Evaluation of Relative Promoter Strength in Primary Hepatocytes Using Optimized Lipofection

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ABSTRACT

For most genetic deficiencies manifested in the liver, maximization of gene expression in hepatocytes will be an important factor in achieving successful gene therapy. A rapid, highly efficient, and nontoxic method for transfecting DNA into hepatocytes was used to compare directly promoter strengths of various cellular and viral promoters. Conditions are described here for transfecting 5–10% of primary hepatocytes using the positively charged liposomes, Lipofectin. Cells are not damaged by this method as they continue to transcribe genes controlled by liver specific promoters and can survive for over 2 weeks in culture. We find that the cytomegalovirus, SRα, and β-actin promoters are more active than the SV40, RSV, RNA polymerase II, albumin, α1-antitrypsin, or phosphoenolpyruvate carboxykinase promoters. A simple TK promoter and a TK promoter with the polyoma enhancer (MCI) were almost completely inactive. This information will be useful in the construction of vectors designed to express genes efficiently in primary hepatocytes for purposes of gene therapy, although the stability of expression from these promoters will need to be demonstrated in hepatocytes in vivo.

OVERVIEW SUMMARY

For most genetic deficiencies manifested in the liver, the ability of an individual liver cell to express the therapeutic gene at high levels will be critical for success of gene therapy. A variety of viral and cellular promoters were tested for their relative efficiency of expression in primary hepatocytes in order to identify those that exhibit the highest level. Further experiments will need to be performed to verify that a similar hierarchy of promoter strength will be observed from vectors after transfer into hepatocytes in vivo.

INTRODUCTION

HEPATOCYTES are an increasingly attractive target for gene therapy for several reasons, including the large number of genetic diseases manifested in the liver. Two general approaches toward hepatic gene therapy are theoretically feasible. The first is to deliver DNA directly to hepatocytes in vivo, using DNA–ligand complexes which are targeted for liver-specific receptors (Soriano et al., 1983; Wu and Wu, 1988; Wu et al., 1989; Kaneda et al., 1990). The second approach involves in vitro transduction of isolated hepatocytes (Ledley et al., 1987; Wolff et al., 1987; Wilson et al., 1988a) followed by reimplantation (Anderson et al., 1989; Ponder et al., 1991). For most genetic diseases, the levels of expression per cell will need to be maximized for either approach to overcome the inability to genetically modify all hepatocytes. Unfortunately, hepatoma cells do not reproduce normal hepatic transcription patterns (Clayton et al., 1985), making it useful to analyze promoters in hepatocytes to get a more accurate measure of their in vivo potency.

Information regarding the relative strengths of promoters in primary hepatocytes may yield information that will facilitate maximization of the level of expression per cell in hepatocytes of...
intact animals. Most previous studies have investigated the relative efficiencies of various promoters by using viral constructs in cultured hepatocytes (Friedman et al., 1986; Ledley et al., 1987; Wilson et al., 1988b), or by analyzing liver expression in transgenic mice (Pinkert et al., 1987; Sifers et al., 1987; McGrane et al., 1988; Shen et al., 1989). Production of retroviral packaging cell lines requires several weeks, however, and inequality of titers between different packaging lines and/or influence from the retroviral long terminal repeats (LTRs) can complicate the results. Analyses using transgenic mice are even more time consuming, and the site of integration and copy number can dramatically influence the levels of expression from a promoter (Palminter and Brinster, 1986).

An efficient, rapid, and consistent method for transfecting DNA into hepatocytes would facilitate studies to directly compare the relative strengths of different promoters. Although direct transfer of DNA into hepatocytes using electroporation (Tur-Kaspa et al., 1986), asialoglycoprotein-polyslysin: DNA complexes (Wu and Wu, 1987), or liposomes with lactosylceramide (Soriano et al., 1983) has been achieved, these methods have been of relatively low efficiency thus far.

Recently, techniques have been developed for in vitro transfer of DNA into primary hepatocytes using positively charged liposomes (Lipofectin; Felgner et al., 1987; Jacoby et al., 1989) or calcium phosphate (Pasco and Fagan, 1989; Rippe et al., 1990). However, the efficiency of transfection, the effects upon liver-specific transcription, and the effects upon long-term growth have not yet been evaluated. We report optimized conditions for lipofection into primary hepatocytes. A total of 5–10% of the cells can be transfected, and they continue to exhibit liver specific function as evidenced by expression from liver-specific promoters directing expression of reporter gene.

