Hepatocyte growth factor and transforming growth factor β regulate 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene expression in rat hepatocyte primary cultures

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Hepatocyte growth factor (HGF) and transforming growth factor β (TGF- β) are believed to be of major importance for hepatic regeneration after liver damage. We have studied the effect of these growth factors on fructose 2,6-bisphosphate (Fru-2,6- P_2) levels and the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/Fru-2,6-BPase) in rat hepatocyte primary cultures. Our results demonstrate that HGF activates the expression of the 6PF2K/Fru-2,6-BPase gene by increasing the levels of its mRNA. As a consequence of this activation, the amount of 6PF2K/Fru-2,6-BPase protein and 6-phosphofructo-2-kinase activity increased, which was reflected

by a rise in Fru-2,6- P_2 levels. In contrast, TGF- β decreased the levels of 6PF2K/Fru-2,6-BPase mRNA, which led to a decrease in the amount of 6PF2K/Fru-2,6-BPase protein and Fru-2,6- P_2 . The different actions of HGF and TGF- β on 6PF2K/Fru-2,6-BPase gene expression are concomitant with their effect on cell proliferation. Here we show that, in the absence of hormones, primary cultures of hepatocytes express the F-type isoenzyme. In addition, HGF increases the expression of this isoenzyme, and dexamethasone activates the L-type isoform. HGF and TGF- β were able to inhibit this activation.

INTRODUCTION

Activation of the glycolytic flux is a common feature of growing cells [1,2]. During this process the levels of Fru-2,6- P_2 , a potent allosteric stimulator of 6-phosphofructo-1-kinase activity [3–5], have been found to be elevated in several cell systems [6–10]. Although this metabolic effect is clear, the mechanism by which it is achieved is not. Fru-2,6- P_2 content is controlled by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/Fru-2,6-BPase), which catalyses both its synthesis and its degradation [11–13]. Different 6PF2K/Fru-2,6-BPase isoenzymes have been described, depending on the tissue and cell specificity. Liver, muscle and F-type isoforms are encoded by the same gene arising from distinct promoters [14–16]. These isoenzymes differ with respect to their N-terminal sequences, regulation by phosphorylation and kinetic properties. Hepatic 6PF2K/Fru-2,6-BPase is also subject to a complex pattern of hormonal regulation at the transcriptional level. Bifunctional enzyme gene expression is increased by insulin in diabetic rats [16], by tri-iodothyronine in hypothyroid rats [17] and by glucocorticoids in adrenalectomized animals [18]. In primary cultures of hepatocytes, insulin and thyroxine act synergistically with glucocorticoids to induce 6PF2K/Fru-2,6-BPase mRNA [19]. In rat hepatoma cells, insulin and glucocorticoids also increase the mRNA of the bifunctional enzyme, but cyclic AMP represses glucocorticoid activation [20–22].

Regenerating liver is often used as a system for studying the metabolic changes that occur during cell proliferation *in vivo*. Previous results obtained by our group showed that 6PF2K/Fru-

2,6-BPase gene expression is modulated during liver regeneration [23,24]. In the replication period of liver regeneration, the levels of 6PF2K/Fru-2,6-BPase mRNA increase to a maximum at 48 h and return to normal values by 96 h. This replication period is associated with hepatocyte DNA synthesis and the major wave of cell division. Various hormones and growth factors are known to stimulate liver regeneration. One such factor, hepatocyte growth factor (HGF), has been shown to be the most potent mitogen for hepatocytes in primary culture [25] and seems to function as a hepatotrophic factor for liver regeneration [26,27]. Another growth factor, transforming growth factor β (TGF- β), is a potent inhibitor of DNA synthesis in primary rat hepatocyte culture [28]. Evidence from experiments *in vivo* implicates TGF- β as an inhibitor of DNA synthesis in liver regeneration [29,30].

The aim of this study was to determine whether HGF and/or TGF- β can modulate Fru-2,6- P_2 levels and/or the expression of 6PF2K/Fru-2,6-BPase gene in rat hepatocyte primary cultures. In addition, we have characterized the expression of different 6PF2K/Fru-2,6-BPase isoenzymes in this system.

