Universitat de Barcelona

Estudio de la regulación del metabolismo hidrocarbonado durante la regeneración hepática

FARMA

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Fructose 2,6-bisphosphate and 6-phosphofructo-2-kinase during liver regeneration

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Glycogen and fructose 2,6-bisphosphate levels in rat liver decreased quickly after partial hepatectomy. After 7 days the glycogen level was normalized and fructose 2,6-bisphosphate concentration still remained low The 'active' (non-phosphorylated) form of 6-phosphofructo-2-kinase varied in parallel with fructose 2,6-bisphosphate levels, whereas the 'total' activity of the enzyme decreased only after 24 h, similarly to glucokinase The response of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase from hepatectomized rats (96 h) to *sn*-glycerol 3-phosphate and to cyclic AMP-dependent protein kinase was different from that of the enzyme from control animals and similar to that of the foetal isoenzyme.

INTRODUCTION

In the liver tissue which remains after partial hepatectomy, marked changes in the composition and the energy metabolism develop even before the occurrence of increased mitotic activity The hepatic metabolism is shifted from a predominant utilization of carbohydrates to an increased utilization of lipids (Simek *et al*., 1967) An increase in gluconeogenesis and a decrease in glycolysis take place in order to maintain the glucose homoeostasis during the phase of rapid cell proliferation (Leffert *et al*., 1979, Katz, 1979) The changes in the main carbohydrate metabolic pathways occur in parallel with an increase in glucagon (Morley *et al*., 1975, Bucher & Weir, 1976, Leffert *et al*., 1979), corticosterone (Leffert *et al*., 1979) and catecholamines (Cruise *et al* 1987) and with a slight decrease in insulin in periportal blood (Morley *et al*., 1975, Bucher & Weir, 1976, Leffert *et al*., 1979) These hormonal variations suggest that the rapid change in the glycolytic/gluconeogenic flux is probably due to an increase in cyclic AMP (Koide *et al*., 1978), which induces phosphorylation of the enzymes susceptible to modification by the cyclic AMP-dependent protein kinase However, Fru-2,6-P_2 could also play an important role in this system Fru-2,6-P_2, which is the most potent allosteric activator of 6-phosphofructo-1-kinase and inhibitor of fructose-1,6-bisphosphatase, has a significant function in the regulation of the glycolytic/gluconeogenic pathway in the liver The synthesis and the breakdown of Fru-2,6-P_2 are produced by the bifunctional enzyme PFK-2/FBPase-2 The regulation of this enzyme is a complex function of the influence of substrates and effectors as well as its phosphorylation state via cyclic AMP-dependent protein kinase, in addition to the control of the enzyme levels (Hue & Bartrons, 1985, Van Schaftingen, 1987, Hue & Rider, 1987, Pilkis & El-Maghrabi, 1988)

During hepatic regeneration, some transitions from adult liver-type isoenzymes to other isoenzyme patterns corresponding to a less differentiated state have been described This is the case for glucokinase (Sato *et al*., 1969) and L-type pyruvate kinase (Bonnet *et al* 1973 Garnett *et al*., 1974) which undergo transitions to isoenzymic forms similar to the foetal enzymes

In this experimental model, we have analysed the variations in glycogen and Fru-2,6-P_2 levels and in PFK-2 activity during the first week of liver regeneration We have also determined the changes in the main regulatory properties of PFK-2/FBPase-2, in order to detect a possible isoenzymic transition similar to that described in hepatoma cells (Loiseau *et al*., 1988)

EXPERIMENTAL

Chemicals

Enzymes and biochemical reagents were from either Boehringer Mannheim or Sigma All other chemicals were of analytical grade

Animals

Fed male Sprague–Dawley rats (180–220 g) were subjected to a 12 h-light/12 h-dark cycle (light periods starting at 08 00 h) Partial hepatectomy (comprising laparotomy and removal of two-thirds of the liver) or sham operation (laparotomy) was performed between 08 00 and 10 00 h, under diethyl ether anaesthesia and by the procedure described by Higgins & Anderson (1931) Control rats were not subjected to either anaesthesia or surgery The animals were killed by decapitation and the livers were removed and quickly freeze-clamped in liquid N_2

Metabolite assays

Fru-2,6-P_2 was extracted and measured as described by Van Schaftingen *et al* (1982) Liver glycogen was isolated as described by Carrol *et al* (1956), and the amount of glucose produced by acid hydrolysis was determined as described in Kunst *et al* (1984) Glycerol 3-phosphate was extracted and measured as described by Lang (1984) Concentrations of glucose 6-phosphate and fructose 6-phosphate were measured fluorimetrically in 0.5 M-HCl extracts by the method of Lang & Michal (1974) Cyclic AMP was measured in these extracts with the cyclic AMP assay kit from Amersham Ribonucleotide concentrations were determined with a h.p.l.c. system The liver was homogenized in 10 vol. of 5% (w/v) trifluoroacetic acid and, after

Abbreviations used Fru-2,6-P_2, fructose 2,6-bisphosphate, PFK-2 6-phosphofructo-2-kinase (EC 2.7.1.105), FBPase-2 fructose-2,6-bisphosphatase (EC 3.1.3.46)

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centrifugation for 5 min at 12000 *g*, the supernatant was neutralized with 0.5 M-acetic acid/acetate buffer, pH 4.0. Then 20 μl of the neutralized sample was used for the determination (Itakura *et al.*, 1986).

Enzyme assays

The 'total' PFK-2 activity and the 'active' PFK-2, corresponding to the activity of the non-phosphorylated enzyme, were measured as described by Bartrons *et al.* (1983). FBPase-2 activity was measured by the production of [^{32}P]P$_i$ from [2-^{32}P]Fru-2,6-P_2, which was synthesized as described by El-Maghrabi *et al.* (1982). The reaction was carried out at 30 °C in 50 mM-Hepes buffer (pH 7.5) containing 50 mM-KCl, 5 mM-KH_2PO_4, 2 mM-EDTA, 1 mM-dithiothreitol, 2 mM-$MgCl_2$, 0.1 mM-$NADP^+$, 5 μM-[2-^{32}P]Fru-2,6-P_2 (200000 c.p.m./assay), 9 units of phosphoglucoisomerase/ml, 4 units of glucose-6-phosphate dehydrogenase/ml, and in the presence or absence of *sn*-glycerol 3-phosphate. Blanks typically did not exceed 0.1%, of the applied radioactivity. Hexokinase activity was calculated as the glucose-phosphorylation capacity at 0.5 mM-glucose, and glucokinase activity as the difference between the glucose-phosphorylation capacity at 100 mM- and at 0.5 mM-glucose, by using the continuous assay described by Davidson & Arion (1987).

One unit of enzyme activity represents the activity that catalyses the formation of 1 μmol of product/min under the assay conditions except for the catalytic subunit of cyclic AMP-dependent protein kinase which is defined (Sigma P-2645) as the activity that transfers 1 pmol of phosphate from [γ-^{32}P]ATP to hydrolysed and partially dephosphorylated casein/min at pH 6.5 and 30 °C.

Partial PFK-2 purification

Liver was homogenized in 10 vol. of 20 mM-KH_2PO_4 buffer, pH 7.1, containing 10 mM-EDTA, 100 mM-KF and 1 mM-dithiothreitol. After centrifugation at 27000 *g* for 30 min, the supernatant was fractionated with polyethylene glycol (6–21%). The pellet was resuspended in 1 ml of homogenizing medium and used to measure PFK-2 activity. To measure FBPase-2 activity the enzyme was purified by the same procedure, but in 50 mM-Hepes, 50 mM-KCl, 5 mM-KH_2PO_4, 0.1 mM-EDTA, 1 mM-dithiothreitol, 0.5 mM-phenylmethanesulphonyl fluoride, at pH 7.5 in homogenizing medium.

Other methods

Proteins were measured as described by Bradford (1976) with bovine serum albumin as standard. Statistical significance of differences was assessed by Student's unpaired *t* test.

RESULTS

Fru-2,6-P_2 and glycogen contents during liver regeneration

As shown in Fig. 1, after partial hepatectomy both the level of Fru-2,6-P_2 and glycogen stores decreased very quickly to very low values. By 7 days after hepatectomy liver weight and glycogen content were nearly normalized (91% and 76% respectively), whereas Fru-2,6-P_2 levels still remained low (40%). In the sham-operated group of animals, the metabolites diminished to a much lesser extent. Fru-2,6-P_2 content was restored after 6 h, whereas glycogen stores needed 24 h to regain control values, coinciding with the refeeding period of the animals (dark cycle).

Time course of PFK-2 activity

In order to explain the decrease observed in Fru-2,6-P_2 concentration after partial hepatectomy, we have determined the activity responsible for the synthesis of the metabolite using kinetic measurements of the 'total' and the 'active' (non-phosphorylated) form of the enzyme. As shown in Fig. 2, after partial hepatectomy the total PFK-2 activity decreased only after 24 h, attained significantly lower values at day 4, and did not recover to normal levels during the period studied. In sham-operated animals the 'total' PFK-2 activity was not modified with respect to control rats. The 'active' PFK-2 activity rapidly decreased in hepatectomized animals, reached 23% of sham-operated activity after 6 h, and recovered moderately, 56%, after 7 days of liver resection. In sham-operated animals, the 'active' PFK-2 varied significantly only after 24 h.

In hepatectomized animals the 'active'/'total' PFK-2 activity ratio paralleled the changes in the 'active' form of the enzyme, reaching minimum values 6 h after the surgical resection, and was normalized after 7 days.

The levels of cyclic AMP rapidly increased in the remnant liver after resection (Fig. 3). This suggests that the decrease in the 'active' PFK-2 activity after partial hepatectomy is mainly a consequence of the phosphorylation of the enzyme by the cyclic AMP-dependent protein kinase.

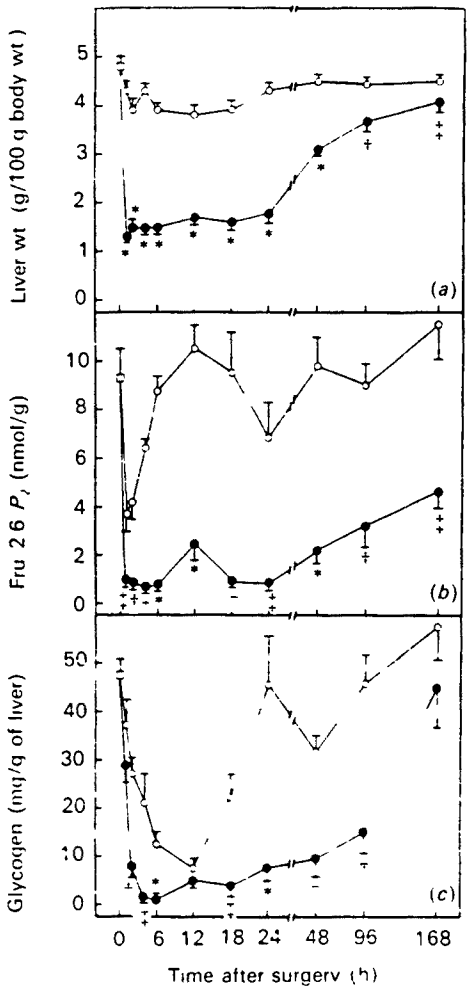

Fig. 1. Fru-2,6-P_2 and glycogen contents during liver regeneration

Liver weight (*a*), Fru-2,6-P_2 (*b*) and glycogen (*c*) concentrations from hepatectomized (●) and sham-operated (○) animals and controls (□) are shown. Each point shows the mean ± S.E.M. for 4–9 rats. Statistically significant differences between the hepatectomized and sham-operated animals are indicated by * $P < 0.001$, + $P < 0.01$ and ‡ $P < 0.05$.