A comparison of a variety of viral, liver-specific, and nonspecific cellular promoters demonstrates that the CMV, SR&alpha, and &beta-actin promoters are stronger than all others tested. Finally, this method does not appear to affect the viability of hepatocytes, as they can be maintained in culture for over 2 weeks and appear morphologically identical to control cells. Thus, lipofection may also prove to be useful for transferring DNA based vectors into primary hepatocytes for the purposes of gene therapy.

**MATERIALS AND METHODS**

**Construction of plasmids**

Standard cloning techniques were as described (Sambrook et al., 1989). 486-ALB-CAT: The human albumin genomic clone &kappa;halb 18 (J. Kelly, D.R. Wilson, K.M. Huber, M. Wilde, and G.J. Darlington, unpublished data) was isolated by hybridization to the human albumin cDNA clone F47 [a gift of R. Lawn (Lawn et al., 1981)]. To create p486-ALB-CAT, a 503-nucleotide SpI-I-Hinc II fragment of the human albumin promoter (includes nucleotides from -486 to +17 relative to the transcriptional start site (Minghetti et al., 1986; Urano et al., 1986)) was used to replace the SV40 enhancer and promoter of pSV&beta;-CAT (Gorman et al., 1982a). AAT-5&beta;-Gal: PAT-CATS contains a 1246-nucleotide fragment (−1,200 to +46 relative to the transcription initiation site) of the natural human α,α-tryptacin (AAT) promoter and enhancer, which drives transcription of a chloramphenicol acetyl transferase (CAT) gene (Shen et al., 1989). The CAT gene (but not the splice and polyadenylation site) was removed from PAT-CATS by cleavage with Kpn I and Bal I, followed by blunt-end formation with the Klenow fragment of DNA polymerase I, and insertion of the blunt-ended 3,500-nucleotide Nor I fragment of E. coli β-gal (MacGregor et al., 1987).

**Plasmid DNA preparation**

Plasmid DNA was grown in the SCS1 strain of E. coli (Strategene, La Jolla, CA) and isolated using the lysozyme lysis method (Sambrook et al., 1989) and two cesium chloride gradients. DNA was treated with DNase-free RNase (Boehringer Mannheim, Indianapolis, IN), extracted once with phenol, twice with chloroform, and ethanol-precipitated. The DNA concentration was quantitated after resuspension in sterile TE (10 mM Tris 7.5, 1 mM EDTA) using a DNA fluorometer (Labarca and Paigen, 1980). The source and description of the plasmids used is described in the figure legends.

**Hepatocyte isolation**

Hormones used for hepatocyte culture were obtained from Sigma (St. Louis, MO) unless otherwise stated; dibutyryl cAMP was obtained from Boehringer Mannheim (Indianapolis, IN). Rat hepatocytes (>80% viability as assessed by Trypan Blue exclusion) were isolated from male Sprague-Dawley (Harlan, Indianapolis, IN) rats weighing 175–200 grams using 0.3 mg/ml type B collagenase (Boehringer Mannheim, Indianapolis, IN; 0.179 units/mg) and the perfusion technique first described by Berry and Friend (1969). Dog hepatocytes (>90% viability) were isolated from a single lobe of a 5- to 10-kg dog by perfusing at 100 ml/min with 0.3 mg/ml collagenase for 20 min. Hepatocytes were plated at 1.5 × 10⁶ cells per 6-cm-diameter Primaria (Falcon, Lincoln Park, NJ) plate in medium containing 75% minimum essential medium (MEM), 25% Waymouths, 10% fetal calf serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 2 mM glutamine, 10 μg/ml insulin (24 IU/mg), 50 ng/ml epidermal growth factor (EGF, Collaborative Research: Bedford, MA), and 100 μM hGH (2.4 IU/mg). Four to six hours later, the cells were rinsed twice with phosphate-buffered saline (PBS); then the medium was replaced with a hormonally defined medium designated SUM (Darlington et al., 1987), which contains 75% tyrosine-free MEM (Hazelton, St. Lenexa, KS), 25% tyrosine-free Waymouths (Hazelton), and the following hormones in their final concentration: bovine insulin at 10 μg/ml, EGF at 50 ng/ml, hGH at 100 μU/ml, transferrin at 10 μg/ml, dexamethasone at 1 μM, 3.5,3′-triiodo-L-thyronine (T₃) at 1 μM, and selenium at 3 × 10⁻⁸M. Medium was changed daily.

**Lipofection**

A modification of the lipofection method described by Jacoby et al. (1989) was used. Four to six hours after plating the hepatocytes to high density (1.5 × 10⁶ cells) in a 6-cm-diameter plate, cells were washed twice with PBS, then 2 ml of SUM was added. Unless otherwise specified, 20 μg of Lipofectin (GIBCO
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BRL, Gaithersburg, MD) in 20 μl of water was mixed well in a polystyrene tube with 3 μg of DNA, which had previously been diluted to 20 μl with sterile water. Samples were incubated for 15 min at room temperature, then added dropwise with gentle mixing to a plate. After 16 hr in a 5% CO₂ incubator maintained at 37°C, the medium was aspirated and replaced with fresh SUM.

