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DNA-Mediated Gene Transfer into Adult Rat Hepatocytes in Primary Culture

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Proliferation-competent and differentiation-competent adult rat hepatocytes in primary culture were investigated for their ability to express reporter genes (firefly luciferase, bacterial chloramphenicol acetyltransferase, and bacterial β-galactosidase) driven by tumor virus or eucaryotic promoters that vary in transcriptional efficiency and tissue specificity. Supercoiled plasmid DNA molecules were introduced into the cells by the calcium phosphate coprecipitation protocol of C. Chen and H. Okayama (Mol. Cell. Biol. 7:2745–2752, 1987). Reporter gene expression was virtually restricted to hepatocytes and was efficient (2 to 20% of the cells). The patterns and absolute levels of reporter gene expression depended on assay conditions employed (plasmid concentration [optimal at 2.4 μg of DNA per ml] and duration of exposure [optimal between 5 and 10 h]), culture growth cycle stages (lag, log, or stationary phase), properties and tissue specificity of the promoter(s) tested, and composition (and timing of fluid change) of the culture medium with or without the hepatocyte mitogen human transforming growth factor-α. Initial observations suggest that during hepatocellular growth transitions, human transforming growth factor-α differentially regulates exogenously introduced promoters associated with hepatocyte-specific function and proliferation. These findings provide a simple, fast, and powerful approach to analyzing the molecular and cellular biology of hepatocyte growth control.

Genetic mechanisms controlling hepatic proliferation and differentiation have come under intensive analysis recently (7, 11). Primary hepatocyte cultures (30, 32) in chemically defined media (1, 3, 19, 20, 22, 26) have played increasingly prominent roles in such experimental investigations (for a review, see reference 27). These cultures have been limited for molecular genetic analyses, since the ability to genetically manipulate normal hepatocytes has been restricted either to indirect (somatic cell hybridization [43]), inefficient and transient (electroporation-mediated [46]), or laborious (but stable and efficient) gene transfer procedures (transduction by retroviral vectors [25, 47, 48]). Partially differentiated hepatoma cell lines (7, 11, 18), though not ideally suited for studies of normal hepatic growth control, have been used widely to fill this molecular genetic gap, since they have generally responded to standard procedures for eucaryotic DNA-mediated gene transfer by calcium phosphate coprecipitation (15). However, it is doubtful that the hepatoma cell lines utilized in such gene transfer experiments reflect normal hepatocyte gene expression, particularly since the genetic background of such recipient cells is abnormal.

A simple system for efficient and regulatable gene transfer into normal hepatocytes, especially under defined conditions, would provide a model for studies of normal hepatocyte gene expression and comparative studies of hepatic gene expression in malignant cell lines. By employing the modified calcium phosphate coprecipitation procedure of Chen and Okayama (6), we provide evidence in this report for such a system.

MATERIALS AND METHODS

Preparation and transformation of primary hepatocyte cultures. Hepatocytes from adult Fisher/344 male rats (180 to 200 g) were isolated, plated (10^6 cells per 2 ml per 35-mm dish) and cultured (ornithine-supplemented, arginine-free medium) exactly as previously described (3, 29). For cultures subjected to calcium phosphate coprecipitation, at various times after being plated (see legends to figures), the media were aspirated and the dishes were shifted (without washing) into 1 ml of transformation medium (standard Dulbecco and Vogt modified Eagle medium, porcine monocomponent insulin [5 μg/ml], hydrocortisone-hemisuccinate [50 ng/ml; Sigma Chemical Co., St. Louis, Mo.] and 4% [vol/vol] heat-inactivated [56°C] dialyzed fetal bovine serum [29]) preincubated at 37°C. DNA-mediated gene transfer was then performed as described by Chen and Okayama (6). For each culture plate to be transformed, 2.4 μg of supercoiled plasmid DNA, dissolved in sterile deionized water, was mixed with 50 μl of 250 mM $CaCl_2$ and 50 μl of 2× *N*-,*N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid (6). This solution was incubated in Falcon plastic tissue culture dishes (no. 3001) for 15 min at 21°C. One hundred microliters of the solution was then added to each culture dish, and the cells were returned to a 37°C incubator containing 97% (vol/vol) air and 3% (vol/vol) CO_2. After 5 h, the media were aspirated and each dish received 2 ml of fresh plating medium (3, 29), unless noted otherwise (see Fig. 4). From this time (zero) through 144 h, replicate dishes were sampled in the different plasmid groups (see Table 1) for standard assays of reporter genes. For primary cultures subjected to lipofection-mediated gene transfer (see Table 3), cationic liposomes were prepared according to the method of Felgner et al. (10). Ten microliters of *N*-[1(2,3-dioleyloxy)propyl-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was added to 10 μl of deionized water containing 4 μg of supercoiled plasmid DNA. At the initial times of transformation, the cultures were shifted into 1 ml of fresh OPTIMEM medium (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) (10); the above 20 μl of DOTMA-DNA solution was added to the culture medium, and the dishes were returned to the standard humidified incubators (90% [vol/vol] air, 10% [vol/