MATERIALS AND METHODS

Chemicals

[α - 32 P]dCTP (3000 Ci/mmol), [α - 32 P]UTP (3000 Ci/mmol), [6- 3 H]thymidine (5 Ci/mmol), ECL Western kit and Hybond-N membranes were from Amersham International (Amersham, Bucks., U.K.). The random-primer DNA-labelling kit, restriction

Abbreviations used: Fru-2,6- P_2 , fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; 6PF2K/Fru-2,6-BPase; 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; HGF, hepatocyte growth factor; TGF- β , transforming growth factor β .

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endonucleases, type-I collagen and collagenase came from Boehringer (Mannheim, Germany). Foetal calf serum, Williams E Medium, L-glutamine and gentamicin were obtained from Gibco Laboratories. TGF- β 1, insulin and dexamethasone were purchased from Sigma (St. Louis, MO, U.S.A.). Human recombinant HGF was purified from the conditioned medium of Cos-1 cells transfected with expression vector CDM8 containing the full-size human HGF complementary DNA insert [31]. Other materials and chemicals were of the highest quality available.

Isolation of primary cultures of hepatocytes

Hepatocytes were isolated from fed male Sprague-Dawley rats weighing 200–250 g by the collagenase perfusion method [32]. Cell viability was determined by Trypan Blue exclusion. Hepatocytes were seeded at 25000 cells/cm² in tissue culture dishes coated with type-I collagen and incubated in Williams E Medium supplemented with 5% foetal calf serum, 1 nM insulin, 1 nM dexamethasone, 1 mM L-glutamine and 50 μ g/ml gentamicin for 2 h. Then the medium was replaced by fresh serum-free medium and the cells were cultured overnight in the defined medium before HGF, TGF- β and/or hormones were added.

Measurement of DNA synthesis

Hepatocytes were labelled with [6-³H]thymidine at a final concentration of 1 μ M and 1 μ Ci/ml. After 48 h the medium was removed and the hepatocytes were washed twice in cold PBS. Cold 5% trichloroacetic acid was added for 15 min, and then removed, and the hepatocytes were washed once in cold PBS and once in cold 100% ethanol. Hepatocytes were solubilized in 0.3 M NaOH for 15 min at room temperature. The radioactivity incorporated was determined using a liquid-scintillation β -counter.

Metabolite assays

Hepatocytes were homogenized with 6% HClO₄. After KClO₄ precipitation and neutralization with 3 M KOH/3 M KHCO₃, supernatants were used to measure hexose 6-phosphate levels by the Klotzsch method [33] and ATP was determined as described by Lamprecht and Trautschold [34]. To measure Fru-2,6- P_2 levels, hepatocytes were homogenized in 0.1 M NaOH, heated at 80 °C for 15 min and centrifuged at 12000 g (13000 rev/min) for 5 min. Fru-2,6- P_2 was determined in supernatants by its ability to activate pyrophosphate-dependent 6-phosphofructo-1-kinase from potato tubers, as described by Van Schaftingen et al. [35].

Enzyme and kinetic assays

Hepatocytes were homogenized in cold medium composed of 50 mM Tris/phosphate buffer, pH 8.0, 50 mM KCl, 50 mM KF, 1 mM EGTA, 1 mM dithiothreitol, 1 μ g/ml leupeptin and 1 μ g/ml pepstatin. The enzyme was partially purified by 6–15% poly(ethylene glycol) 8000 precipitation. Under these conditions the *in situ* phosphorylation state of the enzyme is preserved [16]. Total 6-phosphofructo-2-kinase activity was assayed by measuring the rate of Fru-2,6- P_2 production from fructose 6- P and MgATP as described by Bartrons et al. [36].

Western-blot analysis

Immunoblot analysis was performed essentially as described by Burnette [37] with a 1:1000 dilution of a polyclonal antibody raised against rat liver 6PF2K/Fru-2,6- P_2 ase protein [38]. Bound

antibodies were detected by an enhanced chemiluminescence method. The amount of protein was evaluated by densitometric scanning of the autoradiograms.

