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Transcriptional and posttranscriptional regulation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase during liver regeneration

(gene expression/phospho*enol*pyruvate carboxykinase/fructose 2,6-bisphosphate)

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ABSTRACT **The control of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2; EC 2.7.1.105/3.1.3.46) gene expression during liver regeneration was studied. The level of PFK-2/FBPase-2 mRNA decreased to about 5% of the control value 6 hr after partial hepatectomy. Thereafter the mRNA increased to a maximum at 48 hr and returned to normal levels by 96 hr. In sham-operated animals, only a small increase was observed during the first 4 hr. The mRNA was recognized by a 299-base-pair liver-specific cDNA probe but not by a muscle-specific probe. The time course of mRNA modulation was well correlated with PFK-2/FBPase-2 activity and with the amount of bifunctional enzyme protein determined by immunoblotting with an antibody raised against the N-terminal decapeptide of liver PFK-2/FBPase-2. No alteration in the degradation rate of PFK-2/FBPase-2 mRNA was noted after partial hepatectomy. The modulation of PFK-2/FBPase-2 gene expression during liver regeneration involved changes in the transcription rate. The rate decreased by 50% at 6 hr after liver resection. The rate increased thereafter to a maximum at 72 hr and then returned to control values by 96 hr. The transcription rate of albumin did not change, whereas that of phospho*enol*pyruvate carboxykinase increased 12-fold at 6 hr. These results show that PFK-2/FBPase-2 gene transcription is specifically regulated and that this regulation is in part responsible for the alterations in hepatic metabolism seen in regenerating liver.**

The surgical removal of 70% of the liver induces a partially synchronized growth response that leads to the rapid restoration of organ mass. The molecular signals controlling this growth process are being rapidly defined. Several serum factors, nutrient effects, new growth factors with apparent liver specificity, and gene expression patterns for previously known growth factors control the growth process (for review see refs. 1 and 2). In the liver that remains after partial hepatectomy there are marked changes in composition and energy metabolism even before mitotic activity increases. Hepatic metabolism is shifted from utilization of carbohydrates to increased utilization of lipids (3), and gluconeogenesis increases while glycolysis decreases to maintain glucose homeostasis (4, 5). During this process, fructose 2,6-bisphosphate (Fru-2,6-P_2), the most potent allosteric activator of 6-phosphofructo-1-kinase (PFK-1, EC 2.7.1.11) and inhibitor of fructose-1,6-bisphosphatase (FBPase-1, EC 3.1.3.11), is markedly decreased, resulting in an inhibition of PFK-1 and activation of FBPase-1 (6). In this situation, net gluconeogenesis is enhanced, glycolysis is suppressed, and futile cycling of metabolites is avoided.

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Hormonal regulation of hepatic glycolysis and gluconeogenesis is mediated by phosphorylation/dephosphorylation and control of gene expression of several key regulatory enzymes (7). 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2; EC 2.7.1.105/3.1.3.46) is a bifunctional enzyme that catalyzes both the synthesis and the degradation of Fru-2,6-P_2 (7, 8). This enzyme is regulated by substrates and effectors and by cAMP-dependent protein kinase-catalyzed phosphorylation. Changes in the phosphorylation state of the enzyme are responsible for acute hormonal regulation of Fru-2,6-P_2 levels (8–10) and contribute to the changes in this regulatory metabolite during liver regeneration (6).

Hepatic PFK-2/FBPase-2 is also subject to complex multihormonal long-term control through regulation of its gene expression (11). Although bifunctional enzyme gene expression is not decreased in starvation (12), it is increased by insulin in diabetic rats (12) and by triiodothyronine in hypothyroid rats (13). In adrenalectomized animals, the administration of glucocorticoids increases the PFK-2/FBPase-2 mRNA levels by increasing transcription of the gene (14). In primary cultures of hepatocytes, both insulin and thyroxine act synergistically with glucocorticoids to induce PFK-2/FBPase-2 mRNA (15). PFK-2/FBPase-2 gene transcription is also regulated by insulin, glucocorticoids, and cAMP in rat hepatoma cells (16).

Our objective was to determine whether PFK-2/FBPase-2 gene expression is modulated during rat liver regeneration and, if so, to investigate the mechanism(s) of that regulation. We observed a decrease in PFK-2/FBPase-2 mRNA 6 hr after liver resection, followed by a dramatic increase in the mRNA during liver regeneration. These alterations in mRNA level involved changes in the rate of transcription.