Cytochemical stains

Two days after lipofection was completed, cells were rinsed with PBS and then fixed for 5 min with 0.5% glutaraldehyde in cold PBS. The method of MacGregor et al. (1987) was used for β-gal staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Stratagene; La Jolla, CA).

Enzymatic assays

Cells were scraped from the plates 3 days after isolation and an extract prepared by three consecutive freeze/thaws. Protein concentration was determined using the Bradford protein assay (Bradford, 1976) with a kit obtained from Bio-Rad (Richmond, CA). CAT (Gorman et al., 1982a) and β-gal (Nielson et al., 1983) assays were as described. All test samples received an equal amount of protein, which varied from 20 to 150 μg. The activity obtained from an equal microgram amount of extract from control cells that received pGEM4 DNA was subtracted from each sample. CAT activity was quantitated by eluting spots from a silica thin-layer chromatography plate and scintillation counting: 1 unit corresponds to conversion of 1 nmole of chloramphenicol to the acetylated form per minute at 37°C. β-Gal activity was quantitated by measuring the optical density of the reaction at 420 nm; 1 unit is defined as cleavage of 1 nmole of o-nitrophenyl-β-D-galactopyranoside (ONPG) per minute at 37°C. CAT and β-gal standards were obtained from Sigma (St. Louis, MO).

Run-on transcription

Nuclear run-on transcription assays were done precisely as described by Clayton and Darnell (1983) with the following exceptions. DNA probes included pGEM3 (Promega, Madison, WI) and cDNAs for chicken β-actin (Cleveland et al., 1980), mouse albumin [pMalb3 (Kiousis et al., 1981)], and mouse α1-antitrypsin (Sifers et al., 1990). Two micrograms of DNA per slot was applied to nitrocellulose membranes. Hybridization was performed for 72 hr at 42°C in 50% formamide. For each probe, linearity of signal was documented by adding two different concentrations of labeled RNA. Signals were quantitated using an Ambis Computerized Radioanalyzer. The experiment was done twice with duplicate filters.

RESULTS

Transfection of primary rat and dog hepatocytes by lipofection

Primary rat hepatocytes were lipofected with a DNA construct in which a CMV immediate early promoter/enhancer directed transcription of a β-galactosidase gene (CMV-β-gal), and 2 days later the cells were fixed and stained with X-gal as described. Examination by microscopy of the stained cells revealed that approximately 5–10% of the cells turned blue, as shown in Fig. 1A, indicating efficient transfer of the DNA into the cells. Control cells that received Lipofectin and pGEM4 (a DNA construct which did not contain a β-gal gene) did not have a single blue cell, as shown in Fig. 1B.

To demonstrate that efficient transfection could be achieved for hepatocytes from other species, CMV-β-gal was transfected into primary dog hepatocytes and the cells stained with X-gal. Figure 1C demonstrates that 10% of dog hepatocytes can be efficiently transfected, while control cells were again negative (Fig. 1D). One important prerequisite for a transfection method to be used successfully for gene transfer is that the method be nontoxic for hepatocytes. Figure 1E demonstrates that the morphology of hepatocytes 14 days after the completion of the lipofection procedure is identical to that of the nonlipofected control cells shown in Fig. 1F.

Optimization of lipofection conditions

Although lipofection has been successfully used to transfer DNA into primary hepatocytes (Jacob et al., 1989), there are no prior reports of optimal conditions, transfection efficiency, or toxicity. These parameters are important to consider, as the ratio of Lipofectin : DNA varies for different cell lines, as does the amount of Lipofectin that can be added to a given number of cells (Feldner et al., 1987). Figure 2 shows the effect of varying the total amount of DNA added while keeping the amount of Lipofectin constant at 20 μg or 50 μg. When 20 μg of Lipofectin and varying amounts of either CMV-β-gal or AAT-β-gal was added, 3 μg of DNA clearly gave maximal expression. A similar concentration curve was obtained using a CMV–CAT construct (data not shown). Of note is the remarkable inhibition in expression when 5 μg or more of DNA was added with only 20 μg of Lipofectin. When 50 μg of Lipofectin was added with varying amounts of CMV-β-gal DNA, the maximal expression occurred when 6 μg was added. Thus, a mass ratio of Lipofectin : DNA of 6.67 : 1 is optimal. Although adding more Lipofectin : DNA complex increased the level of expression, the larger amount of Lipofectin resulted in toxicity morphologically visible at 3 days after transfection; adding 20 μg of Lipofectin to 1.5 × 10⁶ cells consistently gave hepatocytes that appeared normal in appearance and could be cultured for at least 2 weeks in culture without any apparent toxic effects, and was used for the remainder of the experiments.