RNA isolation and analysis

Total RNA was extracted from hepatocyte tissue cultures by the LiCl/urea method [39]. The abundance of 6PF2K/Fru-2,6- P_2 ase mRNA was measured by Northern-blot analysis as previously described [24]. To detect 6PF2K/Fru-2,6- P_2 ase mRNA a 1.4 kb *Eco*RI fragment from pPKB plasmid was used as a hybridization probe [13]. The level of mRNA was evaluated by densitometric scanning of the autoradiograms and corrected for the amount of 18S rRNA using a ribosomal cDNA probe [40]. RNase-protection assay was essentially performed as described by Cifuentes et al. [20]. A 0.5 kb *Eco*RI 6PF2K/Fru-2,6- P_2 ase cDNA fragment cloned in pBluescript SK(+) was used to detect 6PF2K/Fru-2,6- P_2 ase mRNA [16]. The plasmid was linearized by digestion with *Ava*II, and then used to synthesize RNA using its T3 RNA polymerase promoter, with ribonucleotide triphosphates, including [α -³²P]UTP, and T3 RNA polymerase. The synthesized riboprobe was 390 bases long, complementary to 319 bases of the liver-specific mRNA, including exon 1_L, and 192 bases of common exons of muscle and F-type mRNA. To detect the presence of F-type mRNA a PCR probe was made from a genomic 6PF2K/Fru-2,6- P_2 ase fragment [41] with the following oligonucleotides: forward 1aF 5'-AAAGGCATTGCCGCC CGGAAGTG (which is located 5' upstream from exon 1aF); reverse 1aF 5'-TGTAATACGACTCACTATACAGTTACTCTTCCCAAGTTTTGG (which is located in the 3' of exon 1aF). The nucleotide sequence underlined contains the T7 RNA polymerase motif. The synthesized riboprobe was 292 bases long, complementary to 218 bases of the 1aF exon of F-type mRNA. Samples of 10 μ g of total RNA from rat liver, Rat-1 fibroblast and hepatocyte primary cultures were incubated at 90 °C for 10 min with a molar excess (100000 c.p.m.) of the purified riboprobe. The tubes were cooled slowly to 42 °C, and maintained at this temperature overnight. A mixture of RNase T1 and RNase A was used to degrade single-stranded unhybridized probe. After digestion, the protected fragments were collected by centrifugation (12000 g; 15 min) at 4 °C in the presence of guanidine thiocyanate and propan-2-ol, and subjected to electrophoresis on 6% polyacrylamide/8 M urea DNA-sequencing gels.

Other methods

Protein was determined as described by Bradford [42] with BSA as standard. The statistical significance of differences was assessed by Student's unpaired *t* test.

RESULTS

It is known that hepatocytes can proliferate in tissue culture when they have been seeded at low density on collagen films. To find the optimum proliferative conditions for our culture, we examined the effect of cell density on DNA synthesis by measuring thymidine incorporation after HGF addition. The highest incorporation of thymidine was observed at 25000 cells/cm². At this density, 0.1 nM HGF induced a 3.2-fold increase in thymidine incorporation over control, and 50 pM TGF- β blocked this incorporation. From these results we chose this cell density and these growth-factor concentrations as the most suitable for all the experiments.

Fru-2,6- P_2 levels were determined at different times after HGF

Figure 1 Effect of HGF and TGF- β on Fru-2,6- P_2 levels

Cells were cultured on six-well plates as described in the Materials and methods section. At the indicated times, extracts for Fru-2,6- P_2 quantification were obtained from cultured cells incubated in the absence (○) or presence of 0.1 nM HGF (●), 50 pM TGF- β (■), 0.1 μ M insulin (△) or 0.1 nM HGF and 0.1 μ M insulin (▲). Means $\pm$ S.E.M. of triplicate measurements are shown. Statistically significant differences with respect to control cells are indicated: * $P < 0.05$; ** $P < 0.01$.