EXPERIMENTAL PROCEDURES

Chemicals. [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq), [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]UTP (3000 Ci/mmol), and nylon membranes were from New England Nuclear and Amersham. Enzymes and other biochemical reagents were from Boehringer Mannheim or Sigma. All chemicals were of analytical grade.

Animals. Fed male Sprague–Dawley rats (180–220 g) were subjected to a 12 hr light/12 hr dark cycle (light starting at 8:00 a.m.). To minimize the diurnal variation in liver DNA synthesis (17), partial hepatectomy (laparotomy and removal of two-thirds of the liver) or sham operation (laparotomy) was

Abbreviations: Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK-2, 6-phosphofructo-2-kinase; FBPase-2, fructose-2,6-bisphosphatase; PFK-1, 6-phosphofructo-1-kinase; FBPase-1, fructose-1,6-bisphosphatase; PEPCK, phospho*enol*pyruvate carboxykinase.

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performed between 8 and 10 a.m. under diethyl ether anesthesia (18). Control rats were not subjected to either anesthesia or surgery. The animals were decapitated, and liver and skeletal muscle were removed and quickly freeze-clamped and placed in liquid nitrogen.

Metabolite and Enzyme Assays. Fru-2,6-P_2 was extracted and measured as described (19). PFK-2 activity was measured at pH 8.5 (20) after partial purification of the extract with PEG-6000 (6–21%). FBPase-2 activity was also measured after partial purification, at pH 7.5 (6). Protein concentration was determined (21) with bovine serum albumin as standard.

Western Blot Analysis. Immunoblot analysis was performed essentially as described (22) with a 1:200 dilution of polyclonal antibody raised against a synthetic decapeptide (GELTQTRLQK) corresponding to the N terminus of liver PFK-2/FBPase-2 (23). This antibody was a gift from Louis Hue (Louvain University, Belgium).

RNA Analysis and DNA Hybridization Probes. Total RNA was extracted from frozen rat tissues by the LiCl/urea method (24). Northern blot analysis was carried out by standard procedures (25). To detect PFK-2/FBPase-2 mRNA from adult liver or muscle, a common 1.4-kilobase (kb) *Eco*RI fragment was isolated from the cDNA for PFK-2/FBPase-2 (12). This fragment contained the sequence transcribed from exons 3–14 of the gene (26, 27). To specifically detect the liver form of the mRNA, a 0.3-kb *Eco*RI/*Ban* II fragment that included only sequences from the first exon was isolated and used as a probe. A muscle-specific probe was obtained from a 1.1-kb *Hae* III restriction fragment isolated from a genomic clone in the phage Charon 4A (16). We also examined the abundance of the mRNA for rat serum albumin as a control, because its level was not changed during liver regeneration (28, 29). A 1.1-kb *Pst* I fragment isolated from a cDNA clone (pRSA 13) for the rat protein (30) was used as a hybridization probe.

Isolation of Liver Nuclei and Run-On Transcription Analysis. Liver nuclei were isolated (31) and the final pellet was resuspended in 40 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/0.1 mM EDTA/40% (vol/vol) glycerol and stored at −80°C. After run-on transcription in isolated nuclei (32), labeled RNA products were extracted and hybridized (33) to nylon membrane containing two tissue-specific DNAs for PFK-2/FBPase-2: the muscle-specific genomic 1.1-kb *Hae* III restriction fragment and the liver-specific genomic 1.6-kb *Eco*RI–*Xba* I fragment (16). The phospho*enol*pyruvate carboxykinase (PEPCK, EC 4.1.1.32) DNA was the pPCK-B7.0 genomic clone (kindly supplied by Richard W. Hanson, Case Western Reserve University, Cleveland) (34). The other DNAs were pRSA 13 for albumin and pBS and pBR322 vectors as controls for background hybridization.

Isolation and Incubation of Hepatocytes. Hepatocytes were prepared from male rats 2 hr after partial hepatectomy and from control rats (20). Actinomycin D was added after the preincubation. At the appropriate times, samples of the cell suspension were removed and centrifuged at 350 × *g* for 5 min at 4°C. The supernatants were discarded and the pellets were frozen in liquid nitrogen. RNA extraction and Northern blot analysis were carried out as described above.