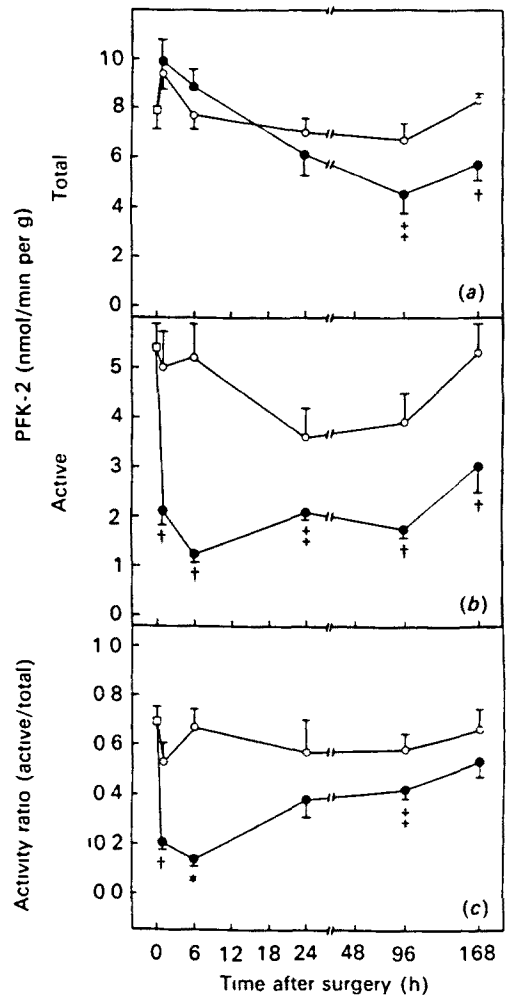

Fig. 2. Time course of PFK-2 activity

Hepatectomized (●), sham (○) and control (□) animals were assayed for (*a*) the 'total' PFK-2 activity (pH 8 5), (*b*) the 'active' form of the enzyme (pH 6 6) and (*c*) the 'active'/'total' activity ratio Each point shows the mean ± S E M for 5–8 rats Statistically significant differences between the hepatectomized and sham operated animals are indicated by * $P < 0.001$, † $P < 0.01$ and ‡ $P < 0.05$

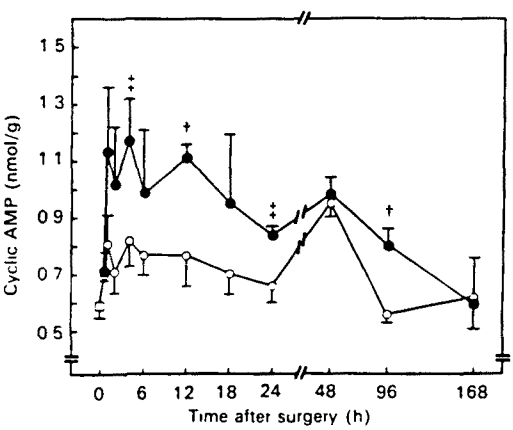

Fig. 3. Cyclic-AMP levels after partial hepatectomy

The concentrations of cyclic AMP were measured in hepatectomized (●), sham-operated (○) and control (□) animals Each point shows the mean ± S E M for 4–10 rats Statistically significant differences between the hepatectomized and sham operated animals are indicated by † $P < 0.01$ and ‡ $P < 0.05$

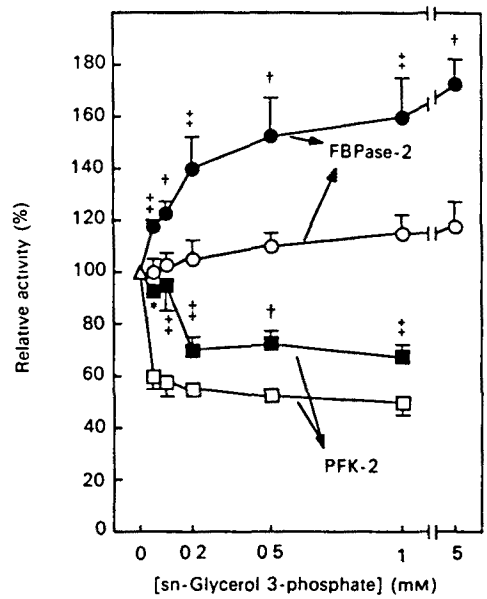

Fig. 4. Effect of *sn*-glycerol 3-phosphate on PFK-2/FBPase-2 activities

Partially purified PFK-2/FBPase-2 from 96 h-hepatectomized (●, ■) and sham-operated (○, □) animals was used PFK-2 was assayed in the presence of 0 1 mM-fructose 6-phosphate and 0 5 mM-ATP-Mg^{2+}, and with the indicated concentrations of *sn*-glycerol 3-phosphate, at pH 7 1 FBPase-2 was determined as reported in the Experimental section The 100% activities (△) for hepatectomized and sham-operated animals were 3 9 ± 1 2 and 15 4 ± 2 9 μ-units/mg of protein for PFK-2, and 9 3 ± 1 2 and 9 2 ± 0 5 μ-units/mg of protein for FBPase-2 The values are means ± S E M for 4–5 rats Statistically significant differences between hepatectomized and sham-operated animals are indicated by * $P < 0.001$, † $P < 0.01$ and ‡ $P < 0.05$

Changes in the effects of *sn*-glycerol 3-phosphate and the catalytic subunit of cyclic AMP-dependent protein kinase on PFK-2 activity

In order to establish if PFK-2 in regenerating liver underwent an isoenzymic transition similar to that found in rat hepatoma cells (Loiseau *et al*., 1988), we have compared the effects of *sn*-glycerol 3-phosphate and the catalytic subunit of cyclic AMP-dependent protein kinase on the hepatic PFK-2 activity from sham-operated and hepatectomized animals It has been reported that PFK-2 isoenzyme from adult liver is more sensitive than the isoenzyme forms from heart (Rider *et al*., 1985), hepatoma cells (Loiseau *et al*., 1988) and foetal liver (Martin-Sanz *et al*., 1987) to *sn*-glycerol 3-phosphate inhibition In contrast with the hepatic isoenzyme, those isoenzymes are not inactivated by cyclic AMP-dependent protein kinase (Hue & Rider, 1987, Loiseau *et al*., 1988, Martin-Sanz *et al*., 1987)

The hepatectomized or sham-operated animals were tested 96 h after surgical treatment. This time corresponded to the highest levels of hexokinase activity observed during the hepatic regeneration (Fig 5) and to the maximal isoenzymic transition described for pyruvate kinase (Bonney *et al*., 1973) As shown in Fig 4, PFK-2 activity from regenerating rat liver was markedly less inhibited by *sn*-glycerol 3-phosphate than was the activity from sham-operated animals In contrast, the FBPase-2 activity was more sensitive and more activated The PFK-2/FBPase-2 activity ratio (at pH 8 5 for the kinase activity, and at pH 7 5 with 5 mM-glycerol 3-phosphate for the phosphatase activity) increased from 5.3 ± 0 9 in hepatectomized to 8 0 ± 1.0 in sham-operated animals ($P < 0.05$)

Incubation with the catalytic subunit of cyclic AMP-dependent

Table 1. Effect of treatment of PFK-2 with the catalytic subunit of protein kinase

Partially purified PFK-2 from 96 h-hepatectomized and sham-operated animals were incubated with 50 m-units of catalytic subunit of protein kinase in a final volume of 0·1 ml containing 20 mM-Hepes buffer pH 7·0 100 mM-KCl 5 mM-$MgCl_2$, 1 mM-ATP Mg^{2+} and 1 mM-dithiothreitol at 30 °C for 10 min. Samples (40 µl) were taken to measure PFK-2 activity. Each value represents the mean ± S.E.M. for 3–5 rats. Statistically significant differences between the hepatectomized and sham-operated animals are indicated by * $P < 0.01$.

Animals	Addition	PFK-2 activity (µ-units/mg of protein) pH 6·6	pH 8·5
Hepatectomized	None	43 ± 8	120 ± 31
	+ catalytic subunit	42 ± 5	131 ± 13
Sham-operated	None	101 ± 5	144 ± 5
	+ catalytic subunit	64 ± 8*	149 ± 4

protein kinase did not modify the 'active' PFK-2 activity from the hepatectomized animals, whereas it inhibited the enzyme from sham-operated animals. The 'total' PFK-2 was not affected in any case (Table 1). One could argue that the absence of inactivation of the regenerating PFK-2 by cyclic-AMP dependent protein kinase could result from the fact that the enzyme was already in a fully phosphorylated form, as suggested by Loiseau *et al.* (1988). However, no activation was observed when regenerating liver extracts were incubated with $MgCl_2$ (2 mM, 10 min at 30 °C) to stimulate phosphoprotein phosphatase activity (Pelech *et al.* 1984).

Time course of glucokinase and hexokinase activities

Several enzymes undergo isoenzymic transitions during liver regeneration (Sato *et al.*, 1969; Bonney *et al.*, 1973). Therefore, we studied the change in the activity of the adult liver glucokinase and the less differentiated hexokinase after partial hepatectomy.

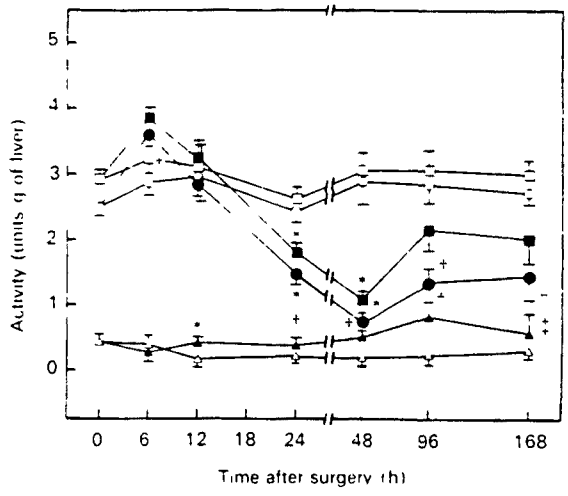

Fig. 5. Time course of glucokinase and hexokinase activities

Hexokinase (▲ △) glucokinase (● ○) and total (glucokinase + hexokinase) activities (■ □) were measured in hepatectomized (▲ ● ■) and sham-operated (△ ○ □) animals. Values at zero time represent control animals. Each point shows the mean ± S.E.M. for 4–6 rats. Statistically significant differences between hepatectomized and sham-operated animals are indicated by * $P < 0.001$ † $P < 0.01$ and ‡ $P < 0.05$.