Table 1 Effect of HGF and TGF- β on hexose 6-phosphate and ATP concentrations

Rat hepatocytes were cultured in six-well plates as described in the Materials and methods section and treated in the absence (control cells) or presence of 0.1 nM HGF or 50 pM TGF- β . At the indicated times, metabolite extracts for hexose 6-phosphate and ATP determination (nmol/mg of protein) were obtained. Means $\pm$ S.E.M. of triplicate measurements are shown. Statistically significant differences with respect to control cells are indicated: * $P < 0.05$; ** $P < 0.01$.

	Hexose 6-P content		ATP content	
	4 h	24 h	4 h	24 h
Control	2.69 $\pm$ 0.39	2.48 $\pm$ 0.10	20.45 $\pm$ 5.47	20.39 $\pm$ 2.62
+ HGF	2.63 $\pm$ 0.32	2.39 $\pm$ 0.04	31.15 $\pm$ 3.68*	30.63 $\pm$ 4.23**
+ TGF- β	1.87 $\pm$ 0.50	2.91 $\pm$ 0.24	24.07 $\pm$ 1.63	28.35 $\pm$ 0.45**

and TGF- β addition. As shown in Figure 1, 0.1 nM HGF increased the Fru-2,6- P_2 content by 80 %, and the levels remained high for the first 24 h. Insulin at 0.1 μ M increased Fru-2,6- P_2 levels, but to a lesser extent than HGF. The effect of insulin and HGF on Fru-2,6- P_2 levels was additive during the first hour but not thereafter. In contrast, 50 pM TGF- β produced a decrease in Fru-2,6- P_2 content. In order to rule out the possibility that the change in Fru-2,6- P_2 concentration was due to changes in substrate concentrations of 6PF2K/Fru-2,6-BPase enzyme, we determined substrate content in the samples. As shown in Table 1, no significant variation was found in hexose 6-phosphate levels. However, ATP concentrations were slightly higher than in controls. The differences in ATP concentration should not affect Fru-2,6- P_2 levels, since 6PF2K/Fru-2,6-BPase enzyme works at saturated concentrations of ATP.

To determine whether the changes in Fru-2,6- P_2 levels were due to variation in the activity of 6PF2K/Fru-2,6-BPase, the activity of the enzyme under V_{max} conditions was measured at 30 min, 4 h, 6 h and 24 h after the addition of HGF or TGF-

Table 2 Effect of HGF and TGF- β on total 6-phosphofructo-2-kinase activity

Rat hepatocytes were cultured in six-well plates and treated or not with 0.1 nM HGF or 50 pM TGF- β . At the indicated times, extracts for determination of total 6-phosphofructo-2-kinase activity were obtained. Means $\pm$ S.E.M. of triplicate measurements are shown. One unit of enzyme activity is equivalent to the formation of 1 μ mol of product/min under the assay conditions. N.D., Not determined. Statistically significant differences with respect to control cells are indicated: * $P < 0.05$; ** $P < 0.01$.

	6-Phosphofructo-2-kinase activity (μ -units/mg of protein)			
	30 min	4 h	6 h	24 h
Control	66.89 $\pm$ 2.62	62.19 $\pm$ 5.40	64.95 $\pm$ 5.51	62.43 $\pm$ 7.95
+ HGF	64.95 $\pm$ 5.48	83.33 $\pm$ 3.52**	80.25 $\pm$ 7.02**	109.25 $\pm$ 3.38**
+ TGF- β	N.D.	39.50 $\pm$ 9.93*	N.D.	32.66 $\pm$ 8.38**

Figure 2 Effect of HGF and TGF- β on the levels of 6PF2K/Fru-2,6-BPase protein

Cells were cultured in collagen-coated 10 cm dishes as described in the Materials and methods section. At the indicated incubation time, 30 μ g of total protein was extracted from cultured cells in the presence of 0.1 nM HGF (●) or 50 pM TGF- β (■), and analysed by Western blot using a polyclonal antibody raised against purified adult liver 6PF2K/Fru-2,6-BPase. An equal amount of protein was tested by Coomassie Blue staining of a gel run in parallel. (a) A representative Western blot is shown. (b) The intensity of the autoradiographic signal was quantified and is presented with respect to time zero, which was considered as one arbitrary unit. Data are means $\pm$ S.E.M. from three experiments.

