Vol. 183, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1992 Pages 184-192

DIFFERENTIAL EXPRESSION OF THE TRANSFECTED LIVER-SPECIFIC α_1-INHIBITOR III GENE IN NORMAL HEPATOCYTES AND HEPATOMA CELLS IN CULTURE

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Received January 14, 1992

Normal and malignant hepatocytes were transfected during log phase culture with a nested series of DNA plasmids containing 5'-flanking regions of the rat liver-specific acute phase plasma proteinase α_1-inhibitor III (α_1I3) gene. Under these conditions, luciferase reporter gene expression in primary adult rat and mouse hepatocytes was 10-fold higher than luciferase expression in hepatoma lines (human HepG2 and Hep3B; rat FAZA). Optimal expression in primary rat hepatocytes required regions stretching 2214 bp 5'-upstream of the transcription start site. Shorter 5'-flanking sequences were optimal for expression in hepatoma cells(-1025 and -186 bp for rat and human lines, respectively) and primary mouse hepatocytes (-225 bp). In contrast, regions from -186 to -225 bp drove luciferase expression in primary rat hepatocytes, but only 20-75% of optimal levels. Qualitative differences were unaccounted for by non-equivalent uptake of plasmid DNA, suggesting that tissue specific gene expression is regulated differently in normal and malignant cells, and with apparent species specificity.

Hepatocyte gene expression is under intensive investigation to understand mechanisms regulating animal cell differentiation and growth (1-3). To date, a standard approach has been analysis of expression following DNA-mediated transfection of differentiated hepatoma cell lines with various nested DNA deletion series of 5'-flanking sequence constructs, linked to reporter genes (4-7). From such experiments, short stretches (50-200 bp) of 5'-flanking regions of several genes have been shown to exert tissue-specific transcriptional control. For example, Fey and his colleagues, using human Hep3B and rat FAZA hepatoma cells, delineated 4 cis-acting liver-specific transcriptional control elements embedded within the promoter-proximal 5'-flanking region 196 bp upstream from the transcription start site of the acute phase plasma proteinase inhibitor, α_1-inhibitor III (α_1I3) gene (8-11). In attempts to extend these findings, preliminary observations in our laboratory (12) led us to question how different α_1I3 promoter-regulatory sequences, defined biologically by

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0006-291X/92 $1.50

DNA-mediated transfection of hepatoma cell lines, might affect luciferase (luc) expression in normal hepatocytes. To investigate this further, we performed comparative transfection studies with primary cultures and several established human or rat hepatoma cell lines.

METHODS

Preparation and transfection of cell cultures.

Primary cultures. Hepatocytes from adult Fisher/344 male rats (180-200g) or C57BL/6N male mice (15-20g) were isolated, plated (1 x 10^6 cells/2 ml/35-mm dish) and grown (orn-supplemented [0.2 mM], arginine-free medium) as described (13,14). DNA-mediated transfection was performed by $CaPO_4$ coprecipitation for 5-8 h two d post-plating. Under these plating conditions (media containing 15% v/v dialyzed fetal bovine serum, and 10 μg per ml each of insulin, inosine and hydrocortisone), hepatocytes are in early log phase (13,15). Transfections, unless noted[1], were made as reported previously (14).

Hepatoma cell lines. PPLO-free human HepG2 and Hep3B, and rat FAZA cells were provided by B. Knowles and G. Fey, respectively. The cells were plated (1 x 10^5 cells per 2 ml per 35-mm dish) and cultured like primary cells, with some exceptions: 0.4 mM l-arg replaced orn; insulin, inosine and hydrocortisone were omitted; and, fluid changes to fresh plating media were made 2 and 4 d post-plating. DNA-mediated transfections were performed, as above, 3-4 d post-plating. Under these conditions, hepatoma cells were also in log phase. Transfections, unless noted[1], were made as described above.

