Nucleic Acids Research, Vol. 20, No. 16 4205–4211

Cationic lipid-mediated transfection of liver cells in primary culture

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Received May 27, 1992; Revised and Accepted July 16, 1992

ABSTRACT

We describe transfection of DNA into parenchymal and individual non-parenchymal cell populations from adult rat liver in early primary culture, using cationic lipid as the carrier. All cell populations were transfectable, although lipid requirements varied by cell type and, for hepatocytes, with the age of the culture. For hepatocytes in early primary culture (2–10 hours after plating), pure DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) was strikingly more effective than commercial formulations (Lipofectin® or TransfectACE®) containing components in addition to, or other than DOTMA . For hepatocytes fully adapted to culture (~48 hours after plating), pure DOTMA and Lipofectin® were similarly effective. Under optimal conditions, about 10% of hepatocytes expressed the transfected reporter gene. CAT expression in hepatocytes doubled from 48 hours to 7 days after transfection. The effect of culture substratum on transfection efficiency also was examined. The presence of basement membrane-like matrix (EHS gel) reduced uptake of the DNA-lipid complex. However, cells in early culture that were transfected on collagen and then replated on EHS gel, displayed significantly greater reporter gene activity than did cells maintained throughout on collagen. In contrast to hepatocytes, non-parenchymal cells (lipocytes, Kupffer cells and endothelial cells, respectively) were transfected most efficiently by Lipofectin; DOTMA alone was inactive. The methods described will facilitate studies of gene regulation in individual liver cell populations.

INTRODUCTION

Transfection of DNA into cultured cells is a valuable technique for examining gene regulation. However, the existing methods have had limited use for transfecting epithelial cells in primary culture. Viral vectors are non-toxic and efficient but cumbersome to prepare (1,2). Electroporation (3) and calcium phosphate-DNA coprecipitation (4,5) have been described but in our hands are associated with cellular toxicity. An alternative method for transfection uses a complex of DNA and cationic lipid and has been shown to be less toxic than calcium phosphate treatment for some cell lines (6). Its use for transfecting primary epithelial cultures has been explored but with commercially available Lipofectin® only (7).

In examining transfection of epithelial cells, we have kept in mind a major rationale for the use of primary cultures, namely, the study of normal cellular function. In this regard, an important consideration is the well-known phenomenon of culture-related phenotypic change, which emerges as the cells age in culture and broadly affects the expression of liver-specific function (8). For this reason, our studies of DNA transfection have focussed on hepatocytes in very early primary culture, when the cellular phenotype is most similar to that *in vivo*. In preliminary work, we found that early cultures were not readily transfected by Lipofectin, despite recently published data (7). We therefore turned our attention to other carriers, surveying a range of cationic lipids. The results indicate that the lipid requirement for transfecting hepatocytes in early culture differs strikingly from that of culture-adapted cells and non-parenchymal cells. We have also evaluated transfection in the presence of a culture substratum that stabilizes the hepatocyte phenotype (9).

MATERIALS AND METHODS

1. Cell isolation and culture

Hepatocytes were isolated from male Sprague-Dawley rats by in situ perfusion of the liver with collagenase (Boehringer-Mannheim, Indianapolis, IN) followed by centrifugal elutriation, as previously described (10,11). Culture substrata, prepared in the laboratory, consisted of either type I collagen from rat tail tendon (11) or an extract of basement-membrane protein from the Engelbreth-Holm-Swarm mouse (EHS) sarcoma (9). Routinely, hepatocytes were plated on 60mm collagen-coated culture dishes at a density of $4-6\times10^6$ cells per dish. In some studies, cells were plated at the same density on EHS extract (EHS gel), as described previously (9). Cultures were incubated in modified medium 199 (12) with 5% bovine calf serum and penicillin (100U/ml). Hepatic lipocytes were isolated by in situ perfusion of the liver with pronase and collagenase, followed by centrifugation on a discontinuous Stractan gradient (13). Kupffer

cells and sinusoidal endothelial cells, taken from the same gradient, were further purified by centrifugal elutriation (12,14). Lipocytes and Kupffer cells were plated on uncoated 60mm plastic culture dishes at a density of $2-3\times10^6$ cells per dish. Endothelial cells were plated at the same density on collagen-coated 60mm dishes. The non-parenchymal cells were incubated in medium 199 with 20% serum (10% calf and 10% horse). For long term culture (>48 hours) media were supplemented with gentamicin (10μg/ml) and amphotericin B (2.5μg/ml).

