer cells present between individual hepatocyte preparations may account for some variability in the levels of TNF-α, IL-1α and IL-1β noted between experiments. Studies that used gadolinium chloride to deplete Kupffer cell populations have demonstrated a change in the balance of inflammatory cytokine expression, including a decrease in IL-10 induction and a sustained overexpression of TNF-α.[32,33] In attempts to ablate Kupffer cell activity in our culture system, we attempted the use of gadolinium chloride. However, at concentrations required for Kupffer cell toxicity, the agent also compromised the highly sensitive hepatocyte phenotypic endpoints of PB induction and albumin gene expression, and thus these treatments were not successful in rescuing the normal patterns of gene responsiveness in the primary cultures (Figure 8).

The results of this investigation point to several issues that will require consideration before baculovirus-mediated gene transfer becomes an effective tool in human therapy. For example, the antagonistic effects of the virus on CYP induction processes and expression profiles may well alter the detoxification capabilities of the liver. Similarly, the up-regulation of cytokine production upon baculovirus exposure has several implications. For instance, TNF-α expression appears to increase the levels of c-fos and c-jun proteins and to enhance their functional interaction with an AP-1 element in the CYP19 aromatase gene.[34] Interestingly, it has been suggested that the effects of TNF-α on CYP2B expression are mediated through nitric oxide;[35,36] if so, the induction of nitric oxide in the liver will certainly have multiple consequences. Future studies addressing the effects of baculovirus on cytokine expression in the liver are required to characterize more fully the impact of baculovirus vectors and their potential role in human gene therapy.

Materials and methods

Materials

PB was obtained from the University of Washington Hospital Pharmacy Services (Seattle, WA, USA). Dexamethasone and gadolinium chloride were purchased from Sigma (St Louis, MO, USA). Forskolin and Ro 20–1724 were purchased from the Alexis Corporation (San Diego, CA, USA). Recombinant human TNF-α was purchased from Genzyme Diagnostics (Cambridge, MA, USA). KU-1 antibody was a generous gift from Dr Jonathan Reichner (Rhode Island Hospital and Brown University, Providence, RI, USA). A high-titer preparation of purified adenovirus vector harboring a β-galactosidase gene expression construct was a gift from Dr Mark Kay (Stanford University). All insect and hepatocyte cell culture material and Trizol were obtained from Life Technologies (Grand Island, NY, USA). Matrigel, ITS+, and NuSerum were obtained from Collaborative Biomedical Products (Bedford, MA, USA).

Virus construction

A TK-luciferase (TK-luc) construct was constructed by inserting the TK promoter of the pBRAMSCAT2 vector into the PGL2 basic vector (Promega, Madison, WI, USA) at the *Hin*dIII site. Viral constructs were made using the pFastBac1 plasmid and the Bac-To-Bac Baculovirus Expression System (Life Technologies). To make the luciferase–FastBac (Lfast) construct, the PGL2 basic cloning vector was digested with *Mlu*1 and *Sal*1 to isolate the luciferase cassette. Fragments were separated by electrophoresis in agarose then excised, treated with Agarase (Boehringer Mannheim, Indianapolis, IN, USA), and then purified with QIAquick PCR columns (Qiagen, Valencia, CA, USA). The purified fragment was ligated to the pFastBac vector, which was previously digested with *Bss*HII and *Sal*1. For TK-Luciferase–FastBac (TKLfast) construction, TK-luc was digested with *Mlu*1 and *Sal*1 to isolate the TK promoter and the luciferase cassette. The isolated fragment was purified and ligated into the pFastBac vector as described for the Lfast construct. All final constructs were confirmed with restriction digestion and PCR with at least three different primer sets. Once the recombinant FastBac constructs were made they were individually transformed with competent DH10Bac *E. coli* cells (Life Technologies) to make the recombinant Bacmid. Correct orientation and sequences of the recombinant Bacmid DNA constructs were confirmed with PCR and cycle sequencing. Sf9 insect cells (ATCC, Rockville, MD, USA) were transfected with the recombinant Bacmid DNA using Lipofectin (Life Technologies) and virus particles were isolated.[16] Viral DNA was extracted with 1% SDS and two phenol–chloroform extractions. Viral DNA sequences were again confirmed using multiple PCR primer sets.