Liver-specific gene expression and hormonal responsiveness in transfected hepatocytes

Some early studies suggested that liver-specific transcription is inhibited in hepatocytes in culture (Clayton and Darnell, 1983), although more recently it has become clear that this apparent inhibition was due to extreme sensitivity of primary hepatocytes to components of the culture medium such as fetal calf serum (Enat et al., 1984; Jefferson et al., 1984; Friedman et al., 1986; Guillouzo, 1986; Reid et al., 1986). Thus, to validate any results regarding relative promoter efficiency, it is imperative to demonstrate that our culture conditions allow
FIG. 1. X-gal staining of lipofected hepatocytes. A. Primary rat hepatocytes were lipofected with CMV-β-gal DNA, and 2 days after completion of the lipofection procedure were stained with X-gal for 6 hr as described in the Materials and Methods. B. Control rat cells lipofected with pgEM4 DNA (which does not contain a β-gal gene) were stained as in A. C. Primary dog hepatocytes were lipofected with CMV-β-gal DNA and stained with X-gal as in A. D. Primary dog hepatocytes were lipofected with pgEM4 and stained with X-gal as in A. E. Primary dog hepatocytes were lipofected with CMV-β-gal DNA and maintained in SUM medium for 14 days, and photographed without X-gal staining. F. Nonlipofected dog hepatocytes were maintained in SUM medium for 14 days and photographed without X-gal staining. All magnifications, 100×.

liver-specific transcription, and to show that newly introduced liver-specific promoters can be expressed.

We first addressed whether or not our culture conditions, which differ considerably from those used by Enat et al. (1984), could support transcription of liver-specific genes. Figure 3 shows the results of a runon transcription assay. Transcriptional rates are demonstrated for hepatocytes that were freshly isolated (lane 1), plated for 30 hr in FCS (lane 2), or plated for 30 hr in
FIG. 2. Effect of DNA and Lipofectin concentration on efficiency of lipofection. Primary rat hepatocytes in 6-cm plates were lipofected with either 20 or 50 μg of Lipofectin, as described in the Materials and Methods section. Some plates received 20 μg of Lipofectin and 1–6 μg of CMV–β-gal DNA (open squares) or AAT–β-gal DNA (diamonds) in 20 μl of water; other plates received 50 μg of Lipofectin mixed with 2–10 μg of CMV–β-gal DNA (closed squares) in 50 μl of water. Cell extracts of each sample were prepared, then 20 μg of protein tested in an ONPG–β-gal enzymatic assay and specific activity determined.

SUM (lane 3). After normalization to the β-actin signal, the endogenous albumin and AAT promoters were transcribed 17% and 88%, respectively, as efficiently in cells which had been plated in SUM, as compared with freshly isolated hepatocytes. In contrast, liver-specific transcription was only 9% (albumin) and 21% (AAT) as high when the cells were grown in FCS, as compared with freshly isolated hepatocytes. Thus, liver-specific transcription is qualitatively maintained under our conditions, albeit at levels somewhat less than occurs in vivo. A decrease in rates of liver-specific transcription has been observed in all culture systems of primary hepatocytes (Reid et al., 1986).

We next determined if liver-specific promoters directing expression of a CAT gene could be expressed after lipofection into primary rat hepatocytes. Three constructs containing liver-specific promoters are depicted in Fig. 4A: (i) a 503-nucleotide human albumin promoter (contains nucleotides −486 to +17 relative to the transcriptional start site), which we find to direct transcription in two hepatoma cell lines (Hep3B2 and HepG2), but not nonhepatic cell lines (EJ and HeLa) (D. Wilson and G. Darlington, unpublished data); Fain et al. (1990) have also recently demonstrated expression from a similar construct in hepatoma cells. (ii) A 563-nucleotide fragment of the PEPCCK promoter (contains nucleotides −490 to +73) generously provided by J.S.Liu and R. Hanson. This promoter exhibits an appropriate inhibition by insulin, and stimulation by cAMP and glucocorticoids in hepatoma cells and livers of transgenic mice (Hue and Girard, 1986; Short et al., 1986; Wynshaw-Boris et al., 1986; McGrane et al., 1988). (iii) A 778-nucleotide fragment of the human AAT promoter (containing nucleotides −732 to +46), which was previously shown to be expressed in hepatoma cells and livers of transgenic mice (Ciliberto et al., 1985; Shen et al., 1987, 1989).