RESULTS

Effect of Partial Hepatectomy on Hepatic PFK-2/FBPase-2 Activities. We reported previously (6) that after liver resection Fru-2,6-P_2 decreased compared with the levels in control animals and did not increase until after the seventh day. These different levels of Fru-2,6-P_2 could be due to changes in the concentration of the bifunctional enzyme and/or to covalent modification. cAMP increased immediately after partial hepatectomy, and cAMP-dependent protein kinase-catalyzed phosphorylation accounted, in part, for the decrease in Fru-2,6-P_2 (6). To determine whether the amount of protein changed after liver resection and during liver regeneration, we measured PFK-2 activity under conditions where phosphorylation had no effect. Under these conditions, activity measurements reflect the amount of enzyme protein (12). We observed a 30% decrease in PFK-2 activity after 12 hr, and activity remained low for 7 days but was restored by 10 days (Fig. 1). Activity was measured after partial purification of the extract by PEG-6000 (6–21%) precipitation with saturating concentrations of Fru-6-*P* and ATP (V_{max} conditions) (9, 20). We have also measured FBPase-2 activity after PEG-6000 fractionation at pH 7.5, under conditions that reflect phosphorylation-induced changes in enzyme activity. There was a decrease in the kinase/bisphosphatase ratio, with a minimum at 12 hr that suggests an increase in the FBPase-2 activity as a consequence of the phosphorylation of enzyme by cAMP-dependent protein kinase, concomitant with an increase in cAMP (6). After 12 hr, the activity ratio was not significantly changed compared with normal values. The results indicate that the decrease in Fru-2,6-P_2 during hepatic regeneration correlates both with a decrease in enzyme protein and with an

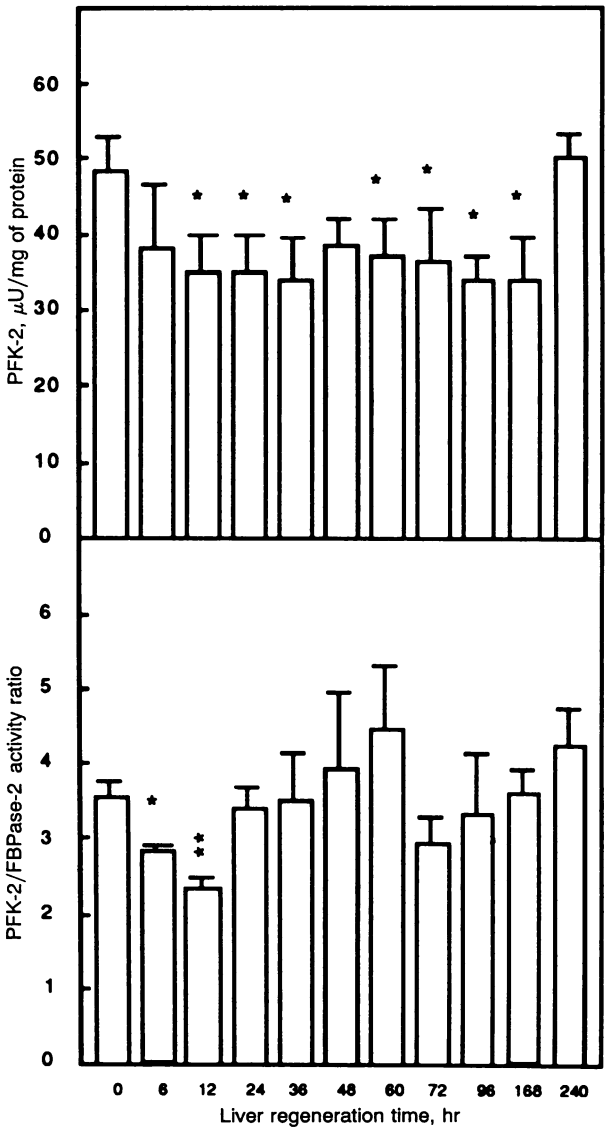

FIG. 1. Effect of partial hepatectomy on PFK-2/FBPase-2 activities. Each bar represents the mean ± SEM for three to five rats. Statistically significant differences between hepatectomized and control animals are indicated: *, $P < 0.05$; **, $P < 0.01$.

increase of enzyme phosphorylation that enhances bisphosphatase activity and inhibits kinase activity.

To confirm that the assay conditions employed to measure kinase activity (Fig. 1) reflected the amount of enzyme protein, we used immunoblotting with an antibody raised against the N-terminal decapeptide of liver PFK-2/FBPase-2 (23). The amount of immunodetectable protein (55 kDa) correlated with kinase activity (Fig. 2).