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TABLE 1. Plasmid, promoter, and reporter gene constructs

Plasmid	Promoter[a]	Reporter gene	Source	Reference
$p\alpha_1I3$	α_1I3 (−2214 to +58)	Firefly luciferase	G. Fey	2, 38
pSV2AL	SV40 early plus enhancer	Firefly luciferase	S. Subramani	9
pRSV-CAT	RSV-5'LTR	*E. coli* CAT	B. Howard	13
pCH126	Promoterless	*E. coli* β-galactosidase	F. Lee	16
pCOLβ-GAL	Collagen α1(I) plus 5' regulatory region	*E. coli* β-galactosidase	D. Brenner	Brenner et al., in press
pRSVβ-GAL	RSV-5'LTR	*E. coli* β-galactosidase	D. Brenner	—[b]
pFOS-CAT	Human c-*FOS*	*E. coli* CAT	M. Karin	41

[a] SV40, Simian virus 40; RSV-5'LTR, Rous sarcoma virus 5' long terminal repeat.
[b] —, See Materials and Methods.

vol] CO_2) for 18 h at 37°C. Reporter gene activities in these cultures were determined as described below.

Preparation, transformation, and culture of cell lines. Human AF2 fibroblasts (passages 7 to 16; 4) and HepG2 cells (18) were cultured in Dulbecco and Vogt modified Eagle medium containing 10% fetal bovine serum under an atmosphere of 95% (vol/vol) air and 5% (vol/vol) CO_2 (4). The cells were plated into plastic tissue culture dishes (10 cm in diameter) (Falcon; no. 3003) at a concentration of 9×10^5 cells per ml. DNA-mediated transformation was performed by the calcium phosphate coprecipitation protocol as described above.

Reporter gene expression assays. Standard procedures were used to quantify firefly luciferase (EC 1.13.12.7) (9; expressed as light units per 10 s per mg of protein) at 21°C, with a Monolight 2001 Luminometer (Analytical Luminescence Labs, La Jolla, Calif.) or chloramphenicol acetyl aminotransferase (CAT) (EC 2.3.1.28) (14; expressed as picomoles per minute per milligram of protein), by quantifying acetylated species in thin-layer chromatography plates scanned with an Ambis Computerized Radioanalyzer (Ambis, San Diego, Calif.). Culture extracts were prepared by washing the dishes three times with 2 ml of Tris-buffered saline (pH 7.4) (29); either 1 ml (luciferase) or 0.5 ml (CAT) of lysis buffer (0.5% [vol/vol] Triton X-100, 100 mM $KHPO_4$ [pH 7.8], 1 mM dithiothreitol, in triple-distilled water) was then added to each dish. After 10 min at room temperature, the extracts were centrifuged for 10 min at 21°C with an Eppendorf Microfuge (model no. 5414). The supernatants were collected and frozen at −20°C until enzyme and protein assays (33) were performed. Parallel mock transformations (minus plasmid DNA) were made; reporter gene activity levels in these extracts never exceeded 50 to 100 light units (for luciferase) or 0.2 pmols/min per mg (for CAT). The background values were subtracted from experimental and control determinations. Measurement errors between replicate samples were from 5 to 10%.

For CAT assays, the cell extracts were diluted in deionized H_2O and made 162 mM Tris (pH 7.8) in a final volume of 129 μl. The extract solution was heated at 65°C for 10 min, after which 1.0 μl of [^{14}C]chloramphenicol (0.025 μCi/μl; 57 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) and 20 μl of a 4 mM acetyl coenzyme A (Pharmacia Fine Chemicals, Piscataway, N.J.) solution were added. At incubation times of 1 and 2 h, 10 μl of 4 mM acetyl coenzyme A was added. After a total of 3 h of incubation at 37°C, the reaction was stopped and the mixture was extracted with 1.0 ml of ethyl acetate (EM Science, Cherry Hill, N.J.). The ethyl acetate was collected and dried overnight in a fume hood. The dried sample was suspended in 20 μl of ethyl acetate, spotted onto a thin-layer chromatography plate (Kodak 13179 silica gel without fluorescent indicator; Eastman Kodak Co., Rochester, N.Y.), and subjected to ascending chromatography in chloroform-methanol (95:5). The plates were air dried and radioanalyzed as described above.