Figure 4B demonstrates expression from these liver promoters and an RSV control, and the response of each to cAMP stimulation. The 486-nucleotide human albumin promoter (lane 3) and the 732-nucleotide AAT promoter (lane 7) were expressed in primary rat hepatocytes, achieving an average level of expression 89% and 25%, respectively, that of RSV-CAT (lane 1). Expression from both ALB-CAT (lane 4) and RSV-CAT (lane 2) showed a slight stimulation upon the addition of dibutyryl cAMP, while the AAT promoter was slightly less active. In contrast, the PEPCCK promoter had almost no detectable transcriptional activity in the basal state but was stimulated on average eight-fold by addition of dibutyryl cAMP (lanes 5 and 6). This demonstrates that appropriate hormonal responsiveness was maintained for this liver-specific promoter.

Comparison of cellular and viral promoters in primary hepatocytes

We compared the transcriptional strength of a variety of promoters in primary hepatocytes. For the following reason, a single reporter gene was transfected into cells without a second construct used for normalization purposes. In initial studies, a series of constructs in which different promoters directed expression of one reporter gene (e.g., CAT) were co-transfected into hepatocytes along with a second construct in which one promoter directed expression of a second reporter gene (e.g., CMV–β-gal or SV40–β-gal). We found that different control constructs (e.g., β-gal) could dramatically affect the expression the first gene, which suggested to us that competition for transcription factors might be occurring. Therefore, we chose to examine the transcriptional activity of a single construct. These results are only valid if transfection efficiency is similar for all DNA constructs. For three reasons, we feel that this was the case: (i) Duplicate samples in a single experiment usually varied by less than 20%; (ii) In all cases, each construct was tested at least three times in duplicate, with the same relative order of transcriptional activity obtained, although the absolute activity
FIG. 4. Expression of liver promoters introduced into rat hepatocytes by lipofection. A. Schematic representation of liver-specific promoters driving a CAT gene are shown. The promoter is indicated by the open box; numbers at the edge of the open box refer to the position relative to the transcription start site for the endogenous gene directed by that promoter, with (−) indicating distance upstream, and (+) indicating distance downstream. The CAT gene is indicated by a stippled box. PEPCK-CAT contains a rat PEPCK promoter and was generously provided by J.S. Liu and R. Hanson (unpublished data); 486-Alb-CAT is derived from the human albumin promoter (see Materials and Methods); and 732-AAT-CAT is derived from the natural human α1-antitrypsin promoter, and was generously provided by R.F. Shen. B. Expression of liver promoters in primary rat hepatocytes. Rat hepatocytes were changed into SUM medium 4 hr after isolation and lipofected with 3 µg of RSV-CAT (lanes 1 and 2), 486-Alb-CAT (lanes 3 and 4), AAT-CAT (lanes 5 and 6), PEPCK-CAT (lanes 7 and 8), or pGEM4 (Cont, lanes 9 and 10) and 20 µg of Lipofectin per plate. After 16 hr, cells were rinsed twice with PBS and changed into regular SUM (lanes 1, 3, 5, 7, and 9) or into SUM containing all other hormones except insulin (lanes 2, 4, 6, 8, and 10). The cells that received the medium lacking insulin were stimulated with 0.5 mM dibutyryl cAMP and 1 mM theophylline for 2 hr, then the medium was replaced with regular SUM. Stimulation was repeated 20 hr later, then cell extracts prepared after an additional 22 hr. A total of 25 µg of protein was used in a 60-min CAT assay as described in Materials and Methods and acetylated chloramphenicol (3 AcCM and 1 AcCM) separated from nonacetylated (CM) on a TLC plate, then visualized by autoradiography. Lanes 11–13 show samples containing 0, 25, or 100 mUnits of purified CAT enzyme. Quantitation was done for two assays performed in duplicate.

(as determined by calculating the units of enzyme per mg of total protein in the extract) varied as much as fourfold from one experiment to another; (iii) Experiments in which a one-half × amount, or a 2× amount of DNA was added gave the same relative results; and (iv) Similar results were observed when either a CAT or a β-gal reporter gene was used with the same promoter.

Figure 5 shows the transcriptional activity of viral and cellular promoters directing CAT expression. Figure 6 shows the average relative CAT activity of the viral and nonspecific cellular promoters depicted in Fig. 5, as well as the average activity of the liver-specific promoters depicted in Fig. 4. Figure 7 shows the average transcriptional activity of promoters controlling a β-gal gene. The specific CAT activity after lipofection with the CMV–CAT construct ranged from 0.4 to 2.0 U/mg, while the β-gal activity of an extract prepared from cells lipofected with the CMV–β-gal construct ranged from 4 to 20 U/mg in repeat assays.