Reporter gene expression assays. Luc activity (EC 1.13.12.7), bacterial ß-galactosidase activity (β-gal) (EC 3.2.1.23) and cellular proteins were quantified by standard procedures (14). Assays were performed either 24 or 48 h after the 5-8 h transfection period in hepatoma or primary cultures, respectively, when reporter gene activity was maximal (14). Basal levels of luc and ß-gal activity in mock infected cultures were 50 light units/10 secs/mg proteins and 0 stained hepatocytes (or, hepatoma cells)/dish, respectively.

Plasmid isolation, purification and characterization. Table 1 lists plasmids employed. Standard procedures were used to grow, isolate, purify and characterize them (14,17). The *E.coli* host strains were: JM83($p\alpha_1$I3 series) and JM109(pRSV-ßGal, pFOS-Luc, pJUN-Luc). Construction of the $p\alpha_1$I3 deletion series, pRSV-ßGal, pFOS-Luc and pJUN-Luc were reported previously (8,9,12,14,18).

Data analysis. Paired Student's t-tests and data histograms were calculated and plotted using SigmaPlot 4.1 (Jandel Scientific, Corte Madera, CA).

RESULTS

Primary cultures. Previous work with Hep3B and FAZA cells (8,9) indicated that optimal luc expression was obtained with -2214+58 bp control region constructs and near-optimal luc activity obtained with progressive 5'-deletions

[1]The 4-hormone "cocktail" (serum-free arg-free medium, supplemented with 0.2 mM orn [or, arg, for hepatoma lines], and 50 ng/ml insulin[I], glucagon[G], epidermal growth factor [EGF] and hydroxycortisone-succinate [Cx]) was used because normal hepatocyte growth, function and viability depend on these factors (glucagon receptors are, however, absent from hepatoma cell [16]). Reporter gene expression 4 d post-plating for primary rat hepatocytes transfected on day 2 with promiscuous promoters in serum-free media containing 4 hormones was: 2-4 x 10^5 and 2-4 x 10^6 light units/10 secs/mg proteins (pFOS-Luc and pJUN-Luc, respectively), and 350-450 ß-gal stained hepatocytes/dish.