Insect cell culture, virus amplification and virus purification

Sf9 cells were cultured at 28°C in ambient air. Cells were grown and maintained in Graces media supplemented with 9% fetal bovine serum, 90 μg/ml NaCl, 0.1 mg/ml streptomycin sulfate, 0.16 mg/ml penicillin G, and 0.56 μg/ml Amphotericin B. Virus was titered in Sf9 cells using the plaque assay technique as described by King and Possee[16] with minor variation. Cells were maintained in supplemented Graces media and after applying the agarose overlay the cells were incubated for 6 days before staining plaques. To amplify the virus, a multiplicity of infection (MOI) of 0.1 was used. Viral stocks were always titered and stored at 4°C in the dark. To purify virus, cell debris was removed and centrifuged for 10 min at 2000 r.p.m. Cleared virus was layered over 27% sucrose and centrifuged at 24000 r.p.m. for 75 min in SW28 tubes. The pellet was resuspended in TE, pH 8, sonicated on ice for 20 s, and then layered over a 20–60% sucrose gradient in SW 27.1 tubes. After centrifugation at 27000 r.p.m. for 155 min, the purified viral band was isolated using a syringe filter with a 20-gauge needle. To concentrate the virus and remove any residual sucrose, the virus was mixed with TE and pelleted in SW 27.1 tubes by centrifugation at 27000 r.p.m. for 150 min. The final pellet was resuspended in TE, pH 8, sonicated briefly on ice, filtered through a 0.45-μm syringe filter and stored at 4°C in the dark. Purified virus was titered using the plaque assay technique.

Primary hepatocyte isolation and culture

Rat hepatocytes were isolated by a modification of the two-step collagenase perfusion *in situ*[37] and cultured with modifications[2,38] of the protocols as described previously. A dilute concentration (233 μg/ml final concentration) of

ECM (Matrigel) was added as an overlay 16 h after initial plating of the cells or as stated. Medium changes were conducted on a daily basis and a 25 nM dexamethasone concentration was maintained in all media.

Cell lines

HuH-7 cells were cultured at 37°C in 95% air/5% CO_2. Cells were maintained in DMEM/F-12 media supplemented with 2.2 mg/ml tissue culture grade sodium bicarbonate, 5% heat inactivated fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Treatments

PB (1.0 M) was dissolved in tissue culture grade water as a stock solution and stored in aliquots at –20°C. Forskolin (100 mM), dexamethasone (25 μM), β-naphthoflavone (βNF) (12 μg/μl) and Ro 20–1724 (500 mM) were dissolved in DMSO as stock solutions and stored at –20°C. TNF-α was diluted in complete Williams E media[2] and stored at –80°C in 10 ng/μl aliquots. Typically, cells were cultured for 24–48 h before the addition of any treatments. In the case of gadolinium chloride treatments, this agent was added to the cells in media immediately after the 3–4 h attachment period, then removed after 14 h of treatment via a fresh medium change, prior to subsequent exposures to viruses or PB. Final concentrations of each treatment were: PB, 500 μM; forskolin, 100 μM; βNF, 22 μM; and Ro, 20–1724 μM; unless otherwise stated. TNF-α treatments were variable and were as stated in the Figure legends. For all analyses, representative data are shown from multiple studies performed independently with different hepatocyte preparations.

Transfections

Lipid-mediated transfections were carried out using Lipofectin (Life Technologies). Liver cells (primary hepatocytes or HuH-7s), were washed and placed in serum-free media for transfections. For primary hepatocytes, transfections were performed in 60-mm dishes 16 h after plating and before the addition of Matrigel. Ten μl of plasmid DNA plus 2.5 μl of a CMV-secreted alkaline phosphatase plasmid or a CMV-β-galactosidase plasmid were cotransfected into cells in 3 ml of media (with PB treatments as noted) as suggested by Life Technologies. After 6 h, 3 ml of serum containing media and Matrigel were added to each dish to stop the transfection. After overnight incubation, the media were replaced with fresh media. For cell lines, serum containing media was added instead of Matrigel to stop the transfections. Cells were harvested 48 h after transfection for either RNA or protein. For β-galactosidase or luciferase staining, the cells were fixed with 3% paraformaldehyde. For viral transfections, cells were grown in 35-mm dishes at a confluency of 1.5×10^6 cells per dish and transfections were performed 16 h after plating, before the addition of Matrigel. Virus, at a MOI of 10–200 was added to the cells in 1.5 ml of complete media and was left on the cells and incubated at 37°C for 2 h. After this period, 1.5 ml of complete media were added and the cells were incubated overnight. The next morning, the virus and media were removed as fresh media, with or without stated treatments, were added in addition to Matrigel. Forty-eight hours after the addition of the virus the cells were harvested for RNA and protein, or fixed for luciferase, β-galactosidase, and KU-1 staining.