In some studies, hepatocytes were transfected on collagen, then removed by incubation with Dispase® (neutral protease, Collaborative Research, Bedford, MA), pelleted by centrifugation (500×g), washed twice with phosphate-buffered saline (L-15) and replated on either collagen (as initially) or on EHS gel. The reattachment and survival of hepatocytes was >95% at 24 hours (unpublished observation).

2. Plasmid DNA

The plasmid constructs used in this study are outlined in Table 1. The mouse albumin enhancer region from −10.5kb to −8.5kb fused upstream of a 322bp promoter sequence (−300 to +22) was a gift from L.E.Babiss and J.E.Darnell (Rockefeller University, New York). Using homologous primers with 5′ HindIII and 3′ XbaI restriction sites, the fragment was amplified by PCR and directionally cloned into the pCAT-Enhancer plasmid (Promega, Madison, WI), containing a 202bp SV40 enhancer sequence and the chloramphenicol acetyl transferase (CAT) reporter gene. A previously reported negative regulatory region from −8.7kb to −8.5kb in the albumin gene (18) was removed by restriction digestion with PstI and the cohesive ends religated. The region around the transcriptional start site (from −200 to +22) was sequenced and was identical to the published sequence (20).

Plasmid DNA was amplified in the JM 109 strain of *E.coli*, isolated by alkaline lysis and separated from high molecular weight RNA by precipitation with 4M LiCl, followed by banding in a cesium chloride/ethidium bromide gradient (21,22). DNA purity was assessed by agarose gel electrophoresis.

3. Cationic liposomes

DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl-ammonium chloride) was a gift from Syntex Corp., Palo Alto, CA. DOPE (dioleoyl-phosphatidylethanolamine) was purchased from Avanti Polar Lipids Inc., Birmingham, AL. DOTAP (1,2-dioleoyloxy-3-(trimethyl ammonio)propane was a gift from Dr. John Silvius (McGill University, Montreal, Canada). L-PE (lysinylphosphatidylethanolamine) and CEBA (cholesterol ester of β-alanine) were gifts from Dr. Luke Guo (Liposome Technology Inc., Menlo Park, CA). DOTAP/DOPE (1:1 molar ratio) and L-PE/CEBA (6:4 molar ratio) were synthesized by dissolving the individual lipids in chloroform or ethanol and evaporating to dryness on a rotary evaporator. Liposomes were prepared by suspending the lipids in 1 ml of sterile deionized water and sonicating for 20 minutes in a bath sonicator (Laboratory Supply Co., Hicksville, NY). Lipofectin® (DOTMA/DOPE) and TransfectACE (DDAB/DOPE) were purchased from GIBCO-BRL (Gaithersburg, MD). All liposomes were stored under argon at 4°C.

4. Transfection

DNA and lipid were mixed together in sterile polystyrene tubes; 1ml serum-free plating medium was added and the mixture was incubated at 25°C for 15 minutes. After aspiration of the medium, cells were washed twice with L-15, and 1ml serum-free medium was added. The transfection mixture was then added, and the cells were placed at 37°C, 2% CO_2. Transfections were stopped by aspiration of the transfection mixture and addition of complete medium. The cells were then incubated as above until harvest. 60mm culture dishes and a final volume of 2ml per dish were used for all transfections. Cells maintained in culture longer than 24 hours underwent a daily medium change.

5. Reporter gene assays

One 60mm plate was used for each data point. After two washes with L-15, hepatocytes were removed from the culture dishes enzymatically using Dispase. The released cells were transferred to microcentrifuge tubes, pelleted by brief centrifugation, washed twice with L-15 and then resuspended in 125μl of 0.1M Tris, pH=7.8. Non-parenchymal cells were washed as above and scraped directly into microcentrifuge tubes. Cell extracts were obtained by three consecutive freeze/thaw cycles followed by centrifugation at 14,000 rpm for 5 minutes. For the CAT assay, samples were heated at 60−65°C for 10 minutes to inactivate endogenous deacetylase activity (5), followed by centrifugation at 14,000 rpm for 10 minutes. The supernatant was placed in a fresh tube and stored at −20°C. A volume of extract corresponding to 20μg protein (23, Protein Assay Kit, Bio-Rad, Richmond, CA) was mixed with 10μl of ^{14}C-chloramphenicol (25μCi/ml, Amersham, Arlington Heights, IL) and 20μl of acetyl Co-enzyme A (3.5mg/ml stock). The final volume was adjusted to 130μl with 0.25M Tris, pH=8, and the reaction mixture was incubated at 37°C for 1 hour. After extraction with ethyl acetate, the reaction products were resolved by thin-layer chromatography ($CHCl_3$:CH_3OH, 95:5), which separates mono- and diacetylated chloramphenicol from the unmodified compound (24). Radio-labeled product was visualized by autoradiography and quantitated by liquid scintillation spectroscopy. For the β-galactosidase assay, the cells were harvested in the same manner except that the 65°C incubation step was omitted, as this inactivates the enzyme; β-galactosidase activity was measured using an assay protocol provided by the manufacturer (Promega, Madison, WI). In studies comparing the relative strength of various promoters, cells were co-transfected with the CMV-β-galactosidase construct and the CAT constructs of interest; CAT expression was normalized to β-galactosidase activity. The promoterless CAT plasmid served as a negative control. Because co-transfection usually reduced the level of reporter gene expression, a 2 hour CAT assay time was used in these experiments.