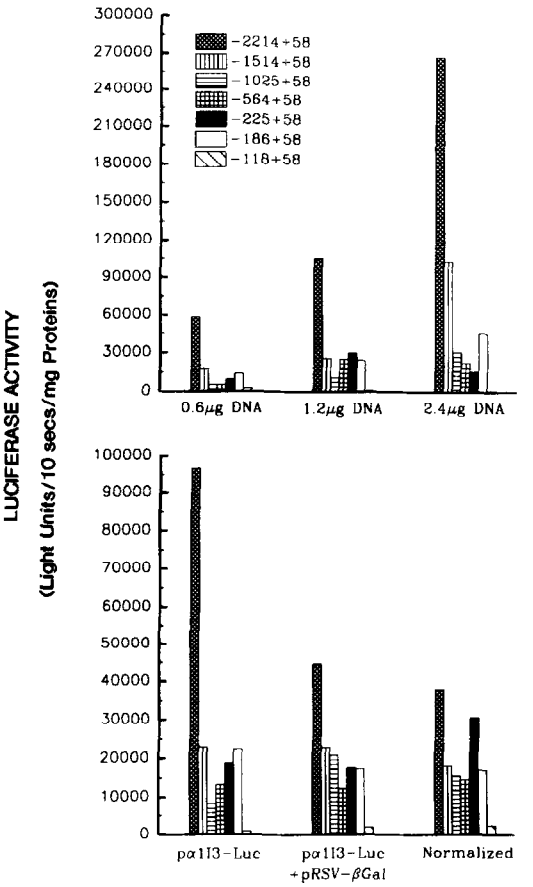

FIG. 1. Luc expression in primary adult rat hepatocytes transfected with $p\alpha_1I3$ deletion constructs. Upper. Dependence on DNA concentration. Data are pooled from 8 platings: 1 at 0.6 μg DNA /dish (N=1 dish); 3 at 1.2 μg (N=4); and, 4 at 2.4 μg (N=5-8). Post-transfection fluid changes were made in 1 case (2.4 μg DNA) into serum-free media containing 4 hormones[1]. At assay, there were 2-4 x 10^5 cells/dish (200-400 μg proteins/dish). Lower. Co-transfections with pRSV-ß-Gal. DNA concentrations were 1.2 μg ($p\alpha_1I3$ series) or 2.4 μg ($p\alpha_1I3$ + pRSV-ßGal, 1.2 μg each). Numbers of ß-gal stained hepatocytes/dish/cotransfectant group (N=3) were 147, 157, 168, 105, 72, 127, and 99 (-2214 through -118+58, respectively). Normalized values were obtained using 147 or 99 set = 1. Significant differences ($P<0.05$): [-118+58] < [all others]; [-2214+58] > [all others]; and, [-186 to -225+58] > [-564 to -1514].

of control constructs as short as -225+58 bp. In Fig. 1 (top panel), rat hepatocytes were transfected with a series of α_1I3 control-region constructs (Table 1). Similar optimal results with the -2214+58 bp construct were observed here under transfection conditions using chemically-defined media.[1] However, in contrast to previous observations with hepatoma cells, constructs containing shorter control sequences, from -186 to -225 bp upstream of the transcription start site, led to sub-optimal luc expression in rat hepatocytes. Further analysis of the entire series revealed a bi-modal histogram of luc expression, with attenuated activity observed with constructs containing α_1I3-promoter sequences in the range -564 to -1514+58 bp (10-50% of optimal), and the lowest

TABLE 1. Plasmid, promoter and reporter gene constructs

Plasmid	Promoter[a]	Reporter Gene	Source	Reference
$p\alpha_1I3$	α_1I3(-2214+58)	Firefly Luciferase	G.Fey	9
$p\alpha_1I3$	α_1I3(-1514+58)	Firefly Luciferase	G.Fey	9
$p\alpha_1I3$	α_1I3(-1025+58)	Firefly Luciferase	G.Fey	9
$p\alpha_1I3$	α_1I3(-564+58)	Firefly Luciferase	G.Fey	9
$p\alpha_1I3$	α_1I3(-225+58)	Firefly Luciferase	G.Fey	9
$p\alpha_1I3$	α_1I3(-186+58)	Firefly Luciferase	G.Fey	9
$p\alpha_1I3$	α_1I3(-118+58)	Firefly Luciferase	G.Fey	9
pRSV-ßGal	RSV-5'LTR	E.Coli ß-galactosidase	D.Brenner	14
pFOS-Luc	FOS(-711+45)	Firefly Luciferase	D.Brenner	14
pJUN-Luc	JUN(2.2-kb SalI)	Firefly Luciferase	D.Brenner	14

[a]RSV-5'LTR, Rous sarcoma 5' long terminal repeat; FOS, human c-FOS; and, JUN, human c-JUN SalI-fragment.

level of expression observed with the TATA box-containing -118+58 construct (0.1-2% of optimal). These patterns were neither artifacts of bell-shaped plasmid DNA infectivity curves (14) nor consequences of non-equivalent DNA uptake since bi-modality was preserved over a 4-fold DNA concentration range (top panel) and when expression levels were normalized to a co-transfected marker like β-gal (bottom panel), respectively.