Time Course of Changes in PFK-2/FBPase-2 mRNA Levels During Liver Regeneration. To determine whether the abundance of PFK-2/FBPase-2 was correlated with the amount of bifunctional enzyme, Northern blot analysis was done with RNA from regenerating liver and a 1.4-kb cDNA probe. The PFK-2/FBPase-2 mRNA had the same size (2.2 kb) as the adult liver form and no other mRNA forms were detected (Fig. 3 *Upper*). The mRNA decreased transiently to ≈5% of the control level within the first 6 hr. The mRNA then increased to a level 4-fold higher than the control, with a maximal mRNA accumulation at 48–60 hr, and returned to near basal level by 96 hr (Fig. 3 *Lower*). This time course correlates very well with the induction of total DNA reported during liver regeneration: within 12–16 hr after hepatectomy, liver cells initiate DNA synthesis and continue to proliferate until the hepatic mass is restored (1, 2).

To study the effect of anesthesia, surgical stress, and/or decreased food intake of hepatectomized animals, we studied the PFK-2/FBPase-2 mRNA accumulation of sham-operated rats by Northern blot analysis (Fig. 4). The PFK-2/FBPase-2 mRNA levels of sham-operated animals were different from those of hepatectomized animals. After a small increase during the first hours, the mRNA returned to basal levels after 6 hr and remained constant for the duration of the experiment.

Since the liver/skeletal muscle PFK-2/FBPase-2 gene encodes two different isozymes by alternative splicing (12, 23, 26), it was of interest to examine which isozyme was expressed during hepatic regeneration. Because the 1.4-kb probe hybridizes with mRNAs of both isozymes, we measured the amount of liver-specific mRNA by using a specific 0.3-kb cDNA probe. This cDNA fragment contains the entire coding region of the first exon of liver-specific transcript, including the nucleotides that encode the decapeptide corresponding to the N terminus of liver PFK-2/FBPase-2. The liver-specific probe hybridized with the RNA from regenerating liver, whereas no hybridization was seen with the RNA from skeletal muscle. The time course of PFK-2/FBPase-2 mRNA accumulation was the same with the 1.4-kb probe and the 0.3-kb probe (data not shown). These results strongly suggest that the isozyme expressed during the hepatic regeneration is the adult liver form.

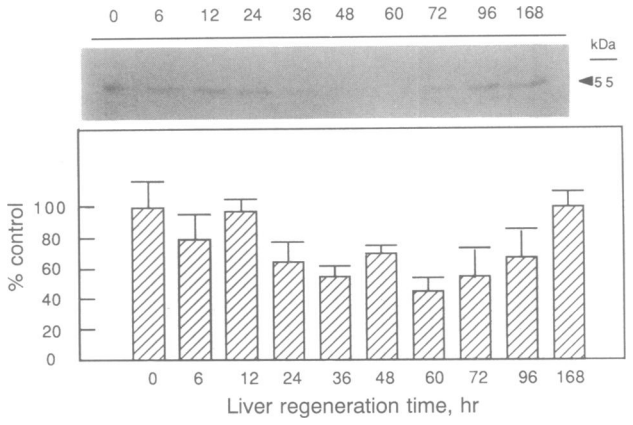

FIG. 2. Amount of PFK-2/FBPase-2 during liver regeneration. Enzyme protein was measured after a fractionation with PEG-6000 (6–21%). Thirty micrograms of protein was used per lane for SDS/12% PAGE and then transferred to nitrocellulose and incubated with the anti-PFK-2/FBPase-2 antibody. (*Upper*) Representative Western blot. (*Lower*) The levels were quantified by densitometric scanning of autoradiograms and expressed relative to the value of normal liver (0 hr), which was taken as 100%. Data are means ± SEM from three to four rats.