Cells expressing β-galactosidase were visualized by staining with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal), as previously described (25). Briefly, transfected cells were fixed directly on the culture dishes with phosphate-buffered 1% glutaraldehyde and then incubated at 37°C with a buffered solution containing 0.2% X-gal. After 1−2 hours the cells were examined under phase-contrast microscopy; cells actively expressing β-galactosidase were readily identified by their intense blue staining. Non-transfected cells served as negative controls and exhibited no blue staining.

6. Toxicity Assay

The cellular toxicity of DOTMA and DOTMA/DNA complexes for intact cells was assessed by radioleucine incorporation into peptide (26). Hepatocytes were plated on a collagen-coated 24-well plate. DOTMA or DOTMA/CMV-CAT in serum-free medium was added 4 hours after plating and remained in contact

with the cells for 24 hours. The medium was then replaced with complete medium, and the cells were incubated for an additional 24 hours. After removal of the medium and washing with L-15, the cells were incubated with serum-free, leucine-free medium supplemented with ^{14}C-leucine, 2μCi/ml. After 30 minutes, the radioleucine medium was removed, and the cells were washed once with L-15 followed by a 10-minute incubation with ice-cold 10% trichloroacetic acid (TCA). The cells were then washed twice with TCA, once with cold methanol/ethyl ether (1:1), dissolved in 2% Na_2CO_3/0.1 N NaOH, then sonicated and counted (26).

7. Statistical analysis

The results are reported as picomoles (pmol) acetylated chloramphenicol/μg protein/hour CAT assay incubation time, unless otherwise indicated. Each point represents the mean of duplicate or triplicate observations ± standard error of the mean from at least two different cell preparations, unless otherwise noted. Results with $p<0.05$ by Mann-Whitney U-test were considered significant.

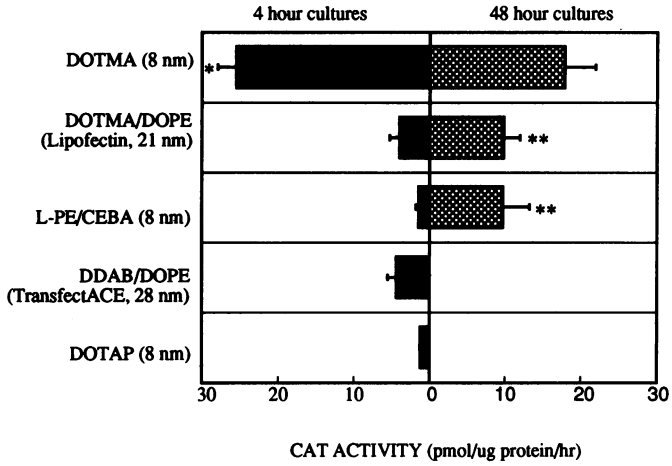

Figure 1. The relative efficacy of different cationic lipids for hepatocyte transfection. Hepatocytes were transfected with CMV-CAT (2.5μg) and the indicated amount of total lipid at 4 and 48 hours after initial plating. Lipofectin® and TransfectACE® were used in amounts suggested by the manufacturer (21nm and 28nm, respectively). The transfection time was 4 hours. Cells were harvested for CAT assay 24 hours after stopping the transfection (DOTMA 4 hrs n=15, 48 hrs n=13; DOTMA/DOPE 4hrs n=11, 48hrs n=9;L-PE/CEBA 4hrs n=11, 48hrs n=6; DDAB/DOPE, DOTAP/DOPE n=6). *$p<0.001$ versus other lipids at 4 hours;**$p<0.05$ versus corresponding 4 hour cultures.