As shown in Fig. 5, glucokinase activity decreased 12 h after hepatectomy, attained a minimum value (25% of sham-operated activity) at 48 h and recovered partially after 96 h. In contrast, the hexokinase activity showed higher activities after 12 h of liver resection and attained a maximal value at 96 h, corroborating the isoenzymic transition. The total (glucokinase + hexokinase) activity did not change in sham-operated animals and followed a parallel pattern to that of glucokinase in hepatectomized rats.

DISCUSSION

It is known that after partial hepatectomy the decrease in liver tissue is accompanied by complex hormonal changes, qualitatively resembling those observed in response to starvation which produce in the liver remnant an increase in glycogenolysis and gluconeogenesis, and a decrease in glycolysis, in order to maintain the blood glucose concentration even during food restriction (Katz, 1979; Petenusci *et al.*, 1983; Holness *et al.*, 1989). After partial hepatectomy, lactate formation from glucose was decreased to less than 20% of the control, whereas lactate formation from fructose remained unaltered (Katz, 1979). In addition, it has been demonstrated that regenerating liver has an abnormal response of hepatic lipid synthesis to glucose in the fed state (Holness *et al.*, 1989). The altered glycolytic rate with glucose as substrate was ascribed to a decrease in hepatic glucokinase (Katz, 1979), whereas the insensitivity of hepatic lipid synthesis to changes in the carbohydrate supply has been attributed to inactivation of the hepatic pyruvate dehydrogenase (Holness *et al.*, 1989).

The results reported herein indicate that, in addition to the glucokinase and pyruvate dehydrogenase restriction, the fructose 6-phosphate/fructose 1,6-bisphosphate cycle can be an important regulatory step, modulating the glycolytic/gluconeogenic flux during liver regeneration. We have observed that in the first 24 h before initiation of DNA synthesis, the contents of glycogen and Fru-2 6-P_2 decreased quickly to very low values (Figs. 1*b* and 1*c*), probably as a consequence of the rise in cyclic AMP levels (Fig. 3) secondary to the changes in the glucagon/insulin ratio (Leffert *et al.*, 1979) and in catecholamine levels (Cruise *et al.*, 1987). Under these conditions, stimulation of glycogen phosphorylase kinase and concomitant inactivation of PFK-2 and activation of FBPase-2, resulting from phosphorylation of these enzymes by cyclic AMP-dependent protein kinase (Bartrons *et al.*, 1983) would cause an increased degradation of glycogen and a decrease in Fru-2,6-P_2 levels. The fast decrease in the 'active' form of PFK-2 observed after hepatectomy (Fig. 2*b*) would agree with this hypothesis.

Other factors involved in PFK-2 inactivation could be the variations in the concentration of different metabolites that modulate the bifunctional PFK-2/FBPase-2. We have found that hexose 6-phosphates decreased transiently after 6 h hepatectomy (from 578 ± 37 nmol/g in sham-operated animals to 100 ± 29 nmol/g, $P < 0.001$). ATP levels did not change significantly (results not shown) and glycerol 3-phosphate content was not significantly altered after 24 h hepatectomy (from 1·2 ± 0·5 µmol/g in sham-operated animals to 0·90 ± 0·07 µmol/g). It has been reported that phosphoenolpyruvate (Schofield *et al.*, 1986) and citrate (Schofield *et al.*, 1987) levels were increased after 24 h of partial liver resection. These changes could be especially important, since both phosphoenolpyruvate and citrate are known to exert a dual inhibitory action on PFK-2: in addition to inhibiting PFK-2 activity directly, they promote the cyclic AMP-dependent phosphorylation of the bifunctional PFK-2/FBPase-2 (Van Schaftingen *et al.*, 1984).

The fall in Fru-2,6-P_2 concentration produced by the mechanisms discussed above could lead to an inhibition of 6-phospho-

fructo-1-kinase and to an activation of fructose-1.6-bisphosphatase In this situation, gluconeogenesis occurs and glycolysis is stopped, so that futile cycling of metabolites is avoided The significance of the restriction of the glycolytic flux at the level of fructose 6-phosphate/fructose 1.6-bisphosphate cycle must be essential during the first day of liver resection, when glycogenolysis is activated mainly to provide glucose in order to maintain glycaemia, and when glucokinase is not yet decreased The role of glucokinase to lower the glycolytic flux should be more important after 12 h of liver regeneration, since it is in this phase when the enzyme begins to decrease

After 24 h of liver resection the stimulation of gluconeogenesis seems to be maintained through changes in the concentration of regulatory enzymes, in addition to the enzymic cyclic AMP-dependent phosphorylations As reported herein 'total' PFK-2 (Fig 2*a*) and glucokinase (Fig 5) activities remained decreased, even 7 days after hepatectomy Pyruvate kinase and pyruvate dehydrogenase activities were also decreased in regenerating liver (Schofield *et al*., 1986), whereas phosphoenolpyruvate carboxykinase and glucose-6-phosphatase activities were increased (Katz 1979) All these results can contribute to explain the higher glucose production seen in the regenerating liver (Petenusci *et al*., 1983)

The results reported herein showing that in hepatectomized rats glycogen stores are replenished faster than Fru-2 6-P_2 levels suggest that glycogenesis could be sustained through the indirect pathway, the bulk of liver glycogen being of gluconeogenic origin Fru-2 6-P_2 levels remain low during glycogen repletion as has been found during refeeding (Katz & McGarry 1984 Kurland & Pilkis 1989) In contrast in the sham-operated group of animals Fru-2 6-P_2 is recovered faster than glycogen (Fig 1) This suggests that after the first hours of surgical stress when the active total PFK-2 activity ratio is not significantly modified (Fig 2) glycogen breakdown could produce the observed recovery of Fru-2 6-P_2 levels by providing substrate for its synthesis Glycogen seems to be deposited essentially during the refeeding period (after 12 h) in the dark phase and in the presence of Fru-2 6-P_2 This would indicate that in this experimental condition either the direct pathway is prevailing or factors other than Fru-2 6-P_2 are influencing the glycogen deposition A similar situation has been described after sucrose refeeding (Kuwajima *et al* 1986, McGarry *et al* 1987)

A shift from adult to foetal isoenzyme expression has been described for several glycolytic enzymes in hepatomas (Weinhouse 1983) and during liver regeneration (Sato *et al* 1969 Bonney *et al* 1973 Garnett *et al* 1974) Recently an isoenzymic transition has been found for PFK-2 in rat hepatoma cells suggesting the attractive hypothesis that hepatoma-cell PFK-2 could be the foetal form of the enzyme (Loiseau *et al*., 1988) The results now reported show that regenerating-liver PFK-2 was less inhibited and that regenerating FBPase-2 was more stimulated by *sn*-glycerol 3-phosphate than was the respective enzyme of normal liver In addition regenerating-liver PFK-2 could not be modified by the catalytic subunit of cyclic AMP-dependent protein kinase Taken together these results seem to indicate that regenerating-liver PFK FBPase-2 is different from that of normal liver and possesses a kinetic behaviour similar to that of the foetal isoenzyme (Martin-Sanz *et al* 1987 1989)

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Transcriptional and Post-Transcriptional Regulation of 6-Phosphofructo-2-kinase/Fructose2,6-Bisphosphatase During Liver Regeneration

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by

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Abbreviations used: Fru-2,6-P2, fructose 2,6-bisphosphate; PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); FBPase-2, fructose-2,6-bisphosphatase (EC 3.1.3.46); PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); FBPase-1, fructose-1,6-bisphosphatase (EC 3.1.3.11); PEPCK, phosphoenolpyruvate carboxykinase (EC 4.1.1.32).

ABSTRACT

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

INTRODUCTION

The surgical removal of 70 % of the liver induces a partially synchronized growth response which 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 1 and 2). In the liver which 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, Fru-2,6-P2, the most potent allosteric activator of PFK-1 and inhibitor of FBPase-1, 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.

Hormonal regulation of hepatic glycolysis and gluconeogenesis is mediated via phosphorylation/dephosphorylation and control of gene expression of several key regulatory enzymes (7). PFK-2/FBPase-2, is a bifunctional enzyme that catalyzes both the synthesis and degradation of Fru-2,6-P2 (7,8). This enzyme is regulated by substrates and effectors, and by cyclic AMP-dependent protein kinase-catalyzed phosphorylation. Changes in the phosphorylation state of the enzyme are responsible for acute hormonal regulation of Fru-2,6-P2 levels (8,9,10) and also contribute to the changes in this regulatory metabolite during liver regeneration (6).

There are at least two genes for the mammalian bifunctional enzyme (12,12a). The rat liver/skeletal muscle PFK-2/FBPase-2 gene is at least 55 Kb in length and contains 15 exons (12). This gene encodes at least two isoenzymes in a tissue-specific manner by alternative splicing from two promoters. The skeletal muscle-specific transcript differs from the liver-specific transcript in the first exon. Exons 2 to 14 are common to both messages. The second gene appears to be expressed only in heart (12a).

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 (13), it is increased by insulin in diabetic rats (13) and by triiodothyronine in hypothyroid rats (14). In adrenalectomized animals, the administration of glucocorticoids increases the mRNAPFK-2/FBPase-2 levels by increasing transcription of the gene (15). In primary cultures of hepatocytes, insulin and thyroxine both act synergistically with glucocorticoids to induce mRNAPFK-2/FBPase-2 (16). PFK-2/FBPase-2 gene transcription is also regulated by insulin, glucocorticoids and cyclic AMP in rat hepatoma cells (17).

The objective of the present report 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 report here a decrease in bifunctional enzyme mRNA 6 hours after liver

resection which is followed by a dramatic increase in the mRNA during liver regeneration. Our data also demonstrate that these alterations in bifunctional enzyme mRNA involve changes in the rate of gene transcription.

EXPERIMENTAL PROCEDURES

Chemicals: (γ-32P)ATP (3000 Ci/mmol), (α-32P)dCTP (3000 Ci/mmol) and (α-32P)UTP (3000 Ci/mmol) were from New England Nuclear and Amersham. The random primed DNA labeling kit and restriction endonucleases were from Boehringer Mannheim. GeneScreen and N-hybond membranes were the product of Du Pont-New England Nuclear and Amersham, respectively. Enzymes and other biochemical reagents were either 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 h-light/12 h-dark cycle (light periods starting at 08:00 h). To minimizal the diurnal variation in liver DNA synthesis (46), partial hepatectomy (comprising laparotomy and removal of two-thirds of the liver) or sham operation (laparotomy) was performed between 8 and 10 a. m., under diethyl ether anaesthesia and by the procedure reported by Higgins & Anderson (18). Control rats were not subjected to either anaesthesia or surgery. The animals were killed by decapitation. Liver and skeletal muscle were removed and quickly freeze-clamped and placed into liquid nitrogen.