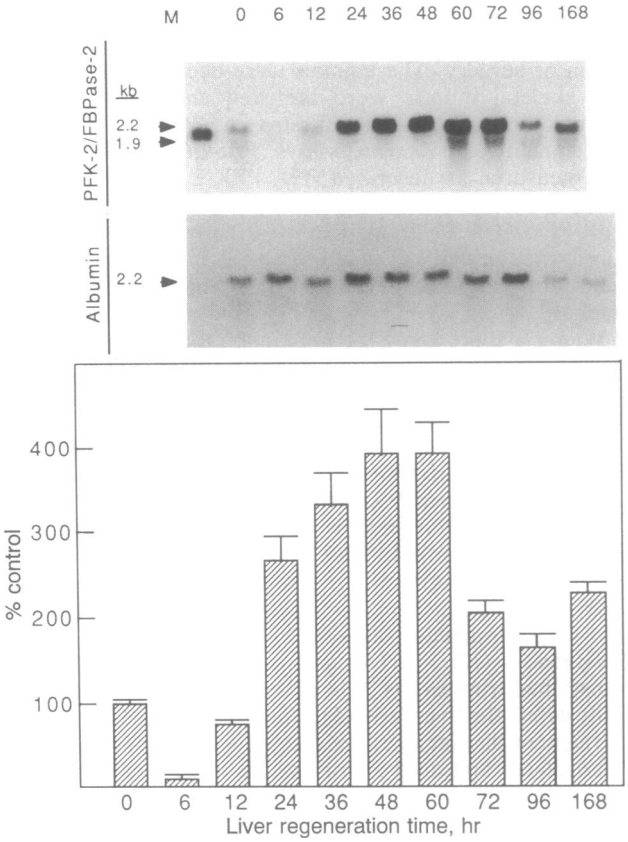

FIG. 3. Expression of PFK-2/FBPase-2 gene during liver regeneration. Total RNA (20 μg per lane) extracted from rat skeletal muscle (M), normal rat liver (0 hr), or regenerating liver after partial hepatectomy was transferred to nylon membranes after electrophoresis and hybridized with cDNA for PFK-2/FBPase-2 (common probe) and serum albumin (pRSA 13). (*Upper*) Representative Northern blot. (*Lower*) The PFK-2/FBPase-2 mRNA level was quantified by densitometric scanning of autoradiograms and corrected for the amount of RNA loaded in each lane by comparison with the bands of the albumin mRNA control. Values are expressed relative to the value of normal liver (0 hr), which was taken as 100%. Data are means ± SEM from three to five rats.

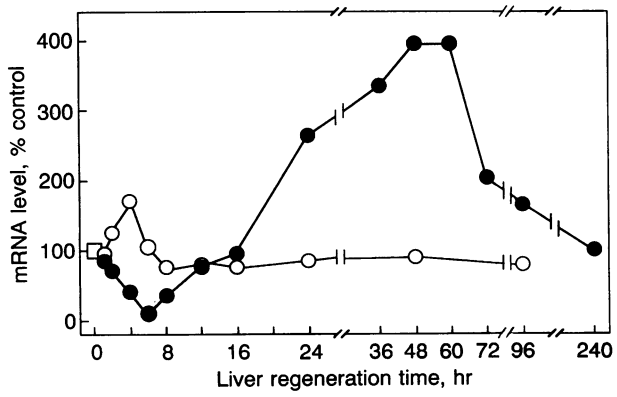

FIG. 4. PFK-2/FBPase-2 mRNA levels of sham-operated and hepatectomized animals during liver regeneration. Northern blot analysis of total RNA (20 μg per lane) extracted from livers of sham-operated (○) and partially hepatectomized rats (●) was done as described for Fig. 3. Data are means from three to five rats.

Transcription Rates of the PFK-2/FBPase-2 and PEPCK Genes During Liver Regeneration. Hepatic proliferation is regulated in a complex manner by hormones, growth factors, and protooncogenes (1, 2). The change in bifunctional enzyme mRNA levels could be due to the action of these factors, through mechanisms involving modulation of transcription, mRNA stability, mRNA processing, transport from the nuclei, or some combination of all these processes. The rate of transcription of the PFK-2/FBPase-2 gene was measured in isolated nuclei from rat liver at 0, 6, 30, 72, and 96 hr after partial hepatectomy. Labeled RNA was extracted and hybridized with two different genomic DNA fragments. These fragments represented the 5′ region of the mRNA for the muscle isozyme, which contains exon 1a, and the 5′ region of the mRNA for the liver isozyme, which contains exon 1b (16). No RNA transcripts were detected by the muscle-specific DNA fragment. In contrast, the rate of transcription was easily measured with the liver-specific DNA (Table 1). Furthermore, significant differences in the rate of gene transcription were observed during the time course of regeneration. The transcription rate reached a minimum of 50% of the control value after 6 hr of partial hepatectomy and then increased to a maximum of 2.3 times the control value at 72 hr (Table 1).