Experiments were performed to see if bi-modality might depend upon a particular mitogen or the hepatocyte species of origin (Fig. 2). If transfected rat hepatocytes were shifted into fluids containing TGFα, a mitogen (17) that stimulates luc expression in rat hepatocytes infected in early log phase with full-length $p\alpha_1I3$ constructs (12,14,18), then bi-modality in luc expression was again observed (top panel). Except for a 50% increase in luc expression in TGFα-treated hepatocytes infected with the full-length construct, no significant differences were observed between control and TGFα-treated cells. If mouse hepatocytes were transfected under identical conditions (bottom panel), then optimal luc expression was observed with constructs containing sequences ranging -225 to -2214 bp. Although expression fell (15-30% of optimal) with constructs containing shorter sequences (-118 to -186 bp), or was <4000 light units/mg with a promoterless construct (data not shown), absolute levels of expression ranged 5-50-fold higher than rat hepatocytes. The quantitative differences might partly reflect unequal transfection efficiency between the 2 species of hepatocytes since mouse cultures showed ≈5-fold higher numbers of ß-gal-stained hepatocytes/dish (1765±120 [N=2]) following transfection with 2.4 μg pRSV-ßGal.

Hepatoma cells. Three differentiated hepatoma lines were transfected with the $p\alpha_1I3$ deletion series constructs under conditions identical to those employed with primary cultures (Fig. 3). Two of these lines (Hep3B and FAZA) were examined previously (8,9), but prior conditions differed from those employed with primary cells (Figs. 1 and 2). Therefore, validation experiments were performed with these lines to determine if their reporter gene expression responses were affected by variable transfection conditions.

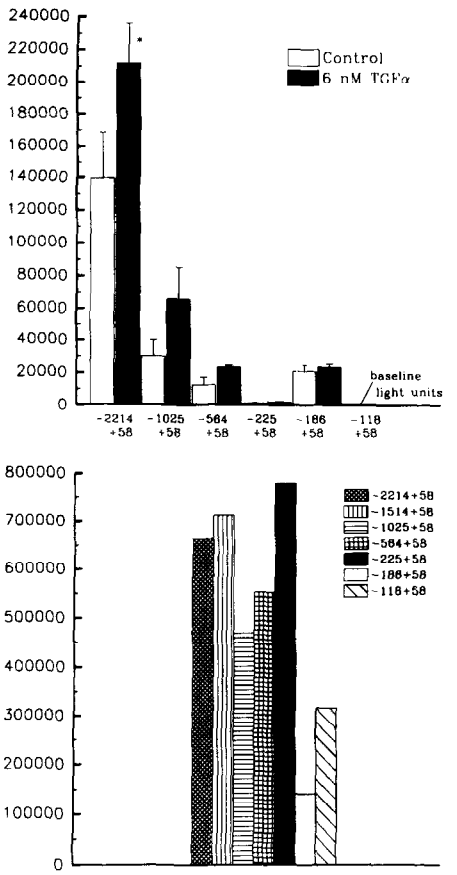

FIG. 2. Effects of TGFα and species difference on luc expression in primary cultures transfected with $p\alpha_1I3$ deletion constructs. Upper. Rat hepatocytes were transfected (2.4 μg DNA) and fluid changes made into plating media without or with 6 nM TGFα. Luc assays were performed as described in Methods (mean+/-SD [N=2]). Significant differences (P<0.05) between TGFα-treated and untreated group (*). Lower. Mouse hepatocytes were prepared, transfected (2.4 μg DNA), and assayed for luc (N=2; errors were 5-15%). Significant differences (P<0.05): [-186+58] < [all others]; and, [-225 through -2214+58] > [-118 to -225+58].

The top and middle panels of Fig. 3 show results of transfection studies with DNA plasmids containing strong tissue non-specific promoters (c-FOS, c-JUN and RSV-5'LTR). When FAZA or HepG2 cells were transfected with pFOS-Luc, under both standard (media containing serum, insulin and hydrocortisone [14]) or chemically-defined conditions, a 5-10-fold greater luc expression was obtained in serum-free media containing insulin, glucagon, EGF and hydrocortisone (top panel).[1] Although glycerol shock augmented luc expression under standard conditions, it was not beneficial under serum-free transfection conditions. These results suggested that serum-free transfection conditions, used with primary hepatocytes, could also be employed without glycerol shock with hepatoma cell lines. Further evidence of this, including Hep3B, was obtained from transfection studies with pJUN-Luc and pRSV-ßGal (middle panel). These results indicated that,