β to primary cultures of hepatocytes. As shown in Table 2, HGF produced an increase in 6-phosphofructo-2-kinase activity, detected 4 h after the addition. In contrast, TGF- β reduced 6PF2K/Fru-2,6-BPase activity to half the control values at 4 and 24 h. To confirm that the assay conditions used to measure kinase activity reflected the amount of enzyme protein, immunoblotting analysis was performed with an antibody raised against the liver

Figure 3 Time course of 6PF2K/Fru2,6-BPase mRNA accumulation after HGF administration

Cells were cultured in collagen-coated 10 cm dishes as described in the Materials and methods section. At the indicated incubation times, total RNA (15 µg/lane) was extracted from cultured cells in the presence (●) or absence (○) of HGF and analysed by Northern blot. The integrity of the RNA was verified by observing the rRNA bands in the ethidium bromide-stained gel under UV irradiation. (a) A representative Northern blot using cDNA for 6PF2K/Fru-2,6-BPase; 18S rRNA is shown. L, Adult liver RNA. (b) The intensity of the autoradiographic signal was quantified and is presented with respect to time zero, which was considered as one arbitrary unit. Data are means ± S.E.M. from three experiments.

isoenzyme. We observed that, whereas HGF increased the amount of 6PF2K/Fru-2,6-BPase, TGF-β decreased it. Under all the conditions, a unique 55 kDa protein band was immuno-detected (Figure 2).

In order to ascertain whether the amount of protein was in agreement with the abundance of 6PF2K/Fru-2,6-BPase mRNA, Northern-blot analysis was performed on RNA from primary cultures of hepatocytes incubated in the presence of HGF or TGF-β. We used a 1.4 kb cDNA probe that recognized the mRNAs of the different isoenzymes. As shown in Figure 3, in the absence of any hormones and growth factors, the levels of 6PF2K/Fru-2,6-BPase mRNA were low. This result agrees with the fact that transcription of this mRNA depends on the presence of glucocorticoids in primary cultures of hepatocytes [19]. However, when HGF was added to the cells, 6PF2K/Fru-2,6-BPase mRNA increased almost 6-fold after 6 h and remained high during the rest of the experiment. At later times, the levels of 6PF2K/Fru-2,6-BPase mRNA decreased, reaching control values at 48 h (results not shown). The 6PF2K/Fru-2,6-BPase mRNA induced by HGF was the same size (2.2 kb) as the adult liver form and no other forms were detected. In contrast, TGF-β had the opposite effect on 6PF2K/Fru-2,6-BPase mRNA levels. As shown in Figure 4, TGF-β caused an almost 3-fold decrease in 6PF2K/Fru-2,6-BPase mRNA abundance in primary

Figure 4 Time course of 6PF2K/Fru2,6-BPase mRNA accumulation after TGF-β administration

Cells were cultured in collagen-coated 10 cm dishes as described in the Materials and methods section. At the indicated incubation times, total RNA (15 µg/lane) was extracted from cultured cells in the presence of 1 µM dexamethasone (control cells) or 50 pM TGF-β and 1 µM dexamethasone (treated cells) at the indicated incubation times and analysed by Northern blot. The integrity of the RNA was verified by observing the rRNA bands in the ethidium bromide-stained gel under UV irradiation. (a) A representative Northern blot using cDNA for 6PF2K/Fru-2,6-BPase; 18S rRNA is shown. (b) The intensity of the autoradiographic signal was quantified and is presented with respect to the control at each time, which was considered as one arbitrary unit. Data are means ± S.E.M. from three experiments.

cultures of hepatocytes when they had been incubated in the presence of dexamethasone.