The changes in transcriptional response of the PFK-2/FBPase-2 gene during the proliferative activation of hepatocytes could be due to changes in hormone concentrations. An increase of plasma glucagon (4, 35, 36), corticosterone (4), and catecholamines (37) and a slight decrease of insulin (4, 35, 36) occur after partial hepactectomy. Since transcriptional activation of PEPCK by cAMP and glucocorticoids has been demonstrated in other systems (38, 39), we also analyzed the transcriptional regulation of this gluconeogenic enzyme during liver regeneration. The transcription rate of this gene increased 12-fold at 6 hr after partial hepatectomy, remained high at 30 hr, and decreased at 72 hr (Table 1). The differential response in the transcription rates of the PFK-2/FBPase-2 and PEPCK genes correlates very well with the opposing role of the two enzymes in the regulation of glycolytic/gluconeogenic flux. No significant differences were found in the transcriptional activity of the albumin gene during hepatic proliferation (Table 1), consistent with a specific effect on transcription of the PFK-2/FBPase-2 and PEPCK genes.

The transcriptional regulation of the PFK-2/FBPase-2 gene does not preclude regulation at other pretranslational levels. Therefore, PFK-2/FBPase-2 mRNA turnover may also play a role in the regulation of this gene's expression during liver regeneration. A half-life of 1.5–3.5 hr has been reported for this mRNA (15, 16). Since the largest decrease in mRNA levels was found at 6 hr, and considering its half-life, we determined the stability of this mRNA in hepatocytes obtained from rats 2 hr after partial hepatectomy.

Table 1. Transcription of albumin, PEPCK, and PFK-2/FBPase-2 genes in regenerating rat liver

Regeneration time, hr	Relative rate of gene transcription					
	Albumin		PEPCK		PFK-2/FBPase-2	
	Unit	Fold	Units	Fold	Unit(s)	Fold
0	0.58	1	1.14	1	1.89	1
6	0.56	1.0	13.60	12.0	0.97	0.5
30	0.46	0.8	4.02	4.3	1.94	1.0
72	0.52	0.9	3.04	1.8	4.38	2.3
96	0.65	1.1	6.16	5.4	2.34	1.2

Run-on transcription was assayed with nuclei from livers of normal (0 hr) and partially hepatectomized (6, 30, 72, and 96 hr) rats. Autoradiographs were scanned by laser densitometry, and the values were expressed as arbitrary units after subtraction of the value obtained for vector DNA. Fold values were calculated by assigning a value of 1 to the rate in the control. Values represent averages of 3 separate experiments. The relative rates of transcription for the three genes cannot be compared with one another.

The degradation rate of PFK-2/FBPase-2 mRNA was estimated from the decay rate of the mRNA in hepatocytes incubated in medium containing actinomycin D (5 μg/ml). The half-life of this mRNA in hepatocytes from control rats did not differ from that in hepatocytes obtained 2 hr after partial hepatectomy (2.5 hr in both cases) (data not shown). Our results suggest that mRNA stability is not involved in the change of PFK-2/FBPase-2 mRNA abundance found after partial hepatectomy. It is likely that PFK-2/FBPase-2 mRNA concentration during the prereplicative state of liver regeneration is regulated principally at the transcriptional level.

DISCUSSION

Regenerating liver provides a good system for studying *in vivo* the metabolic changes that occur during cell proliferation. Partial hepatectomy causes quiescent hepatocytes to enter the G_1 phase of the cell cycle. The sequence of events that occur during liver regeneration can be divided into two phases, an initial hypertrophic stage lasting 10–12 hr and a phase of hyperplasia characterized by a large increase in DNA replication followed by cell division. Metabolic changes associated with these phases reflect the differences in their function (1, 2).