The CMV, SRα, and β-actin promoters were the strongest of those tested for expression in primary hepatocytes. The mouse β-actin promoter achieves a level of expression 33% that of the CMV promoter. The SV40 and RSV-LTR promoters were also well expressed, achieving levels 7.5% and 12%, respectively, that of CMV using a CAT reporter gene, and levels 18% and 19%, respectively, that of CMV using a β-gal reporter gene. RNA Pol II large subunit, albumin, AAT, and PEPCK promoters were intermediate. The 710-nucleotide fragment (HB-I) and a 1,020-nucleotide fragment (HB-II) of the promoter directing transcription of the large subunit of RNA polymerase II resulted in CAT activities of 6.3% and 5.3%, respectively, that of the CMV promoter. The 486-nucleotide albumin promoter was 10.7%, the 732-nucleotide AAT promoter was 3%, and the 490-nucleotide PEPCK promoter (under stimulated conditions) was 15% that of the CMV promoter. For a 1,200-nucleotide AAT promoter directing transcription of the β-gal reporter gene, the specific activity was 18% that of the CMV promoter. Expression from the TK promoter was barely detectable, even when the polynoma enhancer (MCI; Thomas and Capecci, 1987) was placed upstream; β-gal activity was 0.2% and 0.45%, respectively, that obtained using the CMV promoter.
FIG. 5. Expression of viral and cellular promoters driving a CAT reporter gene. A. CAT constructs tested. pSV2-CAT contains the 400-nucleotide fragment of the SV40 early promoter region (Gorman et al., 1982a); RSV-CAT contains the 524-nucleotide fragment of the RSV-LTR promoter and enhancer (Gorman et al., 1982b); CMV-CAT contains the 619-nucleotide fragment of the human CMV immediate early promoter and enhancer (Foeging and Hofstetter, 1986); β-actin–CAT contains a 3-kb fragment of the mouse β-actin promoter, as well as the first exon (Elder et al., 1988); SRα is the construct designated pSRα-CAT196, which contains the 667-nucleotide SRα promoter (Takebe et al., 1988); HB-I-CAT and HB-II-CAT contain 710 and 1020 nucleotides respectively, of the mouse RNA polymerase II promoter (Ahearn et al., 1987) and were obtained in constructs driving CAT from M.L. West and J. Corden (unpublished data). B. Expression in primary rat hepatocytes. Primary rat hepatocytes were lipofected with 0.45 pmols of the constructs described in A, and an amount of pGEM3 to bring the total DNA to 3 μg and cell extracts prepared 2 days after completion of the lipofection procedure. A total of 150 μg of protein was tested in a 90-min CAT assay and reaction products were separated on a TLC plate and autoradiographed. Lane 1 shows the activity obtained with 25 mUnits of purified CAT enzyme. Lane 2 shows the CAT activity present in cells that received pGEM3 only.

**DISCUSSION**

A highly efficient method for transfecting DNA into primary hepatocytes is described in detail. Our results agree with those of Jacoby et al. (1989) in that a ratio of lipofection : DNA of 6.67 : 1 is optimal. However, we found that the larger amounts of Lipofectin (and DNA) utilized in their studies had some toxicity for the hepatocytes, which became morphologically evident after 3–4 days in culture. Under our conditions, long-term (2-week) survival in culture appears to be morphologically identical to that of controls which were not lipofected. Although 5–10% of primary hepatocytes appear to be transfectioned as assessed by X-gal staining at 48 hr after tranfection, this expression is transient, as the levels of the protein expressed from the transfected plasmid rapidly decreased after 60 hr post-transfection, to a level approximately 5% of the initial activity after 10–14 days in culture (K.P.P., unpublished data). This decline is associated with a decrease in the amount of nuclear DNA (K.P.P., unpublished data), a phenomenon that has been observed for most if not all transient expression assays.

We further extend previous studies by demonstrating that constructs in which albumin, AAT, or PEPCK promoters direct transcription of a CAT gene can all be expressed after lipofection into primary hepatocytes, and that the PEPCK promoter exhibits appropriate hormonal control. This indicates that liver-specific transcription is not disrupted by this method.