RESULTS

1. Transfection of hepatocytes in early primary culture

In initial studies, we used the CMV-CAT construct (Table 1.), mixing it with either equimolar amounts of various lipids or in a ratio recommended by commercial suppliers. At 4 hours after plating, hepatocytes were transfected much more effectively by pure DOTMA than by DOTMA/DOPE (Lipofectin®). The difference between DOTMA and DOTMA/DOPE was approximately 7-fold in cells transfected 4 hours after initial plating (Figure 1). DDAB/DOPE (TransfectACE®) was equivalent to Lipofectin® while DOTAP/DOPE and L-PE/CEBA were minimally effective (Figure 1). DOTMA liposomes were used in subsequent studies, unless noted otherwise.

The apparent efficiency of DOTMA transfection varied with the age of the cultures. Reporter gene expression was detectable in hepatocytes transfected immediately after isolation, increased in cells transfected after four hours in culture, then decreased in older cultures (Figure 2A). In parallel studies, transfection with DOTMA/DOPE improved in older cultures such that, at 48 hours, DOTMA/DOPE was about as effective as pure DOTMA (Figure 1).

2. Optimization of transfection

The optimal duration of exposure of the cells to the lipid-DNA complex (transfection time) was examined. Reporter gene expression was detectable with a one-hour exposure to the lipid-DNA complex, increased with a two-hour exposure but was not significantly increased beyond two hours (Figure 2B). In subsequent studies, an incubation time of 2−4 hours was used for hepatocytes plated on collagen; longer transfection times were required for cells plated on EHS gel (data not shown). We also examined a range of DNA/lipid ratios. When various amounts of DOTMA were complexed with 2.5μg of DNA, the optimal amount of lipid varied between 2−16 nmols (the data for 2, 8 and 16 nmols are not significantly different) (Figure 2C). We

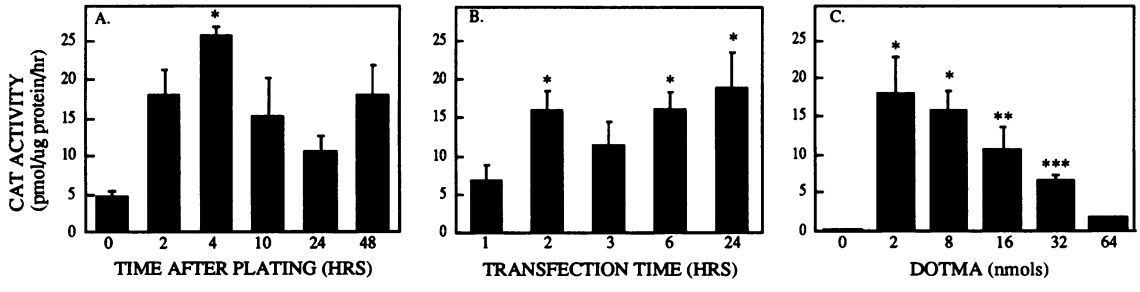

Figure 2. A. Reporter gene expression in hepatocytes transfected at various times after cell plating. Hepatocytes were transfected with CMV-CAT (2.5μg) and DOTMA liposomes (8nm) at the indicated times after initial plating. The transfection time was 4 hours, and the cells were harvested for CAT assay 24 hours after stopping the transfection (0hrs, 2hrs n=9; 4hrs n=15; 10hrs, 24hrs n=8; 48hrs n=13). *$p<0.001$ versus 0 hours and 24 hours, $p<0.05$ versus 10 and 48 hours. B. The effect of transfection time on reporter gene expression. Hepatocytes were transfected with CMV-CAT (2.5μg) and DOTMA liposomes (8nm), 4 hours after initial plating; at the indicated time points the transfection medium was replaced with complete medium. The cells were harvested for CAT assay 24 hours after stopping the transfection (n=4). *$p<0.05$ versus 1 hour. C. The effect of varying DNA/lipid ratios on transfection efficiency. Hepatocytes were transfected with CMV-CAT (2.5μg) and the indicated amount of DOTMA liposomes. The cells were transfected 4 hours after plating; transfection time was 4 hours. The cells were harvested for CAT assay 24 hours after stopping the transfection. The experiment was performed with two different hepatocyte preparations, with comparable results. The data are from one of these studies (n=3). *$p<0.05$ versus 0 nm, 32 nm and 64 nm; **$p<0.05$ versus 0 nm, 64 nm; ***$p<0.05$ versus 64 nm.

Table 1. Promoter-reporter constructs used.