For detection of β-galactosidase-stained cells (EC 3.2.1.23), transformed cultures were washed three times in Tris-buffered saline, fixed 10 min at 21°C in 1% (vol/vol) glutaraldehyde in phosphate-buffered saline (pH 7.4), and then stained at 37°C overnight with 5-bromo, 4-chloro, 3-indolyl-beta-D-galactopuranoside (40). The cultures were washed twice with phosphate-buffered saline (pH 7.4) and examined microscopically.

All experiments were repeated 2 to 6 times. Qualitative trends were identical among different treatment groups intraexperimentally; however, the absolute levels in reporter gene expression varied interexperimentally from 20 to 100%.

Plasmid isolation, purification, and characterization. Table 1 lists plasmids used in this study. Standard procedures were used to grow, isolate, and purify each plasmid by ultracentrifugation through CsCl gradients (35). The plasmid pCOLβ-GAL was constructed by cloning the −2500- to +118-base-pair segment of the murine collagen α_1(I) gene into the unique *Hin*dIII site of the plasmid pCH126; consequently, the β-galactosidase gene is driven by the collagen α_1(I) promoter and 5' regulatory region. The plasmid pRSVβ-GAL was constructed by cloning the *Bam*HI-*Hin*dIII fragment of the plasmid pRSV-cJUN containing the Rous sarcoma virus 5' long terminal repeat into the *Bam*HI-*Hin*dIII fragment of pCH126 containing the *Escherichia coli* β-galactosidase gene; consequently, the reporter gene of pRSVβ-GAL is under the transcriptional control of the Rous sarcoma virus 5' long terminal repeat.

RESULTS

Figure 1 shows the results of initial studies, in which the calcium phosphate coprecipitation protocol for DNA-mediated gene transfer was optimized for primary cultures of adult rat hepatocytes. When 2-day-old cultures (Fig. 1A) were exposed to supercoiled DNA derived from three different plasmids (Table 1), reporter gene expression was detected within 6 (for luciferase) to 18 h (for CAT), peaked within 14 (pSV2ALΔ'5, hereafter called pSV2AL) to 48 h ($p\alpha_1I3$ and pRSVCAT), and declined to 20% of peak levels 144 h later. Although we did not determine whether an optimal cell density per plate might alter these kinetics, when the number of cells plated was reduced 4-fold (2.5×10^5 cells per dish), resulting in a lowered number of cells per culture on day 2 (~80,000), reporter gene expression declined 10-fold (data not shown). Therefore, since conditions of gene transfer employed standard plating conditions (3, 20, 22), standard plating conditions were used in subsequent studies (Fig. 1A).