Recently, the expression of an F-type 6PF2K/Fru-2,6-BPase mRNA has been described in foetal liver, which seems to be expressed in proliferating cells including Rat-1 fibroblasts. This mRNA is the same size as the adult liver mRNA and it arises from a distinct promoter of the same gene [15]. From the results described above we are not able to distinguish whether the 6PF2K/Fru-2,6-BPase mRNA induced by HGF action was the adult liver type (L-type) or another isoform such as F-type. We therefore resorted to the RNase-protection assay. A 6PF2K/Fru-2,6-BPase mRNA-specific riboprobe that coded for the F-exon (1aF exon) was obtained by PCR and hybridized with total RNA from primary cultures of hepatocytes incubated in the presence or absence of HGF and TGF-β. As shown in Figure 5(a), an expected single protected band of 218 bp was observed under all cell culture conditions, including Rat-1 fibroblasts and adult rat liver. The size of this band matches the length of the F-type exon. The results showed that HGF produced an increase in the abundance of the protected fragment, whereas TGF-β had no effect. Indeed, dexamethasone, one of the more potent activators of the expression of the rat liver 6PF2K/Fru-2,6-BPase gene, alone did not affect the levels of the protected 6PF2K/Fru-2,6-BPase mRNA band. However, addition of dexamethasone to primary cultures repressed the activation of 6PF2K/Fru-2,6-BPase mRNA levels by HGF.

Figure 5 RNase-protection assay using an F-type and a liver-specific 6PF2K/Fru-2,6-BPase riboprobe

(a) An F-type-specific 6PF2K/Fru-2,6-BPase riboprobe was obtained by PCR and hybridized with 10 μ g of total RNA from Rat-1 cells, liver tissue and primary cultures of hepatocytes (lanes 1–6), as described in the Materials and methods section. Hepatocytes were cultured for 6 h with: lane 1, control hepatocytes; lane 2, hepatocytes treated with 0.1 nM HGF; lane 3, hepatocytes treated with 50 pM TGF- β ; lane 4, hepatocytes treated with 1 μ M dexamethasone; lane 5, hepatocytes treated with 1 μ M dexamethasone and 0.1 nM HGF; lane 6, hepatocytes treated with 1 μ M dexamethasone and 50 pM TGF- β . (b) The same experiment was performed using a liver-specific riboprobe obtained as described in the Materials and methods section. The arrows point to the RNA-protected fragments.

To investigate the expression of L-type mRNA, we performed the RNase-protection assay using a previously described 6PF2K/Fru-2,6-BPase mRNA-specific riboprobe that contains the L-exon. As predicted (Figure 5b), the liver mRNA showed a major protected fragment 319 bases long corresponding to L-exon, second and third common exons, whereas the major protected fragment in Rat-1 fibroblast was 192 bases long corresponding exclusively to the common exons (results not shown). In primary cultures of hepatocytes, no protected 319-base band was observed in the absence of dexamethasone. When dexamethasone was added to the culture, there was a strong protection of the 319-base band, suggesting that the L-type isoenzyme had been induced. Indeed, HGF and TGF- β inhibited the activation of the L-type isoform by dexamethasone. All these results indicate that, in primary cultures of hepatocyte, HGF activates the expression of the F-type isoenzyme. Dexamethasone activates the L-type isoform and HGF and TGF- β are able to inhibit this activation.

DISCUSSION

We report the modulation of 6PF2K/Fru-2,6-BPase gene expression by HGF and TGF- β . It is known that these growth factors are involved in the mitogenic response after liver damage. HGF has been shown to be the most potent mitogen for hepatocytes and seems to function as a hepatotrophic factor for liver regeneration [25]. The actions of HGF are mediated through a high-affinity receptor, the *c-met* proto-oncogene, which has intracellular tyrosine kinase activity [43]. TGF- β inhibits mitogenesis induced by epidermal growth factor or HGF in primary cultures of hepatocytes. Experiments *in vivo* also implicate TGF- β as an inhibitor of DNA synthesis during liver regeneration [28–30]. The TGF- β receptor belongs to a new class of transmembrane proteins that possess serine/threonine kinase activity [44].

Previous reports have shown that the levels of Fru-2,6- P_2 increase after mitogenic action in various cell lines [6–10]. This increase is consistent with the finding that activation of the glycolytic flux is a common feature of growing cells. Our results demonstrate that HGF activates the expression of the

6PF2K/Fru-2,6-BPase gene by increasing the levels of its mRNA. As a result of this activation, the amount of 6PF2K/Fru-2,6-BPase enzyme and 6-phosphofructo-2-kinase activity rose, which was reflected by an increase in Fru-2,6- P_2 levels. In contrast, TGF- β decreased the levels of 6PF2K/Fru-2,6-BPase mRNA producing a decrease in the amount of 6PF2K/Fru-2,6-BPase enzyme and Fru-2,6- P_2 . The different actions of HGF and TGF- β on 6PF2K/Fru-2,6-BPase gene expression is concomitant with its mitogenic response.