Metabolite and Enzyme assays: Fru-2,6-P2 was extracted and measured as described by Van Schaftingen et al. (19). PFK-2 activity was measured at pH 8.5 as described by Bartrons et al. (20) after partial purification of the extract with PEG-6000 (6-21 %). The FBPase-2 activity was also measured after partial purification at pH 7.5 as described by Rosa et al. (6). The protein concentration was determined according to Bradford (21), using bovine serum albumin as standard. One unit of enzyme activity catalyzes the formation of 1 μmol of product/min under the specified assay conditions.

Western blot analysis: Immunoblot analysis were performed essentially as described by Burnette (22) with a 1:200 dilution of the polyclonal antibody raised against the synthetic decapeptide: GELTQTRLQK corresponding to the N-terminus of liver PFK-2/FBPase-2 (23). This antibody was a kind gift from Dr. Louis Hue (Louvain University, Belgium). The filters were incubated with ^{125}I-labeled Protein A (0.5-1.0 X 10^6 cpm/ml) for 30 min and, after washing, exposed to X-ray film. Bands of the autoradiogram were quantified by laser densitometry.

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 using standard procedures (25). To detect mRNAPFK-2/FBPase-2 from either adult liver or muscle a common

1.4 kb Eco RI fragment was isolated from the cDNA for PFK-2/FBPase-2 (13). This fragment contains the sequence transcribed from the third to fourteenth exon of the gene (12). To specifically detect the liver form of mRNAPFK-2/FBPase-2, a 0.3 kb Eco RI/BanII fragment, which includes 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 as previously reported (17). We also examined the abundance of the mRNA for rat serum albumin as a control because its level was not changed during liver regeneration (27,28). A 1.1 kb Pst I fragment isolated from a cDNA clone (pRSA 13) for the rat protein (29) was used as a hybridization probe. All DNA probes were generated by labeling with (α-32P)dCTP to a specific radioactivity of 1.2-1.5 X 10^9 cpm/μg of DNA by random priming with Klenow DNA polymerase according to Feinberg and Vogelstein (30,31). mRNA levels were evaluated by densitometric scanning of the autoradiograms using an LKB Ultroscan XL laser densitometer and GelScan XL (2.1) software.

Isolation of liver nuclei and run-on transcription analysis: Nuclei were isolated from liver by a modification of the method of Laitinen et al. (32). Fresh livers were homogenized with a Potter-Elvehjem teflon-glass homogenizer in 10 vol. of STM buffer (250 mM sucrose, 50 mM Tris-HCl pH 7.4, 5 mM $MgSO_4$) containing 0.1 mM PMSF and 0.5 μg/ml aprotinin, filtered though four layers of cheesecloth, and centifuged at 800 X g for 10 min at 4°C. The pellet was resuspended in the same buffer and sedimented again at 800 X g for 5 min at 4°C. The pellet was resuspended in RSB buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) containing 0.1 mM PMSF and 0.5 μg/ml aprotinin, and the cells were lysed by stepwise addition of 10 % Nonidet P-40 to a final concentration of 0.5 % with gentle vortexing (≈ 30 s). Detergent extraction was repeated twice. Nuclei were then centrifuged at 800 X g for 5 min at 4°C and washed three times with RSB buffer without detergent. The final pellet was resuspended in nuclei storage buffer (40 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA and 40 % Glycerol) and stored at -80°C. Intact nuclei without cytoplasmic remnants, as revealed by phase-contrast microscopy, were thus obtained. The entire isolation procedure was completed in 90 min. The number of nuclei were measured as described previously (32).

The run-on transcription reaction in isolated nuclei was carried out at 30°C for 20 min using the reaction mixture described (33). Seventeen million nuclei were used per assay in a total reaction volume of 0.2 ml containing 200 μCi of (α-32P)UTP (specific activity 3000 Ci/mmol). Labeled RNA was extracted from the reaction mixture and resuspended in prehybridization solution essentially as described previously (34). Labeled RNA products were hybridized 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 previously described by Cifuentes, et al. (17). For PEPCK the pPCK-B7.0 genomic clone (The clone pPCK-B7.0 was kindly supplied by Dr. Richard W. Hanson, Case Western

Reserve University, Ohio) (26). The other DNAs were pRSA 13 for albumin and pBS and pBR322 vectors as controls for background hybridization. Prior to hybridization, DNAs (4 µg/lane) were incubated for 6 hours at 42 °C in the prehybridization solution. The same amount of radioactivity was added to each hybridization. The filters were washed as described (34). Autoradiography and densitometer scanning were done as with Northern blots.

Isolation and incubation of hepatocytes: Hepatocytes were prepared from male rats two hours after partial hepatectomy and from control rats as previously reported (20). Isolated hepatocytes (4 X 10^6 cells/vial in a final volume of 2 ml) were preincubated with shaking for 30 min at 37°C in Krebs-Henseleit bicarbonate buffer which was equilibrated with O_2/CO_2 (19:1) at pH 7.4 and contained 10 mM glucose and 1% bovine serum albumin. Actinomycin D was added after the preincubation. At the appropiate times, samples of the cell suspension were removed and centrifuged at 350 X g for 5 min at 4°C. The supernatants were discarded and the pellets frozen in liquid nitrogen. RNA extraction and Northern blot analysis were carried out as described above. The viability of hepatocytes was monitored by trypan-blue exclusion, which was always greater than 90%.

RESULTS

Effect of partial hepatectomy on hepatic PFK-2/FBPase-2 activities.

We reported previously (6) that after liver resection Fru-2,6-P2 decreased compared to control animals and did not increase until after the seventh day. These different levels of Fru-2,6-P2 could be due to changes in the concentration of the bifunctional enzyme and/or to covalent modification. Previous studies indicated that the level of cyclic AMP was elevated immediately after partial hepatectomy and that cyclic AMP-dependent protein kinase-catalyzed-phosphorylation accounted, in part, for the decreased Fru-2,6-P2 level (6). In order 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 (13). As shown in Fig.1, we observed a 30 % decrease in PFK-2 activity after 12 hours and activity remained low for 7 days but was restored by 10 days. Activity was measured after partial purification of the extract by PEG-6000 (6-21 %) precipitation with saturating concentrations of Fru-6-P and ATP (Vmax 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 ratio of kinase/bisphosphatase with a minimum at 12 hours that suggests an increase in the FBPase-2 activity as a consequence of the phosphorylation of enzyme by cyclic-AMP dependent protein

kinase, concomitant with an increase of cyclic-AMP (6). After 12 hours, the activity ratio was not significantly changed compared to normal values. The results indicate that the decrease in Fru-2,6-P2 concentration during hepatic regeneration correlates both with a decrease in enzyme protein and with an increase of enzyme phosphorylation that enhances bisphosphatase activity and inhibits kinase activity.

In order to confirm that the assay conditions employed to measure kinase activity (Fig.1) reflect the amount of enzyme protein, we also used immunoblotting with an antibody raised against the decapeptide N-terminus of liver PFK-2/FBPase-2 (23). As shown in Fig.2, the amount of immunodetectable protein (55 KDa) also correlated with kinase activity.

Time course of changes in PFK-2/FBPase-2 mRNA levels during liver regeneration.

In order to determine whether the mRNAPFK-2/FBPase-2 abundance is correlated with the amount of bifunctional enzyme, Northern blot analysis was done with RNA extracted from regenerating liver. The experiment was performed using the 1400 bp probe of the cDNA (see Experimental Procedures). As shown in Fig.3 (top panel), the mRNAPFK-2/FBPase-2 had the same size (2.2 kb) as the adult liver form and no other mRNA forms were detected. The mRNAPFK-2/FBPase-2 level decreased transiently to a value of about 5 % that of the control within the first 6 hours. The mRNA level then increased to values 4-fold higher than the control with a maximal mRNA accumulation at 48-60 hours, and returned to near basal level by 96 hours. This time course correlates very well with the induction of total DNA reported during liver regeneration. It is known that within 12-16 hours after hepatectomy, liver cells initiate DNA synthesis and continue to proliferate until the hepatic mass is restored (1,2).

In order to study the effect of anaesthesia, surgical stress, and/or decreased food intake of hepatectomized animals, we have analysed the mRNAPFK-2/FBPase-2 accumulation of sham rats by Northern blot. As shown in Fig.4, the mRNAPFK-2/FBPase-2 levels of sham animals were different from those hepatectomized. A small increase was observed during the first hours, returning to basal levels after 6 h and resting almost constant during the rest of the time analysed.

Since the liver/skeletal muscle PFK-2/FBPase-2 gene encodes two different isozymes by alternative splicing (12), it was of interest to examine which isozyme was expressed during the hepatic regeneration. Because the 1400 bp probe hybridizes with mRNAs of both isozymes, we measured the amount of liver specific mRNA , using a specific 299 bp cDNA probe (see Experimental Procedures). 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, while no hybridization was seen using this probe with the RNA from skeletal muscle. The time course of mRNAPFK-2/FBPase-2 accumulation was the same using the 1400 bp probe or the 299 bp probe (not shown). These results strongly suggest that the isozyme expressed during the hepatic regeneration is the adult liver form.

Transcription rates of the PFK-2/FBPase-2 and PEPCK genes during the liver regeneration.

It is known that hepatic proliferation is regulated in a complex manner by a large number of factors (hormones, growth factors, proto-oncogenes) (1,2). The change in bifunctional enzyme mRNA levels could be due to the action of these factors, through mechanisms involving modulation of gene transcription, mRNA stability, mRNA processing, transport from the nuclei, or some combination of all these processes. The rate of transcription of PFK-2/FBPase-2 gene was measured in isolated nuclei from rat liver at 0, 6, 30, 72 and 96 hours after partial hepatectomy. Labeled RNA was extracted and hybridized with two different genomic DNA fragments. These fragments represented the 5'region of the RNA from muscle isozyme which contains exon 1a and 5'region of the mRNA from liver which contains exon 1b (17). No RNA transcripts were detected using the muscle specific DNA fragment. In contrast, the rate of transcription was easily measured with the liver specific DNA (Table I). Furthermore, significant differences in the rate of gene transcription were observed during the time course of regeneration. As shown in Table I, the transcription rate reached a minimum level of 50 % of the control value after 6 hours of partial hepatectomy, and increased after this time, reaching a maximal level of 2.3 times the control value at 72 hours.

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. It is known that 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 cyclic AMP 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 hours after partial hepatectomy, remained elevated at 30 hours, and decreased at 72 hours (Table I). The differential response in the transcription rates of the PFK-2/FBPase-2 and PEPCK genes correlates very well with the opposing role of both 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 I), consistent with a specific effect on transcription of the PFK-2/FBPase-2 and PEPCK genes.

Half life of PFK-2/FBPase-2 mRNA. Influence of partial hepatectomy.

The transcriptional regulation of the PFK-2/FBPase-2 gene does not preclude regulation at other pretranslational levels. Therefore, mRNAPFK-2/FBPase-2 turnover may also play a role in the regulation of this gene's expression during liver regeneration. A half life of 1.5 to 3.5 hours has been reported for mRNAPFK-2/FBPase-2 (16,17). Since the largest decrease in mRNA levels was found at 6 hours and considering its half life, we determined bifunctional enzyme

mRNA stability in hepatocytes from rats two hours after partial hepatectomy, and compared the value with that observed in control hepatocytes.