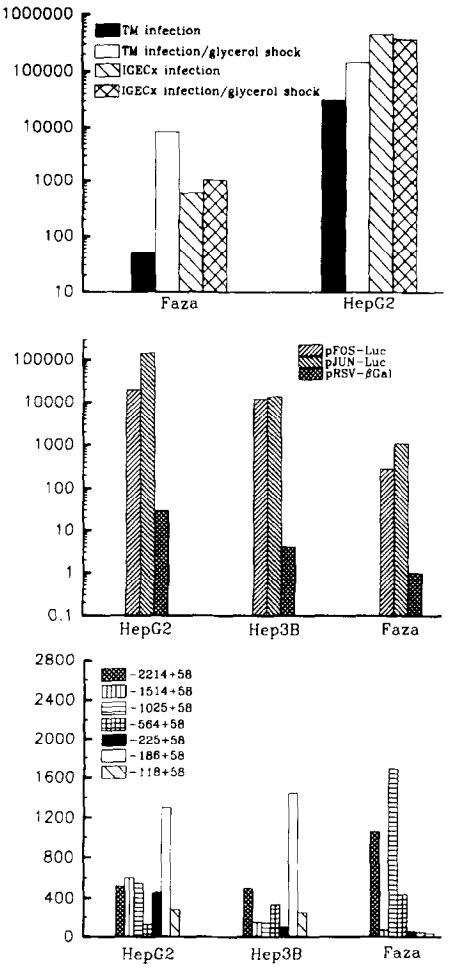

FIG. 3. Luc and β-gal expression in hepatoma cells transfected with plasmids containing promiscuous and tissue-specific promoters. Upper. Variable transfection conditions. Cultures were prepared (media contained 10% serum), transfected (1 μg pFOS-Luc), fluid changed into plating media, and assayed for luc expression (3.8-4.8 x 10^5 cells/dish). For both lines, transfections were performed either in standard media ("TM"[14]) or in serum-free media supplemented with 4 hormones[1]. Following transfection, half of cultures/group (N=5) were subjected to glycerol shock (20% w/v, 1x filter-sterilized BBS [9]) for 2 min. Significant differences ($P<0.05$) were observed in cultures subjected to glycerol shock and standard media, but not under serum-free conditions, following glycerol shock. Middle. Luc and β-gal expression governed by promiscuous promoters. Cultures were transfected (1.2-2.4 μg DNA) in serum-free media containing 4 hormones. Data are pooled from 4-7 platings. Numbers of dishes/group were 14-16 (FOS), 4 (JUN) and 12-13 (RSV; y-axis labels denote stained cells/dish). Bottom. Luc expression governed by $p\alpha_1 I3$ promoter constructs. Cultures were transfected (1.2-2.4 μg DNA) in serum-free media containing 4 hormones (see panels at upper left). Data are combined results of 6-8 platings. The numbers of dishes/group (for each construct) were 22-23 (HepG2), 18-20 (Hep3B) and 19-20 (FAZA). Significant differences ($P<0.05$) in luc activity were observed between the following plasmids or sets of plasmids: for HepG2 and Hep3B, [-186+58] > [all others]; for FAZA, [-1025+58] >[-2214] > [all others], and [-1514] < [-1025] and [-2214].

with respect to all 3 promiscuous promoters, an hierarchy existed of reporter gene expression: HepG2 > Hep3B > FAZA. Expression driven by the JUN promoter tended to exceed FOS expression levels, regardless of cell type.

When transfected with the pα_1I3 series, contrary to findings with rat hepatocytes, human and rat cell lines expressed optimal luc activity with the shorter 5'-flanking sequences (-186+58 and -1025+58 bp, respectively; bottom panel, Fig. 3). Although optimal levels of luc expression were ≈30-fold above the lowest luc levels, and quantitatively similar among the lines, the magnitude of this expression was at least 100-fold below that observed with normal hepatocytes. Statistical analyses of differences among constructs, with respect to each cell line, demonstrated significant uni-modal histograms of luc expression in the human cell lines (histograms for both lines showed rightward shifts, peaking with the -186+58 bp construct) whereas rat FAZA cells displayed a bi-modal pattern, differing from normal hepatocytes, with major and minor peaks obtained from the -1025 and -2214+58 bp constructs, respectively.