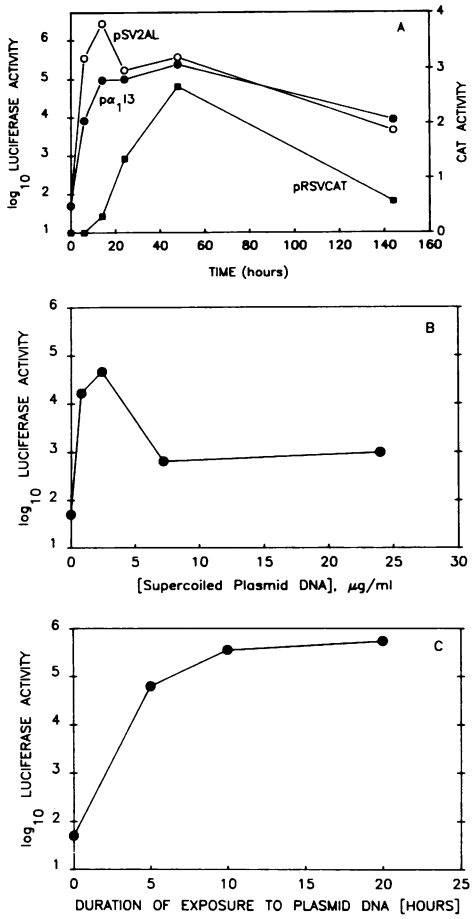

FIG. 1. Experimental variables affecting plasmid reporter gene expression in primary cultures of adult rat hepatocytes. (A) Time courses of expression of luciferase or CAT. Cultures were prepared, transformed by calcium phosphate coprecipitation, and assayed for reporter gene expression with the standard conditions as described in Materials and Methods and Results. The three plasmids are described in Table 1 (2.4 μg of plasmid DNA per ml was used for transformation in each case). Symbols: ○, pSV2AL; ●, $p\alpha_1I3$; and ■, pRSVCAT. (B) DNA-mediated gene transfer: dependence on concentration of supercoiled plasmid DNA. Cultures were prepared and exposed to varying concentrations of plasmid $p\alpha_1I3$ DNA (2 days postplating) as described for panel A. Luciferase assays were performed on cell extracts obtained 48 h later, as described in Materials and Methods. (C) DNA-mediated gene transfer: dependence on time of exposure to plasmid DNA. Cultures were prepared and exposed to plasmid $p\alpha_1I3$ DNA (2 days postplating) as described for panel A, for varying times as indicated on the x axis. Luciferase assays were performed on cell extracts obtained from cultures at 96 h postplating, as described in Materials and Methods.

Since levels of luciferase reporter gene expression driven by the hepatocyte-specific promoter of α_1-inhibitor III ($p\alpha_1I3$; L. J. Abraham, A. D. Bradshaw, B. R. Shiels, G. H. Fey, Mol. Cell. Biol., in press) appeared nearly equal to the levels of expression driven by a non-tissue-specific strong promoter of the simian virus 40 early genes (pSV2AL; Fig. 1A), $p\alpha_1I3$ was used in further validation studies of DNA-mediated gene transfer. Concentrations of 2.4 μg of plasmid DNA per ml of transformation medium (Fig. 1B) and exposure times of between 5 and 10 h were optimal (Fig. 1C) for luciferase reporter gene expression measured 48 h after transformation.

TABLE 2. Reporter gene expression in cultured human fibroblasts and hepatoma cell lines transformed with plasmid DNA

Cell line or strain	Plasmid (reporter gene)	Enzyme activity[a] after infection for:		
		0 h	24 h	48 h
AF2 fibroblasts	pSV2AL (luciferase)	50	136,564	16,963
	$p\alpha_1I3$ (luciferase)	50	1,360	552
HepG2 hepatoma	pSV2AL (luciferase)	50	197,275	593
	$p\alpha_1I3$ (luciferase)	50	1,902	1,476

[a] Enzyme activity units for luciferase and CAT are defined in Materials and Methods.

Cytotoxicity, measured by quantification of numbers of cells per dish or total cellular proteins per dish (5, 30), was not observed under most conditions described above. Slight reductions (10 to 15%) in cellular proteins per dish were seen when plasmid concentrations exceeded 7 μg of DNA per ml of transformation medium (Fig. 1B) or when exposure times were prolonged to 20 h (Fig. 1C). For these reasons, standard conditions of one 5-h exposure to 2.4 μg of plasmid DNA per ml were chosen for subsequent studies.

Characterization of the $p\alpha_1I3$ promoter and its tissue-specific mode of expression were reported recently (Abraham et al., in press), with rat hepatic RNA extracts, human Hep3B (18), and rat FAZA (34) hepatoma cell lines. Results complement these prior findings in several ways (Table 2). First, with the −2214 to +58 $p\alpha_1I3$ construct, the levels of luciferase expression in primary hepatocyte cultures (Fig. 1) were 30- to 100-fold higher than the levels of luciferase expression obtained with human AF2 fibroblasts (Table 2). In contrast, both culture systems displayed high levels of luciferase activity driven by a promiscuous promoter (simian virus 40) (Fig. 1A and Table 2). Second, except for primary hepatocyte cultures transformed on day 2, equivalent levels of luciferase expression were observed in cultures transformed with $p\alpha_1I3$, compared with sister cultures transformed with pSV2AL (Table 3). Third, primary hepatocyte cultures transformed with $p\alpha_1I3$ also expressed higher levels of luciferase activity than those generated by similarly transformed HepG2 cells (compare results in Fig. 1A, 2A, and Table 3 with Table 2).

Results shown in Fig. 1A, Table 3, and Fig. 2 indicated that DNA-mediated gene transfer was obtained during the entire hepatocyte growth cycle (5, 20–24, 30, 31). When the method of Chen and Okayama was followed (6), the observed kinetics and absolute levels of reporter gene expression depended on the plasmid employed as well as on the time of transformation during the growth cycle. For example, reporter gene expression (with pSV2AL, pRSVCAT, or $p\alpha_1I3$) was highest in lag and early log phase cultures (days 2 to 4) transformed and assayed 2 and 4 days postplating, respectively (Fig. 1A and Table 3). Similar trends were observed in cultures transformed with pRSVβ-GAL (Fig. 2C) and pFOS-CAT (Fig. 2E). The observed patterns of increased levels of reporter gene expression were sustained when cultures were transformed during mid-log phase (day 6) with pSV2AL or with the tissue-specific plasmid $p\alpha_1I3$ (Table 3 or Fig. 2A and B).