In the prereplicative state, metabolic adaptation occurs to permit the reduced number of liver cells to maintain hepatic functions necessary for the survival of the animal. When liver mass is reduced to ≈30% of its original value, an increase in gluconeogenesis and a decrease in glycolysis take place to maintain glucose homeostasis (4, 5). Fru-2,6-P_2, a key regulatory metabolite of liver carbohydrate metabolism, remains low during this state (6). Under these conditions, FBPase-1 activity is high and PFK-1 activity low, and the hepatocyte becomes a producer of glucose. Our results suggest that the decrease in Fru-2,6-P_2 is due to regulation of PFK-2/FBPase-2 enzyme at both the transcriptional and the post-transcriptional level. This regulation probably reflects the synergistic action of multiple factors, including circulating hormones and growth factors (1, 2). The changes found in the kinase/bisphosphatase activity ratio and PFK-2 activity (Fig. 1) provide a plausible explanation for the rapid decrease in Fru-2,6-P_2. The rat liver isozyme has a cAMP-dependent phosphorylation site at Ser-32. The weight of evidence now indicates that the enzyme is phosphorylated immediately after liver resection, probably as a result of the increase in cAMP (6). In addition, the decrease in the PFK-2/FBPase-2 mRNA levels observed after partial hepatectomy is consistent with the decline of transcription rate (Table 1). Although direct evidence is lacking, it is reasonable to postulate that elevated cAMP might be responsible for the decline in transcription observed during the first 6 hr, since cAMP has been shown to decrease PFK-2/FBPase-2 mRNA levels in FTO-2B cells by a transcriptional mechanism (16).

While the PFK-2/FBPase-2 decreases in amount and becomes phosphorylated, another gluconeogenic enzyme, PEPCK, increases in amount immediately after partial hepatectomy (5). The coordinated regulation of blood glucose concentration also involves the enhanced expression of the PEPCK gene at the transcriptional level (Table 1). These results are consistent with the increase in PEPCK mRNA observed after partial hepatectomy (40). Presumably, the expression of PFK-2/FBPase-2 and PEPCK genes is regulated in a reciprocal manner by the same factors. In support of this view, insulin inhibits transcription of the PEPCK gene (41, 42) and increases the transcription of the PFK-2/FBPase-2 gene (16). Although it is likely that glucagon and insulin play an

essential role, other hormones and factors may also mediate PFK-2/FBPase-2 gene regulation (1, 2, 40, 43).

The replicative period of liver regeneration starts at about 12 hr. It is associated with hepatocyte DNA synthesis and the major wave of cell division (1, 2). We previously reported an increase in Fru-2,6-P_2 levels in this mitotic state (6). The data shown here demonstrate that this increase correlates with transcriptional activation and PFK-2/FBPase-2 mRNA accumulation and with an increase in the bifunctional enzyme protein. The changes in transcription rate are probably due to a decrease in glucagon/insulin ratio and/or an increase in glucocorticoid levels (4).

One could argue that the differences in the PFK-2/FBPase-2 mRNA accumulation found during liver regeneration were due to surgical stress, anesthesia, and/or differences in nutritional state. The differences in PFK-2/FBPase-2 mRNA accumulation found between sham-operated and hepatectomized animals suggest that stress and anesthesia do not influence the effect of hepatectomy. The modest early increase in PFK-2/FBPase-2 mRNA in sham-operated rats may be a consequence of surgical stress and/or anesthesia, as has been shown for the mRNAs of other proteins (28). A role for nutritional factors during liver regeneration has been reported (1, 17, 44). However, they do not play a role in hepatectomy-induced changes in PFK-2/FBPase-2 mRNA, since there is no difference between the PFK-2/FBPase-2 mRNA levels of fed rats and rats starved for up to 72 hr (12). Our results show that the PFK-2/FBPase-2 mRNA levels of sham-operated rats are almost constant during the circadian cycle (Fig. 4). Also, it does not appear that the increase in PFK-2/FBPase-2 mRNA was a result of the fasted/refed condition, because it has been shown that hepatectomized animals eat, albeit less, during the regeneration process (ref. 45 and data not shown). These results indicate that the changes in the PFK-2/FBPase-2 mRNA levels during liver regeneration are not dependent on the nutritional state of the animals.

Several PFK-2/FBPase-2 isozymes have been described in different tissues (10, 16, 23, 46). These isoforms show differences in their PFK-2/FBPase-2 activity ratio, in their kinetic and antigenic properties, in their sensitivity to phosphorylation by protein kinases, and in their protein molecular weight and mRNA size. The results reported here suggest that the isozyme expressed during liver regeneration is the adult liver form. The kinetic differences previously reported (6) could reflect some posttranslational change.

In conclusion, PFK-2/FBPase-2 gene expression is regulated in response to hepatic insult. This modulation is probably mediated by hormones and other factors that regulate transcriptional and posttranscriptional events and is consistent with the important physiological role of PFK-2/FBPase-2 in the control of hepatic glycolysis and gluconeogenesis.

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