Promoter		Reporter gene	Reference
CMV	760bp major immediate early promoter of human cytomegalovirus.	*E.coli* CAT, β-GAL	15
RSV	5' long terminal repeat of Rous sarcoma virus.	CAT	16
SV-40	Early promoter plus enhancer of simian virus-40.	CAT	5
PRL	250bp bovine prolactin promoter sequence.	CAT	17
ALB	2.3kb murine promoter/ enhancer sequence.	CAT	18
pCAT-EN	Promoterless with SV-40 enhancer	CAT	19

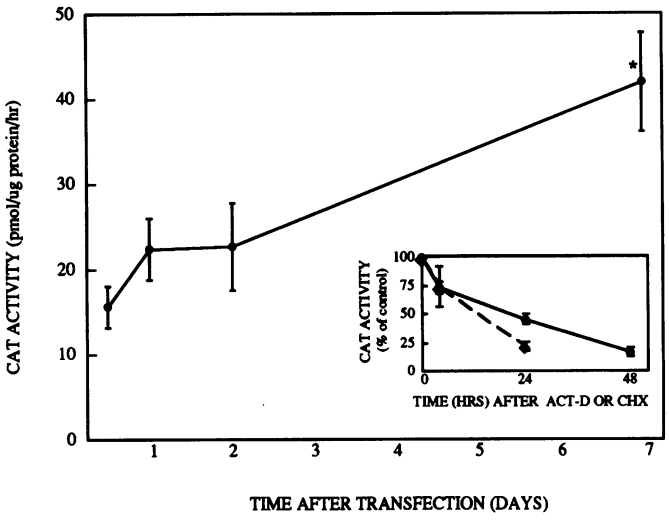

Figure 3. Change in reporter gene expression with post-transfection incubation time. Hepatocytes were transfected with CMV-CAT (2.5 μg) and DOTMA liposomes (8nm) at 4 hours after initial plating. The cells were harvested for CAT assay at the indicated times after stopping the transfection (n=6). *p<0.001 versus 0.5 days, p<0.05 versus 1 and 2 days. *Inset.* Stability of CAT mRNA in hepatocytes. Hepatocytes were transfected with CMV-CAT (2.5μg) and DOTMA (8 nm). 12 hours after stopping the transfection, serum free medium with or without actinomycin D (2μg/ml) or cycloheximide (3μg/ml) was added. The cells were harvested for CAT assay at the indicated times after adding actinomycin or cycloheximide. Results are reported as percent of CAT expression in control cells (not exposed to actinomycin or cycloheximide) harvested at the same time (actinomycin n=4, cycloheximide n=3). Actinomycin (ACT-D) –●–; cycloheximide (CHX) – – ◆ – –.

varied the amount of CMV-CAT from 1 to 8μg added to a fixed amount of DOTMA (8 nm) (data not shown). Peak CAT activity was obtained with 5μg while 2.5μg was the lowest amount of DNA that generated acceptable and reproducible levels of expression. Increasing the amount of DNA in the complex while maintaining a constant DNA:lipid ratio increased CAT expression (not shown). These studies led to the protocol described in the legend to Figure 1 as the 'standard' for transfecting hepatocytes in early culture.

The time-course of reporter gene expression following transfection also was examined. As shown in Figure 3, CAT activity was essentially constant up to 48 hours following transfection, then increased approximately two-fold over the subsequent 5 days. The stability of the CAT mRNA and the CAT enzyme were examined in hepatocytes that had been transfected,

Table 2.

A. Effect of serum on transfection efficiency

Transfection Condition	CAT Activity (% of serum-free control)
Serum-free	100%
2.5% calf serum	58.9 ± 11.3%*
10.0% calf serum	10.6 ± 6.5%*

B. Effect of culture substratum on reporter gene expression

Transfection Condition	CAT Activity (% of Type I collagen control)
Transfected on Type I Collagen; replated on Type I Collagen	100%
Transfected on EHS gel; no replating	45 ± 38.1%
Transfected on Type I Collagen; replated on EHS gel	150 ± 38.1%**

A. Hepatocytes were transfected with CMV-CAT (2.5μg) and DOTMA liposomes (8nm). Transfections were performed in serum-free medium (standard protocol) or in the presence of 2.5% or 10% calf serum; type I collagen substratum was used for all cells. After a transfection time of 4 hours, the transfection medium was aspirated and replaced with growth medium. Cells were harvested for CAT assay 24 hours after stopping the transfection. The experiment was performed with two different cell preparations, with comparable results. The data are from one of these studies (n=3). *p<0.05 versus serum-free conditions.