Similar results were observed under conditions of lipofection-mediated gene transfer (with pSV2AL and $p\alpha_1I3$) (Table 3). However, overall response levels of reporter gene expression obtained with lipofection were about 10-fold lower in magnitude than response levels obtained with the Chen and Okayama procedure of calcium phosphate copre-

TABLE 3. DNA-mediated gene transfer: dependence on growth state of primary cultures of adult rat hepatocytes[a]

Days post-plating of:		Luciferase activity with:				CAT activity with pRSV-CAT and $CaPO_4$
		pSV2AL		$p\alpha_1I3$		
Transfection	Assay	$CaPO_4$	Lipofection	$CaPO_4$	Lipofection	
2	2	50	50	50	50	0.20
	4	305,200	48,960	88,540	38,480	12.10
	8	12,440	5,630	2,359	5,272	2.80
4	4	50	50	50	50	0.20
	6	50	2,441	380	2,037	0.65
	10	25,030	10,240	72,650	9,230	1.00
6	6	50	50	50	50	0.20
	8	50	2,164	50	2,341	0.33
	12	31,180	2,469	49,380	1,602	0.20
8	8	50	50	50	50	0.20
	10	24,190	3,115	17,220	1,442	0.37
	14	1,049	296	2,860	292	0.25

[a] Cultures were prepared and exposed to one of three plasmids (pSV2AL, $p\alpha_1I3$, or pRSV-CAT) as described in the legend to Fig. 1. Five-hour-long exposures were made 2, 4, 6, or 8 days postplating (basal reporter gene activity levels are given in Materials and Methods). Reporter gene expression was monitored for each plasmid at each initial time of transformation and 2 and 6 days later, as described in the legend to Fig. 1. Studies of sister cultures subjected to lipofection-mediated gene transfer were performed, as described in Materials and Methods, with pSV2AL or $p\alpha_1I3$.

cipitation (6). Comparisons between the two methods are difficult, since cytotoxicity generated from lipofection approached 33%, as determined by measurements of cellular proteins per dish and by microscopic examination of the cultures at or beyond 4 days postplating (data not shown). The original procedure of Graham and Van der Eb (15) was not employed, since it caused high levels of cytotoxicity (80 to 85%) in this primary culture system.

In contrast to these trends, progressive attenuation of CAT expression, driven by a Rous sarcoma virus 5′ long terminal repeat, was observed when cultures were transformed 4, 6, or 8 days postplating and then, in each case, monitored over 144 h thereafter (Table 3). Attenuation did not depend on the nature of the reporter gene product whose activity was assayed, since attenuation was also observed when cultures were transformed with pRSVβ-GAL and analyzed for positively stained cells (Fig. 2C and D).

Since primary adult hepatocyte cultures contain 5 to 20% nonparenchymal cells (21, 30), DNA-mediated gene transfer experiments were performed with pRSVβ-GAL in order to identify transformed cells directly. This plasmid contains a strong nonspecific promoter (Table 1) driving a gene whose product can be visualized cytochemically. The results (Fig. 3) indicated that mononucleated and binucleated hepatocytes were the predominant cellular targets; no positively stained nonparenchymal cells were observed (10 dishes scored; 400,000 cells per dish). Microscopic analyses indicated that under these conditions, at least 1 to 2% of hepatocytes expressed β-galactosidase (Fig. 2C). Similar findings were made when hepatocyte cultures were transformed with pCOLβ-GAL (Table 1; D. A. Brenner, J. M. Alcorn, H. L. Leffert, S. P. Feitelberg, and M. Chojkier, Mol. Biol. Med., in press) or when NIH 3T3 fibroblasts were plated and transfected under similar conditions with optimal concentrations (1 μg/ml) of pRSVβ-GAL (data not shown). In contrast, a 10- to 20-fold-lower staining response was obtained when hepatocyte cultures were transformed with a promoterless variant, pCH126 (data not shown).