Our major interest in pursuing this method of high-efficiency transfer of DNA into primary hepatocytes was to enable us to compare directly a variety of promoters to determine which were the strongest. This is important for those interested in gene therapy in hepatocytes, as it is essential for most scenarios that
FIG. 6. Comparison of average CAT activity of liver-specific, viral, and nonspecific cellular promoters. Average transcriptional activities for promoters directing expression of a CAT gene relative to the activity observed for the CMV-CAT construct are shown. These data were determined from averaging the results of at least two experiments with duplicate samples (see Figs. 4B and 5B). The activity for the PEPCK promoter is that which is observed during stimulation with cAMP; the others represent the activity of cells grown in SUM medium.

That the RSV-LTR and SV40 promoters were also well expressed, while the TK promoter was virtually inactive even when the polyoma enhancer was added. These results are in good agreement with those of Wilson et al. (1988b), who determined that a Moloney leukemia virus LTR promoter was at least 70-fold stronger than a TK promoter when both were placed within the context of a retrovirus vector. Our results contradict those of Ledley et al. (1987), who claimed that a TK promoter was active while LTR and SV40 promoters were inactive in primary mouse hepatocytes.

In transgenic mice, viral promoters exhibit tissue specificities that are different from that observed in primary hepatocytes. For example, a CMV enhancer/TK promoter construct (Schmidt et al., 1990), an SV40 enhancer/promoter construct (Palmiter and Brinster, 1986), and an RSV enhancer/promoter construct (Overbeck et al., 1986; Swain et al., 1987) are all inactive in livers of adult transgenic mice. Although in the first case the inactivity might be attributed to the fact that the degree of transcriptional activation by an enhancer is dependent upon the promoter used (Berg et al., 1984), and the TK promoter is known to be virtually inactive in primary hepatocytes, the SV40 and RSV constructs used in the transgenic mice were similar to those used in our study. It is possible that viral promoters might be subject to inactivation during development. For example, although the above-mentioned viral-derived promoters are not expressed in transgenic mice if introduced by injecting into one-cell embryos, the LTR promoter from Moloney leukemia virus is very well expressed in liver cells of adult mice if introduced on the eighth day of embryogenesis (Jaenisch, 1980). Thus, inactivation of viral promoters may occur during the early stages of development and not be operative for adult cells. Alternatively, differentiated cells may also have mechanisms for inactivation of viral genes; Xu et al. (1989) recently reported that loss of proviral gene expression in fibroblasts can occur via a reversible epigenetic mechanism. Further experiments will need to be done to determine if in fact mechanisms exist for inactivation of viral derived promoters in adult hepatocytes.

Cellular promoters

Use of cellular promoters should obviate the possibility of promoter inactivation that occurs with viral promoters. Two promoters which are expressed in all cells were tested for their relative expression in hepatocytes. A mouse β-actin promoter was well expressed, while an RNA polymerase II gene promoter was less active. Although Wilson et al. (1988b) found that a chicken β-actin promoter was expressed only 14% as well as the LTR promoter, their promoter fragment was only 267 bp whereas ours was 3 kb. Species specificity or the inclusion of more upstream sequences may have led to the relative increase in expression in our experiments.

Cellular promoters that exhibit specificity for expression in hepatocytes have a further advantage over nonspecific promoters. Use of such a promoter would guarantee that any expression observed in vivo did in fact derive from expression in liver cells and was not due to transfer into some other cell type. Three liver-specific promoters were tested for expression in primary hepatocytes. The maximal expression of each was considerably less than that of the most active viral promoters. One caveat to these results was the fact that the transcriptional rate of the
FIG. 7. Expression of viral and liver promoters driving a β-galactosidase reporter gene. A. β-gal constructs tested. CMV-β-gal contains the 619-nucleotide human CMV intermediate early enhancer and promoter (MacGregor et al., 1987); SV40-β-gal contains a 525-nucleotide fragment containing the SV40 origin of replication, enhancer, and early promoter region (MacGregor and Caskey, 1989); MCI-β-gal (generously provided by P. Soriano, unpublished data) contains the MCI promoter (Thomas and Capocchi, 1987), which is a 382-nucleotide DNA fragment with the polyoma enhancer and the TK promoter; TK-β-gal (MacGregor and Caskey, 1989) contains a 825-nucleotide fragment of the herpes simplex virus thymidine kinase promoter; RSV-β-gal (MacGregor et al., 1987) contains a 524-nucleotide fragment of the Rous sarcoma virus (RSV)-LTR enhancer and promoter; and AAT-β-gal contains a 1,200-nucleotide fragment of the human AAT promoter constructed as described in the Materials and Methods section. B. Expression in primary rat hepatocytes. Primary rat hepatocytes were lipofected with 0.45 pmoles of the constructs described in A. and an amount of pGEM3 to bring the total DNA to 3 μg. Two days after completion of the lipofection procedure, cell extracts were prepared and 25 μg analyzed in an ONPG assay. The average activity relative to CMV-β-gal is shown, with error bars indicating the standard error of the mean.