The degradation rate of mRNAPFK-2/FBPase-2 was estimated from the decay rate of the mRNA in hepatocytes incubated in media containing actinomycin D (5 μg/ml). As shown in Fig.5, no difference in the half life of mRNAPFK-2/FBPase-2 was found in hepatocytes from control rats compared with hepatocytes obtained two hours after partial hepatectomy. The half life of mRNAPFK-2/FBPase-2 was 2.5 hours, in both cases. Our results suggest that the mRNA stability is not involved in the change of mRNAPFK-2/FBPase-2 abundance found after partial hepatectomy. It is likely that the mRNAPFK-2/FBPase-2 concentration during the pre-replicative 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 G1 phase of the cell cycle. The sequence of events which occur during liver regeneration can be divided into two phases, an initial hypertrophic stage lasting 10-12 hours, 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 pre-replicative 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 approximately 30 % of its original mass, an increase in gluconeogenesis and a decrease in glycolysis take place to maintain glucose homeostasis (4,5). Fru-2,6-P2, 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. The results of this study suggest that the decrease in Fru-2,6-P2 concentration is due to regulation of the bifunctional enzyme at both the transcriptional and post-transcriptional levels. This regulation probably reflects the synergistic action of multiple factors including circulating hormones and growth factors (1,2). The changes found in the activity ratio (kinase/bisphosphatase) and PFK-2 activity (Fig. 1) provide a plausible explanation for the rapid decrease in Fru-2,6-P2. The rat liver isozyme has a cyclic AMP-dependent phosphorylation site located 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 cyclic AMP (6). In addition, the decrease in the mRNAPFK-2/FBPase-2 levels observed after partial hepatectomy is consistent with the decline of transcription rate (Table I). Although direct evidence is lacking, it is reasonable to postulate that elevated cyclic AMP would be the responsable for the decline in transcription observed during the first 6 h, since the cyclic nucleotide has been shown to decrease mRNAPFK-2/FBPase-2 levels in FTO-2B cells by a transcriptional mechanism (17).

While the bifunctional enzyme decreases in amount and becomes phosphorylated, another gluconeogenic enzyme, PEPCK, increases in amount immediately after partial hepatectomy (5). We show in this report that the coordinated regulation of blood glucose concentration also involves the enhanced expression of the PEPCK gene at the transcriptional level (Table I). These results are consistent with the increase of mRNAPEPCK 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 PEPCK gene (42,43) and increases the transcription of PFK-2/FBPase-2 gene (17). Although it is likely that glucagon and insulin play an essential role, other hormones and factors may also mediate bifunctional enzyme gene regulation (1,2,40,44).

The replicative period of liver regeneration starts at about 12 hours. It is associated with hepatocyte DNA synthesis and the major wave of cell division (1,2). In this mitotic state we have previously reported an increase in Fru-2,6-P2 levels (6). The data shown in this paper demonstrate that this increase correlates with transcriptional activation and mRNAPFK-2/FBPase-2 accumulation, and the increase of 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 mRNAPFK-2/FBPase-2 accumulation found during liver regeneration were due to surgical stress, anaesthesia and/or differences in the nutritional state. The differences in the mRNAPFK-2/FBPase-2 accumulation found in sham with respect to hepatectomized animals suggest that stress and anaesthesia do not influence in the specific effect of hepatectomy. The increase of mRNAPFK-2/FBPase-2 in sham rats would be a consequence of surgical stress and/or anaesthesia, as it has been shown by other proteins (27). The importance of nutritional factors during liver regeneration has been reported by different authors (1,45,46). However, it does not seem to be the case for mRNAPFK-2/FBPase-2. As it has been reported by other authors (13,47), no differences in the mRNAPFK-2/FBPase-2 levels are found between fed and fasted rats, and induction is only observed in strong conditions of fasting /refeeding (13). Our results showing the mRNAPFK-2/FBPase-2 levels of sham rats almost constant during the cyrcardian cycle (Fig.4). Also, it does not appear that the increase in the mRNAPFK-2/FBPase-2 levels was a result of strong conditions of fasting/refeeding because it has been shown that hepatectomized animals eat, although less, during this process (48, and results not shown). Taken together, these results seem to indicate that the changes found in the mRNAPFK-2/FBPase-2 levels during liver regeneration are not dependent of the nutritional state of the animals.

Several PFK-2/FBPase-2 isoenzymes have been described in different tissues (10,17,23,41). 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, in

protein molecular weight and mRNA size. The results reported here suggest that the isoenzyme expressed during liver regeneration is the adult liver form. The kinetic differences previously reported (6) could reflect some as yet unidentified post-translational 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 post-transcriptional 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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Legends:

Figure 1. Effect of partial hepatectomy on PFK-2/FBPase-2 activities.

PFK-2 and FBPase-2 activities were assayed as described under "Experimental Procedures". Each point represents the mean ± SEM for 3-5 rats. Statistically significant differences between hepatectomized and control animals are indicated by: * $P< 0.05$ and ** $P< 0.01$.

Figure 2. Amount of PFK-2/FBPase-2 during liver regeneration.

Enzyme protein was measured after a fractionation with PEG-6000 (6-21%). 30 µg of protein was used per lane for PAGE (12% acryamide/SDS), then transferred to nitrocellulose and incubated with the antibody anti-PFK-2/FBPase-2 as described under "Experimental Procedures". A representative blot is shown in the inset. The levels were quantified by densitometer scanning of autoradiograms and expressed relative to the value of normal liver (0 hours), which was taken as 100%. Data are means ± SEM from 3-4 animals.

Figure 3. Expression of PFK-2/FBPase-2 gene during liver regeneration.

Total RNA (20 µg/lane) extracted from normal rat liver (0 hours), regenerating liver after partial hepatectomy and skeletal-muscle (M) was transferred to nylon membranes after electrophoresis and hybridized with cDNA for PFK-2/FBPase-2 (common probe) and serum albumin (pRSA 13) as is described in "Experimental Procedures". A representative Northern blot is shown in the inset. The mRNAPFK-2/FBPase-2 level was quantified by densitometer scanning of autoradiograms and corrected for the amount of RNA loaded in each lane by comparison with the bands of the mRNAalbumin control. The values are expressed relative to the value of normal liver (0 hours) which was taken as 100 %. Data are means ± SEM from 3-5 animals.

Figure 4. mRNAPFK-2/FBPase-2 levels of sham and hepatectomized animals during liver regeneration.

Northern blot analysis of total RNA (20 µg/lane) extracted from normal (□), sham (○) and regenerating (●) livers was done as it is described in the legend of figure 3 and in "Experimental Procedures". Data are means from 3-5 animals.

Figure 5. Time-course of decay of mRNAPFK-2/FBPase-2 in isolated hepatocytes.

Hepatocytes were isolated from control rats (○) and from rats after two hours of the liver resection (●), and incubated at 37°C as described in "Experimental Procedures". Cells were incubated in the presence of actinomycin D (5 µg/ml) during the times indicated. The mRNA levels were quantified by laser densitometry of autoradiograms and expressed relative to the amount present at 0 hours, which was taken as 100 %.

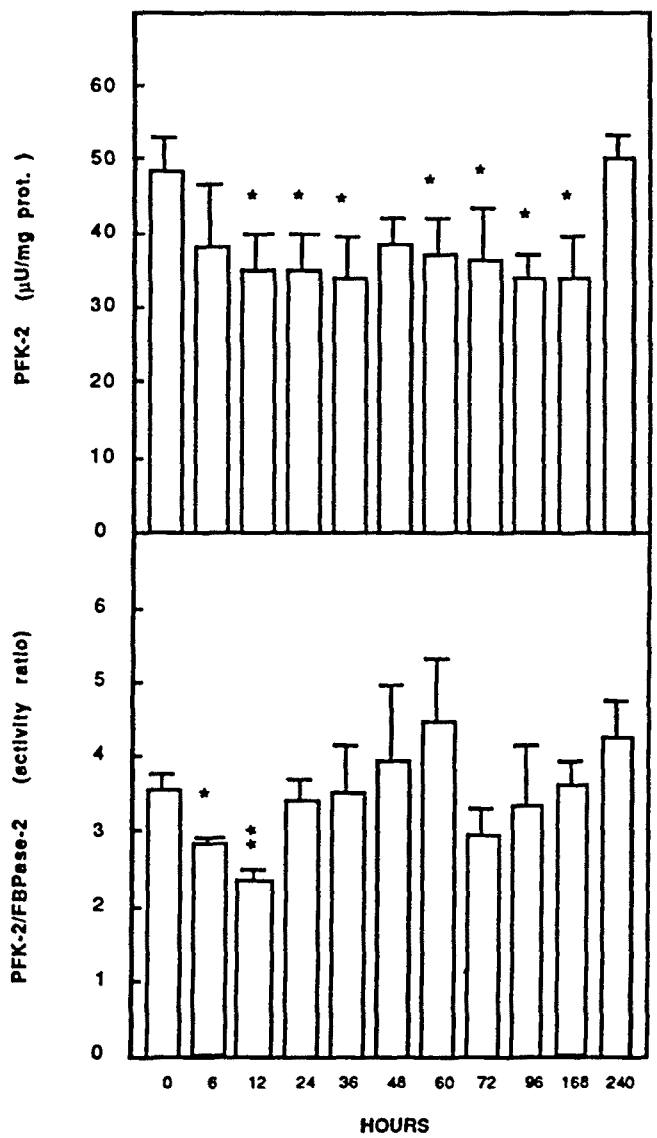
PFK-2 (µU/mg prot.)
60
50
40
30
20
10
0
PFK-2/FBPase-2 (activity ratio)
6
5
4
3
2
1
0
0 6 12 24 36 48 60 72 96 168 240
HOURS