Reporter gene expression responded to humoral factors associated with proliferation of cultured hepatocytes. After the cultures were transformed during the growth cycle either 2 or 6 days postplating (lag or log phase, respectively [5, 24, 30, 31]), human transforming growth factor-α (TGF-α) (8), an hepatocyte mitogen (3, 37), stimulated reporter gene expression in hepatocytes transformed with $p\alpha_1I3$ (20-fold over untreated cultures; Fig. 2A and B), pRSVβ-GAL (4-fold; Fig. 2C and D), or pFOS-CAT (8-fold; Fig. 2E and F). In contrast, when quiescent cultures (transformed 5 days

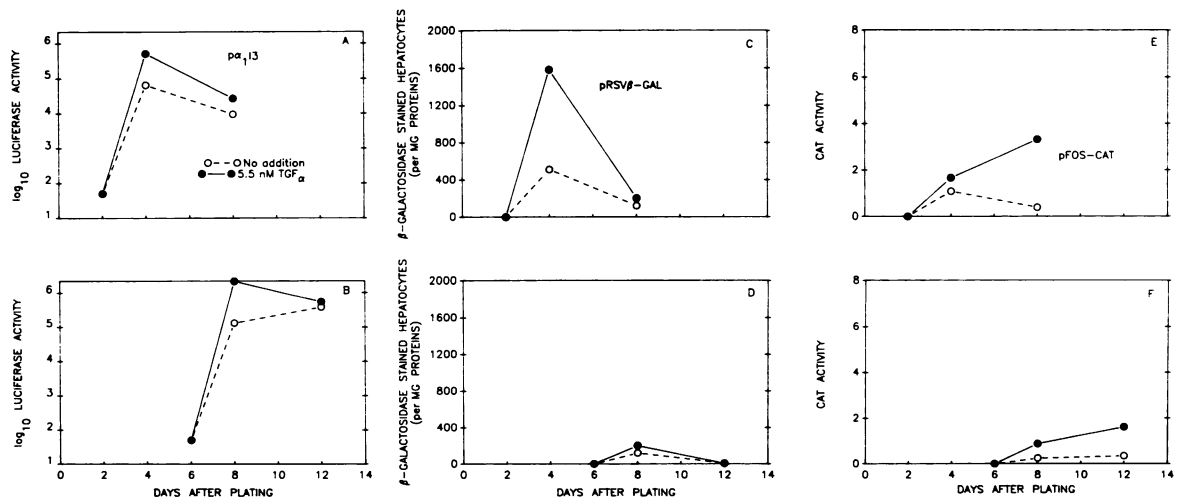

FIG. 2. DNA-mediated gene transfer: dependence of reporter gene expression on TGF-α during the growth cycle of adult rat hepatocytes in primary culture. Cultures were prepared and exposed (5 h; days 2 or 6 postplating) to one of three plasmids ($p\alpha_1I3$ [A, and B], pRSVβ-GAL [C and D], and pFOS-CAT [E and F]), as described in the legend to Fig. 1. After being returned to fresh plating media (days 2 or 6 postplating), the cultures were divided into two groups; one received 5.5 nM TGF-α immediately or immediately and again 4 days later (●), while the other received diluent alone immediately or immediately and again 4 days later (○). Reporter gene expression was monitored for each set of plasmids 2 and 6 days later, as described in the legend to Fig. 1.