B. Hepatocytes on EHS gel were transfected with CMV-CAT (2.5μg) and 16nm DOTMA, with a transfection time of 16 hours (n=3). Cells initially on collagen were transfected according to the standard protocol, using serum-free medium and 8nm DOTMA. When the transfection was ended, the cells were released from the collagen substratum with Dispase® and replated on either type I collagen (n=8) or EHS gel (n=9), as indicated. All cells were harvested for CAT assay 24 hours after stopping the transfection. **p<0.05 versus other conditions.

allowed to develop CAT expression, and then treated with actinomycin D (Boehringer-Mannheim) 2μg/ml or cycloheximide (Sigma Chemical Co.) 3μg/ml in serum-free medium (26). These concentrations of actinomycin and cycloheximide block 95% of radiouridine and radioleucine uptake, respectively (data not shown). Figure 3 (inset) shows that CAT activity decreased to 50% of control levels approximately 13 hours after exposure to cycloheximide and approximately 22 hours after exposure to actinomycin D.

3. Effect of serum and a basement membrane-like substratum

The presence of serum significantly inhibited DOTMA-mediated transfection (Table 2A). When hepatocytes plated on EHS gel substratum were transfected with the CMV-CAT construct, the level of reporter gene expression was not as high as that in cells plated on collagen, although the use of longer transfection times (16–24 hours) narrowed the difference, suggesting that EHS gel inhibited uptake of the DNA-lipid complex. To exclude an effect on the cellular expression of the CAT construct, we plated hepatocytes on collagen and transfected them as above, then replated the cells on either collagen or EHS gel and harvested them at 24 hours. The cells replated on EHS exhibited 3-fold greater CAT expression than the cells transfected directly on EHS and 50% greater expression than the cells replated on collagen (Table 2B).

4. Activity of viral and liver-specific promoters

We evaluated the relative strength of several different promoters (Table 1). The cells were co-transfected with a CMV-β-galactosidase construct, and CAT expression was normalized to

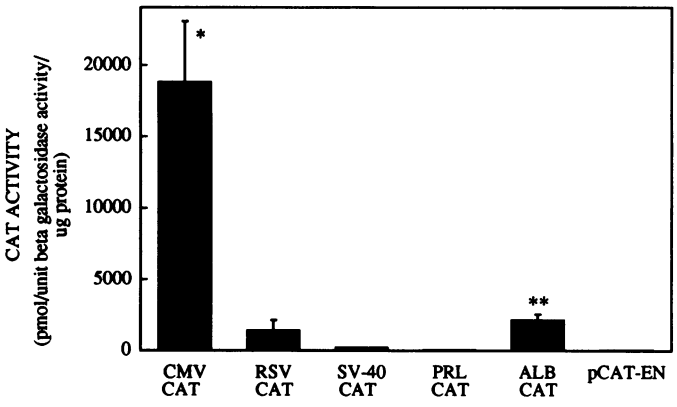

Figure 4. Activity of various promoters transfected into hepatocytes. Hepatocytes were transfected with the indicated plasmid (2.5μg), CMV-β-Gal (2.5μg) and DOTMA (16nm). The cells were harvested 24 hours after stopping the transfection. Results are reported as CAT activity per unit of β-galactosidase activity per μg protein per hour of incubation (n=9 for CMV, RSV; n=3 for SV-40 and PRL; n=6 for ALB; n=3 for pCAT-EN). *p<0.001 versus RSV, p<0.01 versus ALB; **p<0.05 versus RSV. See Table 1 for abbreviations. The concentration of β-galactosidase in the cell extracts varied from 1×10^{-5} to 2×10^{-4} Units/μl, corresponding to 0.02 to 0.4 absorbance units at 420μm.