Fig 1

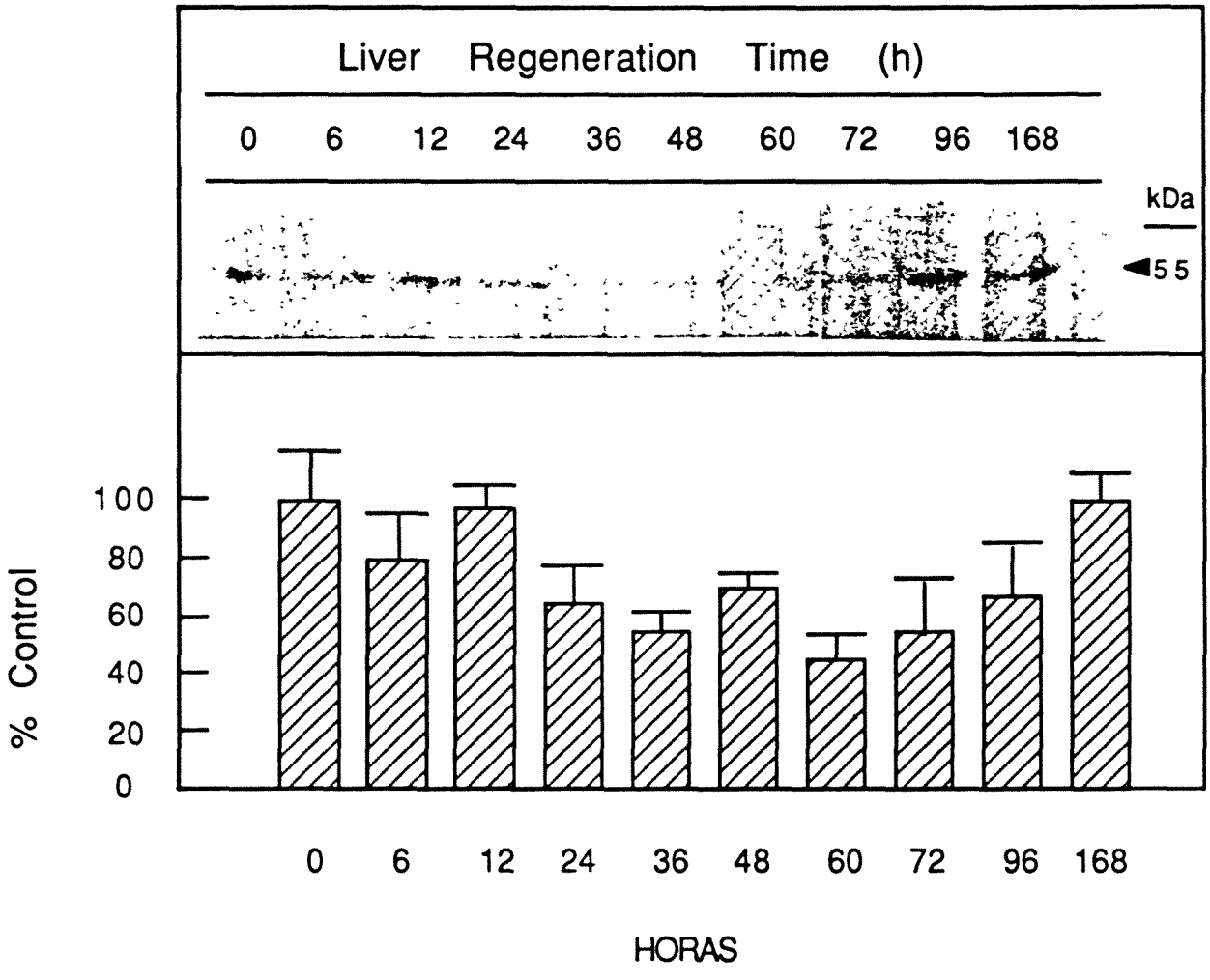
Liver Regeneration Time (h)
0 6 12 24 36 48 60 72 96 168
kDa
55
% Control
100
80
60
40
20
0
0 6 12 24 36 48 60 72 96 168
HORAS

Fig 2

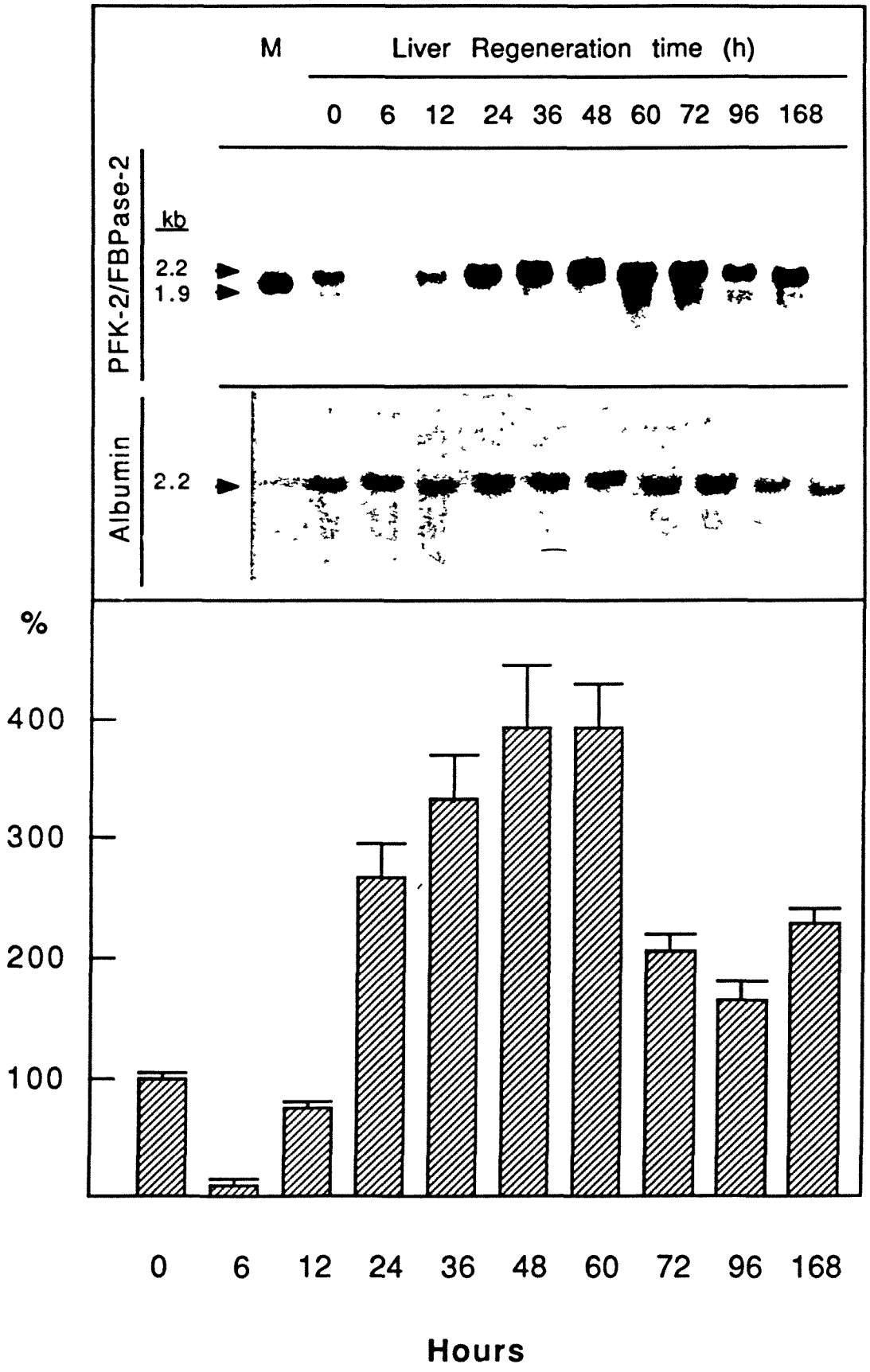
M
Liver Regeneration time (h)
0 6 12 24 36 48 60 72 96 168
PFK-2/FBPase-2
kb
2.2
1.9
Albumin
2.2
%
400
300
200
100
0 6 12 24 36 48 60 72 96 168
Hours

Fig 3

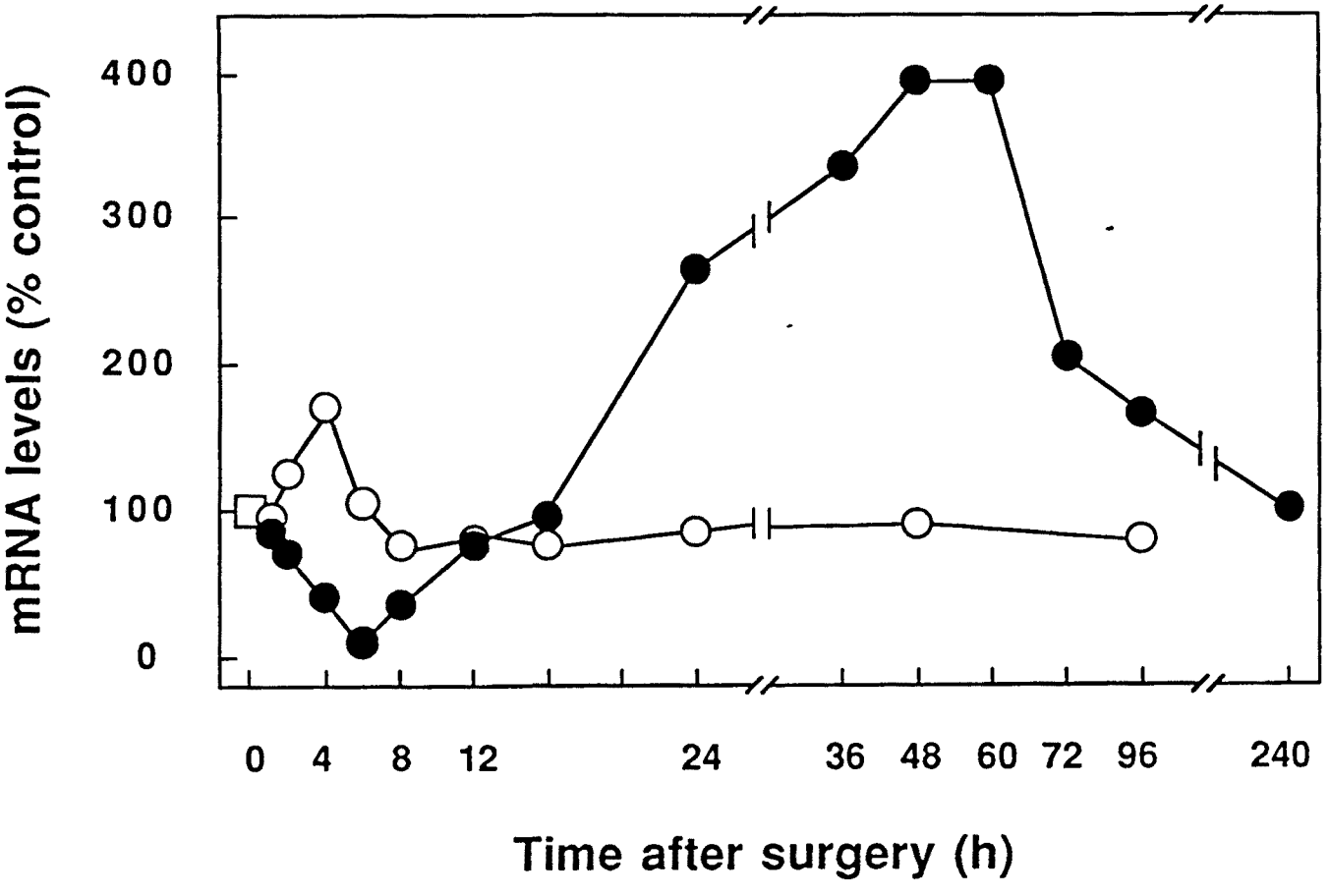
mRNA levels (% control)
400
300
200
100
0
0
4
8
12
24
36
48
60
72
96
240
Time after surgery (h)