Luciferase activity

Cells were scraped in PBS, centrifuged at 500 r.p.m. and resuspended in 100 μl of reporter lysis buffer (Promega). After one freeze–thaw cycle, cellular debris was pelleted and 25 μl of supernatant was measured for luciferase activity using an LB9507 luminometer (EG&G Berthold, Nashua, NH, USA). Promega luciferase assay substrate, 100 μl, was used. Luciferase levels, relative light units (RLU) were normalized to protein levels which were measured using the BCA assay (Pierce, Rockford, IL, USA).

β-Galactosidase measurements and antibody staining

To measure transfection efficiency with β-galactosidase, fixed cells were stained with a potassium ferricyanide/ferrocyanide solution[39] and the stained cells were counted using phase-contrast light microscopy. To examine luciferase expression, fixed cells were permeabilized with 0.1% Triton-X and incubated at room temperature with a 1:200 dilution of anti-luciferase antibody (Promega) in a 1% BSA solution. After 2 h cells were washed with PBS and a goat-anti-rabbit IgG-FITC secondary antibody (Sigma) was added. For detection, nuclei were stained with propidium iodide and cells were examined using scanning laser cytometry on the ACAS 570 laser cytometer (Meridian Instruments, Okemos, MI, USA). For Kupffer cell detection, fixed cells were permeabilized with 0.1% saponin and incubated with a 1:2 dilution of KU-1 antibody in 1% goat serum for 2 h. A goat anti-mouse-FITC-conjugated secondary antibody was then added. For nuclei detection a Hoechst 33342 solution (2 μg/μl) was added immediately before examining the cells with scanning laser cytometry.

cAMP analysis

Intracellular cAMP was measure using the Biotrak cAMP enzyme immunoassay system from Amersham (Arlington Heights, IL, USA). Cells were stimulated for the indicated times and cell extracts were prepared. Briefly, cells were washed twice with cold PBS and then lysed and scraped in 800 μl of ice-cold 70% ethanol. Cell debris was pelleted at 2000 *g* and the resulting supernatant was subsequently lyophilized and stored at –20°C. For analysis, resulting pellets were resuspended in 500 μl of the required assay buffer. Protein concentrations were determined using bovine serum albumin as a standard with the BCA protein assay reagent (Pierce).

Measurement of PKA activity

Protein kinase A activity was assessed by measuring the transfer of ^{32}P-labeled phosphate to a biotinylated Kemptide (LRRASLG) substrate using Promega's Signa-TECT PKA assay system (Madison, WI, USA). At specified time-points after treatments, cells were washed twice and then scraped with cold PBS. Cells were pelleted at 500 *g* at 4°C and resuspended in 100 μl of ice-cold buffer (25 mM Tris pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, and 10 mM $MgCl_2$). The resulting cell suspension was lysed by brief (7 s) sonication on ice, and the cell debris was pelleted at 15000 *g* at 4°C. Five μl of supernatant was assayed for 5 min at 30°C with: 5 μl PKA assay buffer (Promega), 0.1 mM ATP, 5 μl γ-^{32}P ATP (6000 Ci/mmol), and 0.1 mM PKA biotinylated Kemptide substrate, with or without activation by 5 μM cAMP. Mixtures without exogenous

cAMP measured endogenously activated PKA, while mixtures with added cAMP measured the total available PKA in the cells. Reaction termination, spotting on to membranes, and washing were carried out as directed by the manufacturer's protocol. Protein concentrations were determined using bovine serum albumin as a standard with the Bio-Rad Protein assay (Hercules, CA, USA).

RNA analysis

Total RNA was isolated using Trizol as described by the supplier. For slot blot evaluation, 5 μg of total RNA was applied directly to a Genescreen Plus nylon membrane (DuPont, Boston, MA, USA) under vacuum and denaturing conditions using a Minifold II apparatus (Schleicher and Schuell, Keene, NH, USA). The membranes were hybridized with specific ^{32}P-radiolabeled oligonucleotides or cDNA probes for CYP2B1, CYP2B2, CYP3A1, CYP1A1, albumin, and 18S rRNA, as described previously.[11,38] To examine cytokine expression in primary hepatocytes multi-probe RNase protection assays were performed using the rCK-1 template with the Riboquant multi-probe RNase protection assay system (Pharmingen, San Diego, CA, USA). Fifteen μg of target RNA were used for each sample and assays were performed according to the manufacturer's specifications.

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