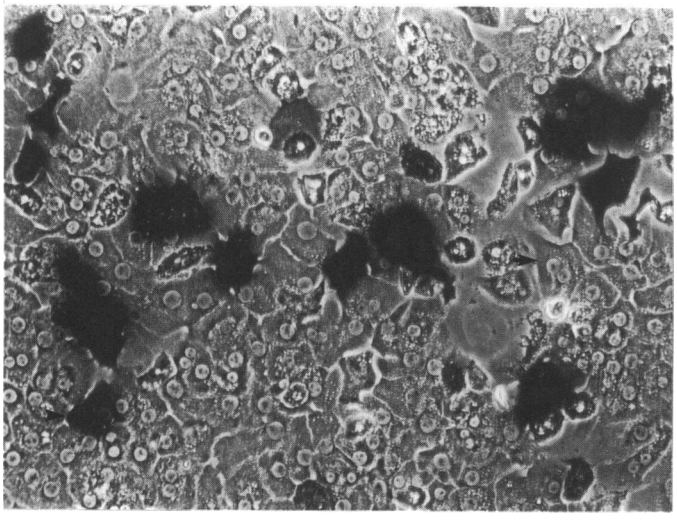

Figure 5. Histologic evaluation of β-galactosidase expression in hepatocytes. Hepatocytes were transfected with CMV-β-galactosidase (2.5μg) and DOTMA (16nm). The cells were transfected 2 hours after plating; transfection time was 4 hours. After 24 hours in complete medium, the cells were fixed and stained with x-gal, as described. Transfected cells strongly expressing the galactosidase reporter gene are identified by their intense blue staining, which appears black in the photo (▶). Occasional less intensely stained cells (pale blue in the original) were seen (→). All of the staining cells are hepatocytes.

protein content and β-galactosidase activity. Figure 4 shows that the CMV promoter yielded the highest level of CAT activity, nearly 9-fold greater that the albumin promoter/enhancer and 14-fold greater than RSV. A construct containing the albumin promoter alone (−300 to +22) yielded no detectable activity; the albumin promoter/enhancer construct was inactive in sinusoidal endothelial cells (not shown).

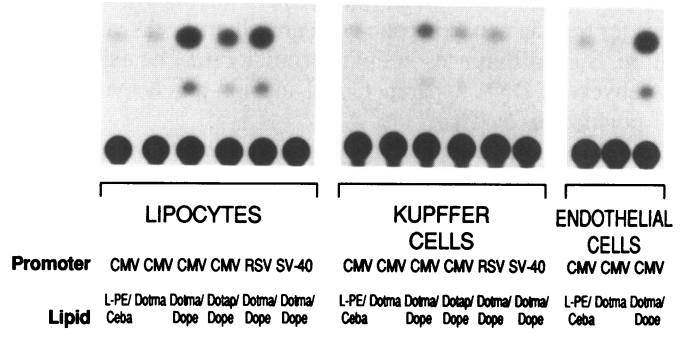

Figure 6. Transfection of hepatic non-parenchymal cells. Cells were transfected with 2.5μg of plasmid DNA and the indicated lipid (8nm). The cells were transfected after overnight incubation in complete medium and were harvested 24 hours after stopping the transfection.

5. Histologic evaluation of transfection

For each of the above experiments parallel transfections were performed using a CMV-β-galactosidase expression vector. Transfected cells were fixed in situ with 1% glutaraldehyde and then stained with x-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Those actively expressing β-galactosidase were readily identified by their dark blue color. In general, the number of positively stained cells paralleled CAT expression. Optimal transfection conditions resulted in approximately 10% positively stained cells (Figure 5).

6. Transfection of hepatic non-parenchymal cells

The various lipids were evaluated for their ability to transfect hepatic non-parenchymal cells. DOTMA/DOPE yielded the greatest transfection efficiency while DOTMA alone was not effective. In lipocytes, the CMV and RSV promoters displayed comparable activity (Figure 6). These results are in contrast to those obtained with hepatocytes.

7. Cellular toxicity studies

We evaluated the toxicity of DOTMA using ^{14}C-leucine incorporation into trichloroacetic acid-precipitable peptide. Neither DOTMA alone (up to 64nm/2 ml) nor DOTMA/DNA complexes caused significant inhibition of this process in hepatocytes (not shown).

DISCUSSION

A novel finding to emerge from these studies is the differing lipid requirement for efficient transfection of hepatocytes at an early time after plating or after full adaptation of the cells to culture. The basis for this is speculative but may involve changes in the composition or structure of the hepatocyte plasma membrane during culture. In a conventional culture system utilizing plastic coated with a thin layer of collagen, hepatocytes undergo rapid flattening and redistribution of integral membrane proteins (27), which suggests changes in membrane fluidity. Cells that are fully adapted to culture may have membranes similar to those of established cell lines, and it is for transfecting the latter type of cell, rather than normal epithelial cells, that the commercial lipid reagents were developed. This may explain the reduced efficacy of Lipofectin® and TransfectACE® for hepatocytes in very early primary culture, before major changes in cell shape have

occurred. On the other hand, Lipofectin® was superior to other lipids for transfection of hepatic non-parenchymal cells in primary culture. Such differences in lipid specificity may be exploitable for delivery of DNA to parenchymal and non-parenchymal liver cell populations *in vivo*.