Fig 4

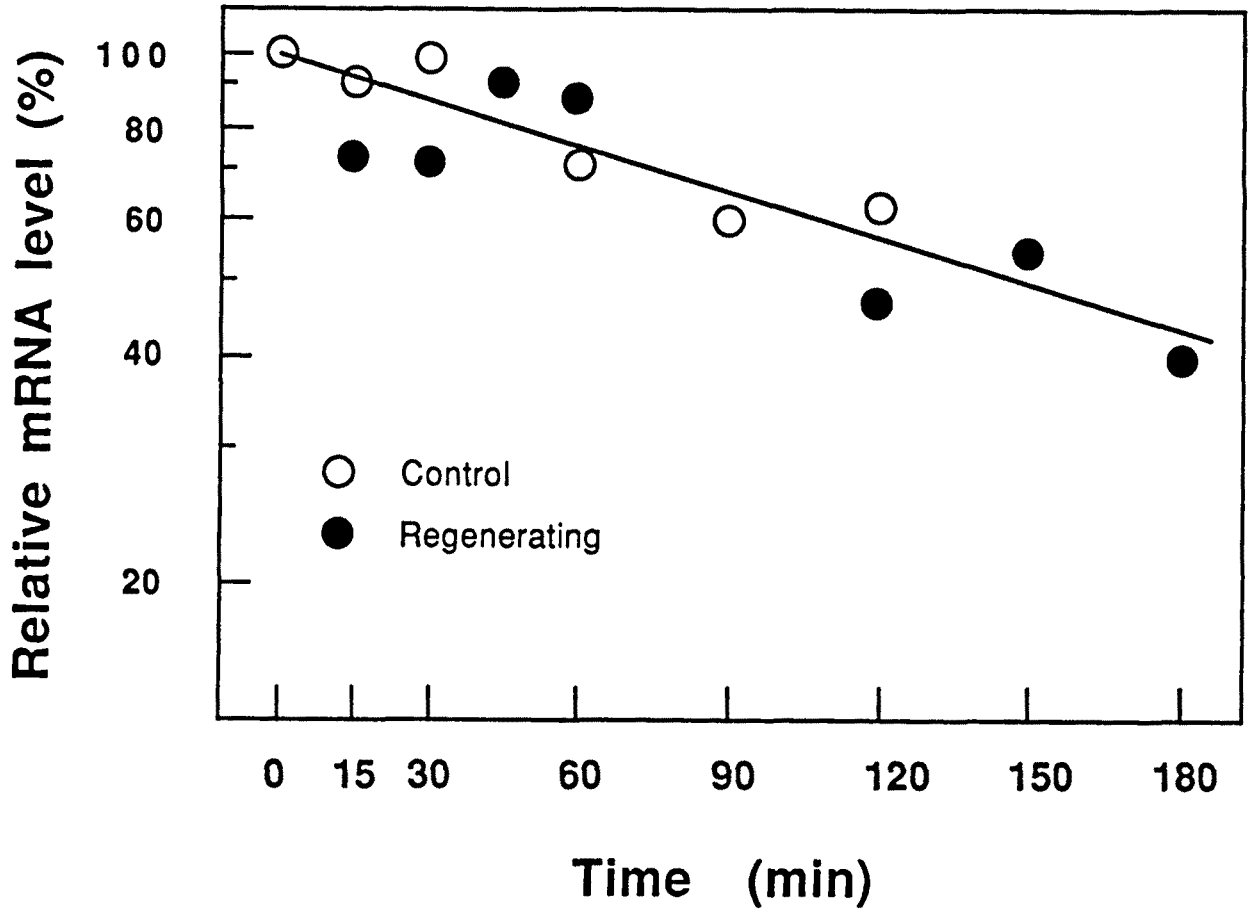
Relative mRNA level (%)
100
80
60
40
20
Control
Regenerating
0
15
30
60
90
120
150
180
Time (min)

Fig. 5

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

Regeneration time (h)	Relative rate of gene transcription					
	Albumin		PEPCK		PFK-2/FBPase-2	
	Arbitrary units	-Fold	Arbitrary units	-Fold	Arbitrary units	-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	4.82	4.2	1.94	1.0
72	0.52	0.9	2.04	1.8	4.38	2.3
96	0.65	1.1	6.16	5.4	2.34	1.2

Run-on transcription assays with nuclei isolated from normal (0 h) and after partial hepatectomy (6, 30, 72 and 96 h) rats were performed as described under "Experimental Procedures". The autoradiographs were scanned by laser densitometry and the values expressed as arbitrary units after subtraction of the value obtained for vector DNAs. - Fold values were calculated by assigning a value of 1 to the transcriptional rate in control rat.

Gene Expression of regulatory enzymes of glycolysis/gluconeogenesis in regenerating rat liver

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by

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Short running title: "Gene expression during liver regeneration".

Section: Regulation of metabolism.

Keywords: (liver regeneration/ gene expression/ glycolysis/ gluconeogenesis/ glucokinase/ L-pyruvate kinase/ fructose 1,6-bisphosphatase/ phosphoenolpyruvate carboxykinase).

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Abbreviations used: GK, glucokinase; L-PK, Liver form of pyruvate kinase; FBPase-1: fructose 1,6-bisphosphatase; PFK-2/FBPase-2, 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase; PEPCK, phosphoenolpyruvate carboxykinase; Fru-2,6-P_2, fructose 2,6-bisphosphate.

Abstract

The mRNA levels of glucokinase, L-pyruvate kinase, fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase were analyzed during liver regeneration. The mRNA levels of glycolytic enzymes (glucokinase and L-pyruvate kinase) decreased rapidly after partial hepatectomy. Glucokinase mRNA increased at 16-24 h with a maximum at 48 h, returning to normal values after this time. L-pyruvate kinase mRNA recovered control levels at 168 h. In contrast, phosphoenolpyruvate carboxykinase mRNA increased rapidly after liver resection and remained high during the regenerative process. However, the levels of fructose 1,6-bisphosphatase mRNA were not modified significantly. These results correlate with reported rates and enzyme levels of increased gluconeogenesis after partial hepatectomy. The effect of stress on the mRNA levels was also studied. All enzymes showed variations in their mRNA levels following the surgical stress. In general, the differences were more pronounced in regenerating than in sham animals, being practically normalized at 24 h.

Introduction

One of the most important functions of the liver is the maintenance of glucose homeostasis. This mechanism operates in different physiological conditions (1,2) even after a drastic reduction of the liver mass (3-5). Indeed, after partial hepatectomy the gluconeogenic capacity of the liver remnant increases. This increase is more acute during the first hours of liver regeneration and it remains high until the initial cellular mass is restored. It has been reported that there is an increase of over 200 % in the gluconeogenic flux from lactate, and a decrease to less than 20 % in the glycolytic flux from glucose in hepatocyte suspensions of regenerating liver (3). In addition, partial hepatectomy produces metabolic changes in the liver remnant, which culminates in coordinate waves of DNA synthesis and mitosis. Therefore, cell proliferation and gluconeogenic flux must to take place in a synchronous manner.

The regulation of the hepatic gluconeogenic pathway is brought about by phosphorylation/dephosphorylation and control of gene expression of several key regulatory enzymes. These enzymes control hepatic glucose production and utilization through regulation of three major substrate cycles: glucose/ glucose 6-phosphate, fructose 6-phosphate/fructose 1,6-bisphosphate and phosphoenolpyruvate/pyruvate. The fructose 6-phosphate/fructose 1,6-bisphosphate substrate cycle is also regulated by a subcycle in which the amount of the regulatory molecule Fru-2,6-P_2 is controlled by the bifunctional enzyme PFK-2/FBPase-2 (1,2,6,7). Recently, changes have been reported in Fru-2,6-P_2 concentration, PFK-2 activity (5) and PFK-2/FBPase-2 mRNA levels* during liver regeneration. No data have been reported for the mRNA levels of other glycolytic/gluconeogenic enzymes such as L-PK, GK and FBPase-1, and the results for PEPCK are contradictory (8,9). The aim of the present paper was to study the changes in the mRNA levels of these enzymes during hepatic regeneration.

Experimental Procedures

Chemicals: (α-^{32}P)dCTP (3000 Ci/mmol) was from Amersham. The random primed DNA labelling kit and restriction endonucleases were from Boehringer Mannheim. GeneScreen and N-hybond membranes were the product of Du Pont-New England Nuclear and Amersham, respectively. Other biochemical reagents were either 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 h-light/12 h-dark cycle (light periods starting at 08:00 h). To minimize the diurnal variation in liver DNA synthesis (10), partial hepatectomy (comprising laparotomy and removal of two-thirds of the liver) or sham operation (laparotomy) were performed between 08:00 and 10:00 h, under diethyl ether anaesthesia following the procedure described by Higgins & Anderson (11). Control rats (0 h) were not subjected to either anaesthesia or surgery. The animals were killed by decapitation and the livers removed and immediately freeze-clamped in liquid nitrogen.

RNA analysis and DNA hybridization probes: Total RNA was extracted from frozen rat tissues by the LiCl/urea method (12). Northern blot analysis was perfomed using standard procedures (13). The following fragments were used as probes: a ≈1.4 kb Eco RI fragment isolated from the cDNA for PFK-2/FBPase-2 (14); a ≈1.6 kb Pst I fragment from cDNA clone (G4) for L-PK (15); a ≈0.65 kb Eco RI fragment from cDNA for FBPase-1 (16); a ≈2.4 kb Eco RI fragment from cDNA for GK (17); a ≈ 2.6 kb Pst I fragment from cDNA clone (pPCK10) for PEPCK (18); and a ≈1.1 kb Pst I fragment from cDNA clone (pRSA13) for rat serum albumin (19). All DNA probes were generated by labelling with (α-^{32}P)dCTP to a specific radioactivity of ~$1.5*10^9$ cpm/μg of DNA by random priming with Klenow DNA polymerase. The level of these mRNAs was measured by densitometric scanning of the autoradiograms using an LKB Ultroscan XL laser densitometer and GelScan XL (2.1) software, and corrected for the amount of albumin mRNA that was used as control (20,21).

Results

In order to determine whether the changes in enzyme activities, previously reported (3,5,22,23), are correlated with mRNA levels, Northern blot analysis were perfomed with RNA extracted from regenerating liver. Values were corrected for the amount of albumin mRNA that was used as control (20,21). As shown in table 1 and Fig.1, the mRNA levels of GK and L-PK decreased transiently to values of about 20 and 10% of control, respectively, within the first 6 hours. At 24 h, the mRNA levels of GK increased, reaching a maximum at 48 h and returning to approximately normal levels after this time. The mRNA levels of L-PK remained low during the first 96 h and reestablished the normal values at 168 h. These profiles are concordant with the decrease in total GK and L-PK activities described during liver regeneration (5,22,23). The L-PK cDNA used as probe showed cross-hybridization with mRNAs of different sizes (3.2, 2.2 and 2.0 kb) (15). However, the pattern was the same in all cases. The mRNA levels of PFK-2/FBPase-2 followed the pattern previously reported*.