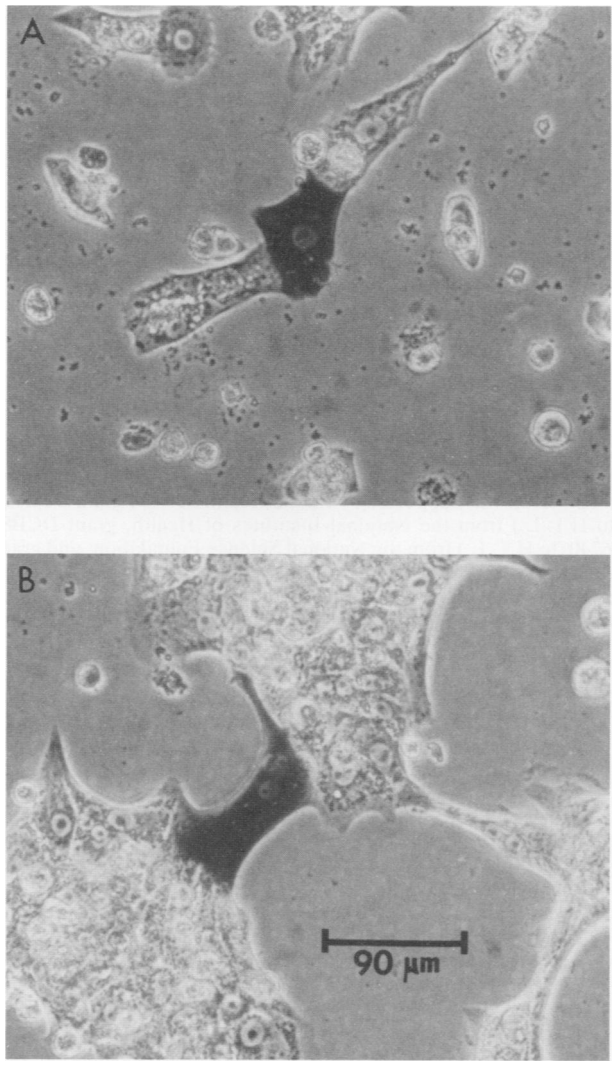

FIG. 3. Morphological identification of cells transformed with plasmid pRSVβ-GAL in primary cultures of adult rat hepatocytes. Cultures were prepared and exposed to plasmid DNA (2 days postplating) as described in the legend to Fig. 1. After 48 h, cultures were harvested and stained for bacterial β-galactosidase, as described in Materials and Methods. Microscopic analyses were performed by using a phase-contrast objective. Positively stained mononucleated (A) and binucleated (B) hepatocytes are shown. Mock-infected cultures showed no detectable positively stained hepatocytes.

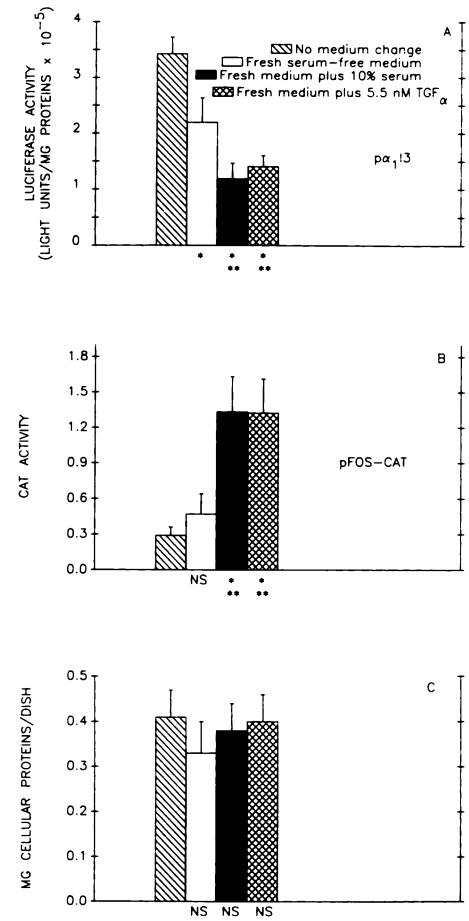

FIG. 4. DNA-mediated gene transfer: dependence of reporter gene expression in quiescent primary hepatocyte cultures on growth reinitiation transitions. Eleven-day-old cultures, exposed to plasmid DNA (pα_1I3 or pFOS-CAT) 6 days postplating (as described in Materials and Methods), were subjected to growth reinitiation conditions by fluid changes into fresh serum-free medium (2 ml per dish) without or supplemented with 10% (vol/vol) dialyzed adult rat serum or 5.5 nM TGF-α. Twenty-four hours later (day 12), reporter gene expression (luciferase [A]; CAT [B]) and cellular proteins (C) were measured in these and nongrowth stimulated control cultures, as described in the legend to Fig. 1 and Materials and Methods. Error bars give standard deviations (n = 4; two independent experiments). Symbols: *, significantly different from control (no medium change; $P < 0.05$); **, significantly different from fluid change into fresh serum-free medium. NS, Not significantly different from control.

previously) were subjected to mitogenic conditions and were assayed for reporter gene expression 24 h later, two different trends were observed. First, luciferase expression driven by pα_1I3 (Fig. 4A) was attenuated, compared with unperturbed 12-day-old control cultures, by shifting the cultures into fresh serum-free medium without (36% decrease) or with serum or TGF-α (66% decreases). Second, CAT expression driven by the FOS promoter in pFOS-CAT (Fig. 4B) was stimulated compared with its controls: 1.6-, 5.4-, and 5.5-fold by serum-free medium without or with serum or TGF-α, respectively. These differential responses could not be attributed to cytotoxicity, since similar amounts of cellular proteins per culture were observed among the four different treatment groups (Fig. 4C).