endogenous albumin promoter in hepatocytes grown in our in vitro culture medium was only 17% that of freshly isolated hepatocytes, as determined by a runon transcription assay, a phenomenon that has been observed in other culture systems (Reid et al., 1986). However, studies in transgenic mice confirm our conclusion that relatively short albumin promoter constructs are not highly active in hepatocytes. A 300-nucleotide mouse albumin promoter directing transcription of a bovine GH (bGH) gene led to low levels of bGH mRNA, although expression remained liver specific (Pinkert et al., 1987). Although it is likely that inclusion of the albumen enhancer would increase expression in primary hepatocytes, as occurs in transgenic mice (Pinkert et al., 1987), we chose not to test it because its size would considerably increase the total size of the promoter/enhancer to over 2 kb, making it more difficult to be accommodated in a retrovirus.

The fact that the runon transcription rate of the endogenous AAT gene in plated hepatocytes was maintained at 88% that of freshly isolated hepatocytes suggests that the AAT promoter is probably not dramatically more active in vitro than was observed in vivo. Indeed, studies in transgenic mice support this conclusion. When the same or similar AAT promoters were used to direct β-gal (K. Ponder, unpublished results) or CAT (Shen et al., 1989) expression in transgenic mice, liver extracts had low specific activities for the appropriate enzyme, as compared with the specific activities obtained after transfecting a CMV-CAT or CMV-β-gal construct into the primary hepatocytes. Thus, these studies suggest that the AAT promoter is not significantly more active in vitro than was observed in vivo. Although there is one report that a retroviral construct containing a 1.2-kb AAT-SV40 hybrid promoter (contains the SV40 transcriptional start site) was well expressed in primary hepatocytes (Peng et al., 1988), there was no quantitative data regarding mRNA or protein levels in these experiments. Subsequent studies in which a natural 1.2-kb AAT promoter (contains the AAT transcriptional start site) directed transcription of a cDNA for the human AAT protein from an internal site in a retrovirus vector resulted in very low levels of expression (D. Armentano and K. Ponder, unpublished data). Furthermore, transfection of constructs in which the 1.2- or 0.7-kb AAT- SV40 hybrid promoter (Shen et al., 1987) directed transcription of CAT resulted in lower CAT activity in primary hepatocytes than was seen with the natural AAT constructs (data not shown).

Of the short liver-specific promoters tested, the PEPCK promoter had the highest specific activity (when stimulated with cAMP): When tested in primary hepatocytes in vitro its transcriptional activity was 15% that of the CMV promoter. A similar construct resulted in high mRNA levels for bGH in livers of some lines of transgenic mice, and serum bGH protein levels of over 1 μg/ml, although a direct comparison with our in vitro results cannot be made. Furthermore, the promoter could be stimulated appropriately by feeding the transgenic mice a high protein diet.

Implications for gene therapy

These results demonstrate that the CMV and SRα promoters are expressed more efficiently in cultured primary hepatocytes than any of the other promoters tested. Of nonspecific cellular promoters tested, a 3-kb β-actin promoter exhibited the highest
level of transcription, although these transcriptional rates may be overestimated if newly plated hepatocytes increase transcription of the β-actin promoter. Furthermore, the large size of the β-actin construct used here (3 kb) may decrease its usefulness for expressing large cDNAs from within a retrovirus vector. Of the fairly short liver-specific promoters tested here, the PEPCK promoter had the highest transcriptional activity, achieving levels 15% (with cAMP stimulation) that of the CMV promoter. Although the liver promoters are less active than the strongest viral promoters, they have the advantage that they can be used to prove that in vivo expression derives from primary hepatocytes, and they may prove to maintain expression longer if mechanisms should exist for inactivation of viral promoters over time in adult hepatocytes. It remains to be proven that data regarding promoter strength of extrachromosomal plasmids will operate after integration of genes into chromosomal DNA. Yet, when these data regarding relative transcriptional activities were incorporated into the construction of a retroviral vector, it was found that a CMV promoter directing expression of a Factor IX gene was well expressed in primary hepatocytes, as predicted (Armentano et al., 1990).

The second major conclusion of this study is that 5–10% of primary hepatocytes can be lipofected using conditions that do not disrupt liver specific functions and that allow survival of cells for at least 2 weeks. This method will permit studies to be performed which analyze epismal vectors for their ability to function in primary hepatocytes. Lipofection may be of sufficient efficiency to transfer genes into hepatocytes for purposes of gene therapy.

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