DISCUSSION

Results in this report suggest that quantitative and qualitative levels of plasmid-directed tissue-specific gene expression, following DNA-mediated transfection of reporter genes in cell culture, reflect not only the ability of the recipient cell to recognize different far upstream regulatory sequences, but also its physiological state and species of origin. Control studies suggest that these trends were not caused by differences in transfection conditions, nor by differences in plasmid DNA uptake, measured indirectly by co-transfection with pRSV-ßGal. However, primary adult mouse hepatocytes produce more α_1-fetoprotein than similar rat hepatocyte cultures (19), a phenomenon that might be related to the species differences observed in Fig. 2. In addition, the degree to which tissue-specific luc expression in normal hepatocytes, driven by the full-length pα_1I3 construct, exceeded luc expression in hepatoma cell lines, is difficult to judge precisely because serum-free co-transfection studies were not performed with hepatoma lines. Nevertheless, these lines were clearly capable of generating high levels of luc expression, governed by promiscuous promoters, precluding the possibility that a general attenuation of plasmid DNA uptake might have accounted for the observed tissue-specific quantitative differences.

Qualitative comparisons between findings here and prior work (8,9) indicate that transfected hepatoma lines produce optimal levels of luc expression with relatively short sequences of the putative α_1I3 promoter (-186 or -1025+58 bp). Unless systematic transfection artifacts were occurring, the findings in Fig. 3 should reflect accurate trends, as the data were averaged from 6-8 independent experiments (N=18-23 per construct) employing 3 independently prepared plasmid preparations.

There are several other problems of interpretation. First, extracellular ligands differentially control hepatic expression of the α_1I3 gene (8,14,20).

For example, in addition to corticosteroids (8), a regulatory role has been demonstrated for the cytokine, IL-6 (20); here, a "TGFα-responsive" region (extending from -1025 to -2214 bp) was suggested (Fig. 2). Second, growing and quiescent cells, whether of normal or malignant origin, respond differently to such ligands (14,21,22). Underlying these differences are combinatorial interactions among regulatory ligands, including cell-type specific differences in the shapes of ligand dose response curves (3,12,21,22). Third, transfection studies with Hep3B and FAZA cells suggest that constitutive levels of tissue-specific expression of α_1I3 depend upon positive and negative regulatory elements embedded within 196 bp upstream of the transcription start site (8,9). Findings in this report show that luc expression in rat hepatocytes, and possibly in rat FAZA cells as well, is attenuated in transfectants harboring plasmids with sequences embedded within the range -564 to -1514 bp in the α_1I3 promoter, implicating additional negative regulatory sites. Lastly, gender differences between hepatocytes were not examined here, but might be expected to be contributory (23).

That studies with normal hepatocytes can reveal the existence of distal regulatory sites in tissue-specific genes has also been suggested, indirectly and directly, from studies of albumin (24) and S_{14}-gene expression (25), respectively, in primary culture. The studies reported here suggest that transfection studies in normal hepatocytes provide evidence for regulatory sites otherwise cryptic in studies of hepatoma lines. This information may be important to an investigation of normal regulation, since there is reason to believe that the normal differentiated state of hepatocytes requires the cooperation of proximal promoter and far upstream enhancer sequences (26) interacting with high cellular levels of tissue-specific transacting factors, such as C/EBP (27).

ACKNOWLEDGMENTS: Supported by grants from the ACS, NIH, NSF, The University of California TDRP, and the United States Veterans Administration. We thank B. Sakamoto and G. Somerano for help with hepatoma cell culture studies and P. Alford for word processing.

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