DISCUSSION

This study demonstrates that adult rat hepatocytes in primary culture can be used easily and reproducibly for molecular genetic analyses via DNA-mediated gene transfer. Standard conditions in these experiments employing a modified calcium phosphate coprecipitation protocol generated levels of CAT reporter gene expression that exceeded by 10-fold those reported earlier in electroporation studies with similar plasmids with short-term primary hepatocyte cultures (46). Furthermore, from morphological observations of hepatocytes expressing a cytoplasmic reporter gene (β-galactosidase), the magnitude of this difference might actually be higher with respect to the percent hepatocytes transformed, since direct measurements were not made with

short-term cultures (46). Thus, 4 days postplating, 1 to 2% of hepatocytes expressed β-galactosidase following a 5-h transfection interval on day 2. A 9- to 10-fold increase in this fraction would be anticipated had 20-h transfection intervals been employed instead (Fig. 1C). In addition, the observed levels of luciferase expression were equivalent to the levels seen in primary hepatocyte cultures expressing firefly luciferase after infection with retroviral vector pLNRLL (48; H. L. Leffert, K. S. Koch, G. G. Brownlee, S. J. Goss, J. Wolff, A. Martinez-Conde, L. Xu, H. Skelly, and T. Friedmann, manuscript in preparation). Therefore, it should be possible to analyze the behavior of any gene (or genetic element) of biological interest after its insertion into a normal hepatocyte. This conclusion seems particularly valid for studies of tissue-specific gene expression, based on comparative observations between $p\alpha_1I3$ expression in primary hepatocyte cultures and human fibroblasts.

Under the conditions used to plate primary cultures in these studies, hepatocytes proliferate (two to three population doublings) and exhibit defined lag (0 to 2 days postplating), log (2 to 8 days postplating), and stationary growth cycle phases (5, 21, 24, 30, 31). During a 10- to 14-day growth cycle, two kinds of intrinsic phenotypic patterns of change in differentiated states occur: transient dedifferentiation (2 to 6 days postplating) followed by partial restoration of mature tissue-specific differentiated function (for example, production of albumin [30, 31], alcohol dehydrogenase [23, 24], and ligandin [31], between days 6 and 12 postplating) and transient retrodifferentiation, whereupon tissue-specific fetal functions (for example, production of alpha-fetoprotein [31]) reemerge (2 to 10 days postplating). These types of developmental changes, accompanied by population growth curves, have been described in detail for both adult rat and adult mouse hepatocyte systems elsewhere (12, 17, 20–24, 27–31, 39, 42, 45; A. S. Gleibermann, E. I. Kudrjartseva, Y. Y. Sharovskaya, G. I. Abelev, Mol. Biol. Med., in press).

The expression of exogenous genes in hepatocytes was examined during lag, log, and stationary growth phases. Both the expression of such genes and the effect of the addition of TGF-α on their expression varied with the growth cycle. Differential efficiencies of transformation among the different plasmids might have produced these findings. This seems unlikely, since similar results were obtained when cultures were cotransfected with plasmids expressing heterologous reporter genes as internal controls (data not shown). Alternatively, chromosomal integration of plasmid DNA and subsequent hepatocyte proliferation, while possibly contributory, would appear unlikely, since the magnitudes of mitogen-stimulated increases in reporter gene expression responses (averaging more than 10-fold above controls) greatly exceeded the limit of hepatocyte population doublings obtained in these experiments (3-fold).

A more plausible interpretation of differential responses of transformed hepatocytes to TGF-α (Fig. 2 and 4) is that intrinsic developmental differences are present during different growth states—a property of this culture system that has been demonstrated repeatedly (27, 28). This conclusion is further supported from observations of reciprocal responses of reporter genes driven by exogenous FOS and α_1I3 promoter elements during growth reinitiation transitions (Fig. 4). For example, with respect to c-*FOS* expression, the behavior of the inserted plasmid gene mimics stimulation of the endogenous c-*FOS* gene in growth-reinitiated hepatocytes (22, 44) and is proportional to different levels of growth stimulation in quiescent cultures shifted into serum-free medium with or without TGF-α (3). The attenuation of hepatocyte-specific $p\alpha_1I3$ expression is provocative, since differentiated hepatic functions generally decline transiently during normal hepatocyte proliferative transitions (27); however, endogenous α_1I3 gene expression measurements have yet to be made under these in vitro conditions. Lastly, although the rapidly declining efficiency of reporter gene expression in pRSV-CAT-transformed cultures is not yet understood, it is notable that similar trends were observed when these types of cultures were infected during their growth cycle with 5′ long terminal repeat-based retroviral vectors (47, 48).

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