The type of 6PF2K/Fru-2,6-BPase isoenzyme expressed in primary cultures of hepatocytes had not been previously characterized. The results of RNase-protection experiments led us to conclude that, in the absence of any hormones, F-type isoenzyme is expressed in primary cultures of adult hepatocytes. It has been reported that, in contrast with the behaviour of the adult rat liver isoform, cyclic AMP stimulated 6PF2K/Fru-2,6-BPase gene expression in primary cultures of adult hepatocytes at later times [19]. Our suggestion that the F-type isoenzyme is expressed in primary cultures of adult hepatocytes is consistent with the finding that cyclic AMP also stimulates 6PF2K/Fru-2,6-BPase expression in primary cultures of foetal hepatocytes (probably F-type) [45]. HGF stimulates 6PF2K/Fru-2,6-BPase expression of the F-isoform. This F-type mRNA has also been found in other proliferative situations such as foetal liver, preterm placenta and established cell lines [15]. Previous characterization of the F-type promoter has demonstrated its association with *Sp1* and *ets* protein families [15]. HGF could probably modify the phosphorylation-dependent activity of these transcription factors, thus regulating the activity of the F-promoter.

We also demonstrate that dexamethasone is essential for the expression of L-type isoenzyme but not for the F-type. Lange et al. [41] reported the characterization of a glucocorticoid-response element in the first intron of 6PF2K/Fru-2,6-BPase liver gene. The inhibitory action of HGF and TGF- β on the activation of 6PF2K/Fru-2,6-BPase expression by dexamethasone could be due to the interaction of the Jun/Fos family with the glucocorticoid receptor. It has been reported that both Jun and Fos are able to dimerize with glucocorticoid receptor and thus repress its action [46,47]. This interaction could be present in our system. As expected, under our hepatocyte culture conditions the expression of Fos was induced by both HGF and TGF- β (results not shown).

The present study could also help us to understand the mechanism of regulation of 6PF2K/Fru-2,6-BPase expression during liver regeneration. As previously shown [24], transcriptional activation and 6PF2K/Fru-2,6-BPase mRNA accumulation were detected during the first few hours of the replication period of liver regeneration. In this period HGF concentration is increased suggesting that this growth factor could be involved in the mitogenic and metabolic response of the liver [25,26]. Moreover, TGF- β remains low in the first few hours of the replication period (until 20 h after hepatectomy) and then rises sharply, peaking at 72 h [29,30]. The time course of HGF and TGF- β accumulation correlates with the increase and decline in 6PF2K/Fru-2,6-BPase mRNA levels. For all these reasons, although other growth factors and hormones could also contribute to the modulation of 6PF2K/Fru-2,6-BPase gene expression during liver regeneration, we believe that HGF and TGF- β could play a role in this regulation.

In conclusion, the data presented may facilitate our understanding of the regulation of Fru-2,6- P_2 metabolism by HGF and TGF- β . We show that, in addition to allosteric and phosphorylation mechanisms, this regulation occurs via the control of mRNA levels, and that the isoform expressed and regulated is the F-type.

We thank Dr. S. J. Pilgis for the 6PF2K/Fru-2,6-BPase antibody. We also thank Dr. F. Ventura, Dr. T. Roig, M. Dalmau and M. del Mont Llosas for their help and for many valuable suggestions during the course of this work. We also thank C. Ortuño for his skilful technical assistance. We thank R. Rycroft for correcting the English. M.J. is the recipient of a research fellowship (F.P.I.) from the Ministry of Education from Spain. C.S. and S.L. are recipients of research fellowships from the Generalitat de Catalunya. This work was supported by DGICYT (PM92-0248) and DGICYT (PB93-0761).

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