CAT expression doubled in hepatocytes from 48 hours to 7 days after transfection (Figure 3). The increase at 7 days appears to represent ongoing transcription since the half-life of the CAT protein in hepatocytes was 12−14 hours (Figure 3, inset), which is about one-fourth that reported in HepG2 cells (28). Thus, the expression of transfected genes in hepatocytes in culture appears to be prolonged relative to that *in vivo*. Genes delivered *in vivo* to hepatocytes via the asialoglycoprotein receptor were expressed at 24−48 hours but declined to zero by 7 days (29). The basis for this difference in the stability of gene expression in culture and *in vivo* is unknown. It could reflect different routes of DNA uptake and, hence, variation in the subcellular disposition of episomal DNA.

The transfection efficiency of hepatocytes was about 10%. This may indicate that only a minor subpopulation is susceptible to transfection. Alternatively, a larger fraction may undergo transfection but express the bacterial enzyme only to the level of the endogenous galactosidase, making detection of the transfected gene difficult. We are currently exploring methods to monitor directly the uptake of plasmid DNA by hepatocytes as well as using other detection methods for assessing reporter gene expression. Serum inhibited transfection of hepatocytes by DOTMA. While this is the general experience (30,31), serum enhances transfection of some cell lines in which the lipid vehicle is DOTMA/DOPE (32).

Among the potential applications of transfection methodology is analysis of the transcriptional regulation of liver-specific genes. Whether and under what circumstances this is possible with hepatocytes in culture is a subject of current investigation. Hepatocytes plated on collagen lose transcriptional activity for several endogenous liver-specific genes, and for some genes such as albumin, the decrease is evident within 12 hours of plating (8). We have explored transfection of cells in very early culture, with the aim of introducing DNA prior to culture-related changes in transcription. Although transfection efficiency was greatest 4−10 hours after plating, it is feasible virtually as soon as cells attach to the substratum. When carried out 2 hours after plating, reporter gene expression was easily detected (Figure 2A). With further incubation for 2 hours to allow for uptake of the DNA/lipid complex (Figure 2B) and another 12 hours for transcriptional and translational events, cultures may be analyzed when less than 16 hours old. Although transcriptional change undoubtedly is taking place during even this brief period, the ability to introduce DNA within the first 2 hours of culture allows sampling of a relatively normal intracellular regulatory environment.

The basis for altered hepatocyte function in culture has been analyzed in detail. An important controlling factor is the culture substratum. Changes in several liver-specific genes, including albumin, are largely prevented if cells are maintained on a basement membrane-like substratum, such as the EHS gel (9). Moreover, transcriptional activity that has declined in conventional culture can be rescued by transferring hepatocytes from a thin layer of collagen (the usual substratum) to EHS gel (33). Therefore, we examined the transfection of hepatocytes in the latter environment. With cells plated on EHS gel, transfection efficiency was reduced relative to that obtained with conventional cultures. This could reflect decreased uptake of the lipid/DNA complex or decreased expression of the transfected reporter gene. To distinguish between these possibilities, we transfected hepatocytes under standard conditions, then released the cells for replating either on collagen (as before) or on EHS gel. The transfected cells transferred to EHS matrix displayed activity greater than that of cells replated on collagen (Table 2B), indicating that the CMV promoter was fully active; indeed, it may be stimulated by basement membrane matrix, as is the transcription of some liver-specific genes (9). Thus, EHS gel appears to block uptake of the DNA/lipid complex. While it is composed mainly of laminin and other matrix proteins (9), it also contains a significant amount of DNA (unpublished observations), which could affect the composition and therefore the uptake of the DNA/lipid complex. The fact that serum reduces transfection efficiency of DOTMA liposomes (Table 2A), suggests that soluble protein in the EHS gel also could be inhibitory.

The CMV promoter was clearly the strongest of the various promoters tested in hepatocytes. The data are generally in agreement with those of Parker-Ponder et. al. (7). Transfection with the albumin construct indicates that liver-specific promoters are active in this system. Reporter gene expression was readily detectable in a construct containing the upstream albumin enhancer (−10.5 to −8.7) but was sharply reduced without the enhancer. These data parallel observations in transgenic mice (30), and support the use of early primary culture as a model of the liver *in vivo*.

ACKNOWLEDGEMENTS

We thank Kun-Bee Chang for expert technical assistance and Dr. Jacquelyn Maher for reviewing the manuscript. This work was supported by NIH grants T32DK07573 (W.R.J.), DK31198 (S.S.W. and D.M.B.), DK26743 (S.S.W and D.M.B), AI26128 (R.J.D) and AI27681 (R.J.D). Additional funds were provided by the State of California and allocated by the University-wide Taskforce on AIDS (R.J.D.).

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