In contrast with the behaviour of glycolytic enzymes described above, the PEPCK mRNA levels increased 13 fold over control at 6 h after hepatectomy. Afterwards, a decrease was observed until 24 h and other peaks of mRNA accumulation occurred at 36 h and at 72 h after liver resection. These results agree with the changes of transcription rates previously reported for this enzyme*. In addition, the increase in the mRNA levels would explain the increase in PEPCK activity observed (3). The FBPase-1 mRNA levels were not modified, which would also explain the unchanging activity reported (3).

From the results described above, it seems clear that it is during the early phase when great changes in the mRNA levels occur. To a most accurate evaluation in this phase, Northern blot analysis were performed with RNA extract from rat liver during the first 24 h after the surgery. To distinguish between the events which are specific for liver regeneration and those which are related to the stress, sham controls (animal subjected to a midventral laparotomy without direct manipulation of the liver) were used. As shown in table 2 and Fig.2, the enzymes showed variation of mRNA levels in sham controls. However, differences were found between the profile of early partial hepatectomy and sham animals. Although, a decrease in the mRNA levels for L-PK was found in sham animals, this was more marked in hepatectomized rats. In both animal groups, the minimal levels were found about 12-16 h, remaining low afterwards only in hepatectomized animals. GK mRNA levels showed a slight decrease during the first 30 minutes after liver resection, which was not observed in sham animals, followed by an acute decrease (minimal value about 8 h) and a large increase at 16 h both in sham and hepatectomized animals. PEPCK mRNA levels fastly increased after partial hepatectomy (7 fold at 30 min) remaining high afterwards. A similar

profile, but less pronounced, was observed in sham animals. FBPase-1 mRNA levels were unchanged in hepatectomized animals and a transient increase between 4-16 h was observed in sham rats. The differences in PFK-2/FBPase-2 mRNA levels found between sham and hepatectomized animals were similar to those previously reported*

Discussion

It is known that after partial hepatectomy the decrease in liver tissue is accompanied by complex hormonal changes which produce an increase in glycogenolysis and gluconeogenesis in the liver remnant, together with a decrease in glycolysis, in order to maintain the blood glucose concentration (3-5). During this process the gene expression of glycolytic/gluconeogenic enzymes would also be modified. Different studies have been reported on the regulation of different key enzymes involved in glucose metabolism during hepatic regeneration. High levels of PEPCK activity have been detected at 24 and 48 hours after partial hepatectomy (3). This increase correlates with a decrease in hepatic GK (3,5,22) and L-PK (22,23) activities. Recently (5), we have shown that Fru-2,6-P_2, the most potent allosteric activator of 6-phosphofructo 1-kinase and inhibitor of fructose 1,6-bisphosphatase (1,2,6,7), changes after liver resection, in part as a consequence of the modification of the phosphorylation state of PFK-2/FBPase-2 (5), the bifunctional enzyme which catalyzes both the synthesis and the degradation of Fru-2,6-P_2. In addition, we have also found that the mRNA levels of the bifunctional enzyme are regulated at transcriptional level* after partial hepatectomy.

The results reported herein suggest that the increase in PEPCK activity found during liver regeneration is probably due to an increase in its mRNA levels. This result agrees with the transcriptional activation of this gene reported previously* and with the fact that PEPCK was found to be one of the immediate-early genes cloned by differential screening of a subtraction-enriched regenerating liver cDNA library (8). However, opposite results were observed by Milland and Schreiber (9), claiming that a certain insulin-like growth factor could de-induce the PEPCK gene expression. Although changes in the mRNA levels have been found for FBPase-1, another gluconeogenic enzyme, in different physiological and hormonal conditions (16); we did not find significant changes during liver regeneration. This fact and the non modification of the glucose 6-phosphatase activity reported (3) point to the major role of PEPCK in the regulation of gluconeogenic flux during this process.

The behaviour of glycolytic enzymes, GK and L-PK, was opposed to the gluconeogenic enzyme PEPCK during the prereplicative phase. The mRNA levels of both glycolytic enzymes, together with PFK-2/FBPase-2, showed a similar pattern. Differences

were found only in the replicative state (>12 h after partial hepatectomy) in which the L-PK mRNA levels reestablished later (at 168 h). This delay in the recovery of the mRNA levels for L-PK and the high levels of PEPCK mRNA found during this time would be related with the suggestion that glycogenesis could be sustained through the indirect pathway, the bulk of liver glycogen being of gluconeogenic origin (5).

The changes in the activities previously reported for the regulatory enzymes of glycolysis/gluconeogenesis (3,5,22,23) correlate with the mRNA levels now reported. Several hormones, growth factors and neuromediators, presumably acting in synergy (24,25), regulate the expression of these genes during liver regeneration. Bearing in mind what it is known about the gene expression of these enzymes in other physiological conditions (26), the following tentative model is suggested. The increase in cyclic AMP found in the earliest phase of regeneration (5,27) would induce the mRNAs that encode gluconeogenic enzymes and repress the glycolytic (26). The decrease in the insulin levels (28,29) would act on the expression of these genes in a similar manner (26). However, the effect of increasing glucocorticoid concentrations (29) would be more restrictive, breaking the coordination between glycolytic and gluconeogenic enzymes during the replicative phase. Therefore, induction of PEPCK and PFK-2/FBPase-2 genes would take place, whereas L-PK would be inhibited (26).

Footnote:

(*) J.L. Rosa, A. Tauler, A.J. Lange, S.J. Pilkis and R. Bartrons, submitted to Proc. Natl. Acad. Sci. USA.

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Legends

Figure 1. **Gene Expression of regulatory enzymes of glycolysis/ gluconeogenesis during liver regeneration.**

To see the legend of table 1.

Figure 2. **Effect of stress in the gene expression of regulatory enzymes of glycolysis/gluconeogenesis after partial hepatectomy.**

To see the legend of table 2.

mRNA	Liver Regeneration (% control)									
GK	100	21	18	102	59	139	86	90	96	160
L-PK	100	10	15	10	9	10	14	27	41	92
PFK-2/FBPase-2	100	8	78	182	334	393	394	207	165	228
FBPase-1	100	117	94	96	115	101	95	129	92	168
PEPCK	100	1300	776	496	680	540	620	1300	1200	760
	0	6	12	24	36	48	60	72	96	168
	Time (h)									

Table 1. Gene expression of regulatory enzymes of glycolysis/ gluconeogenesis during liver regeneration.

Total RNA (20 μg/lane) extracted from normal (0 h) and regenerating rat livers at different times were transferred to nylon membranes after electrophoresis in 1.5 % agarose and hybridized with GK, L-PK, PFK-2/FBPase-2, FBPase-1, PEPCK and albumin cDNAs as it is described in "Experimental Procedures". The level of these mRNAs was measured by densitometric scanning of the autoradiograms and corrected for the amount of albumin mRNA. The values represent means of 2-4 different experiments. Representative Northern blots are shown in figure 1 where the mRNA size is indicated.

mRNA	Liver Regeneration (% control)								
GK	Hep 100	51	46	127	96	18	18	274	102
	Sham 100	96	75	79	26	20	71	868	108
L-PK	Hep 100	31	73	74	100	72	15	11	10
	Sham 100	96	112	52	126	50	33	37	52
PFK-2/FBPase-2	Hep 100	92	88	72	40	36	78	93	182
	Sham 100	86	93	126	171	78	80	78	85
FBPase-1	Hep 100	118	116	106	101	112	94	99	96
	Sham 100	94	138	102	147	252	186	227	105
PEPCK	Hep 100	758	913	737	672	608	776	310	496
	Sham 100	320	660	600	100	630	360	140	400
	0	0.5	1	2	4	8	12	16	24
	Time (h)								

Table 2. Effect of stress in the gene expression of regulatory enzymes of glycolysis/gluconeogenesis after partial hepatectomy.

Total RNA (20 μg/lane) extracted from normal (0 h), sham and regenerating (Hep) rat livers at different times were transferred to nylon membranes after electrophoresis in 1.5 % agarose and hybridized with GK, L-PK, PFK-2/FBPase-2, FBPase-1,PEPCK and albumin cDNAs as it is described in "Experimental Procedures". The level of these mRNAs was measured by densitometric scanning of the autoradiograms and corrected for the amount of albumin mRNA. The values represent means of 1-4 different experiments. Representative Northern blots are shown in figure 2.

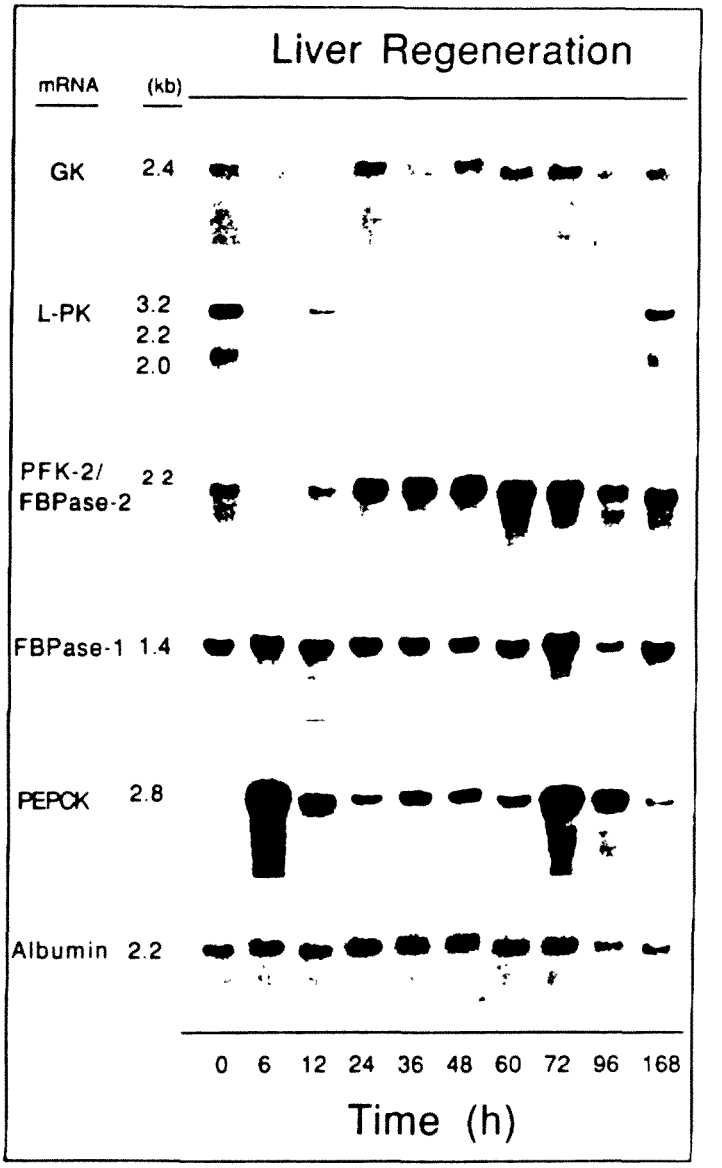
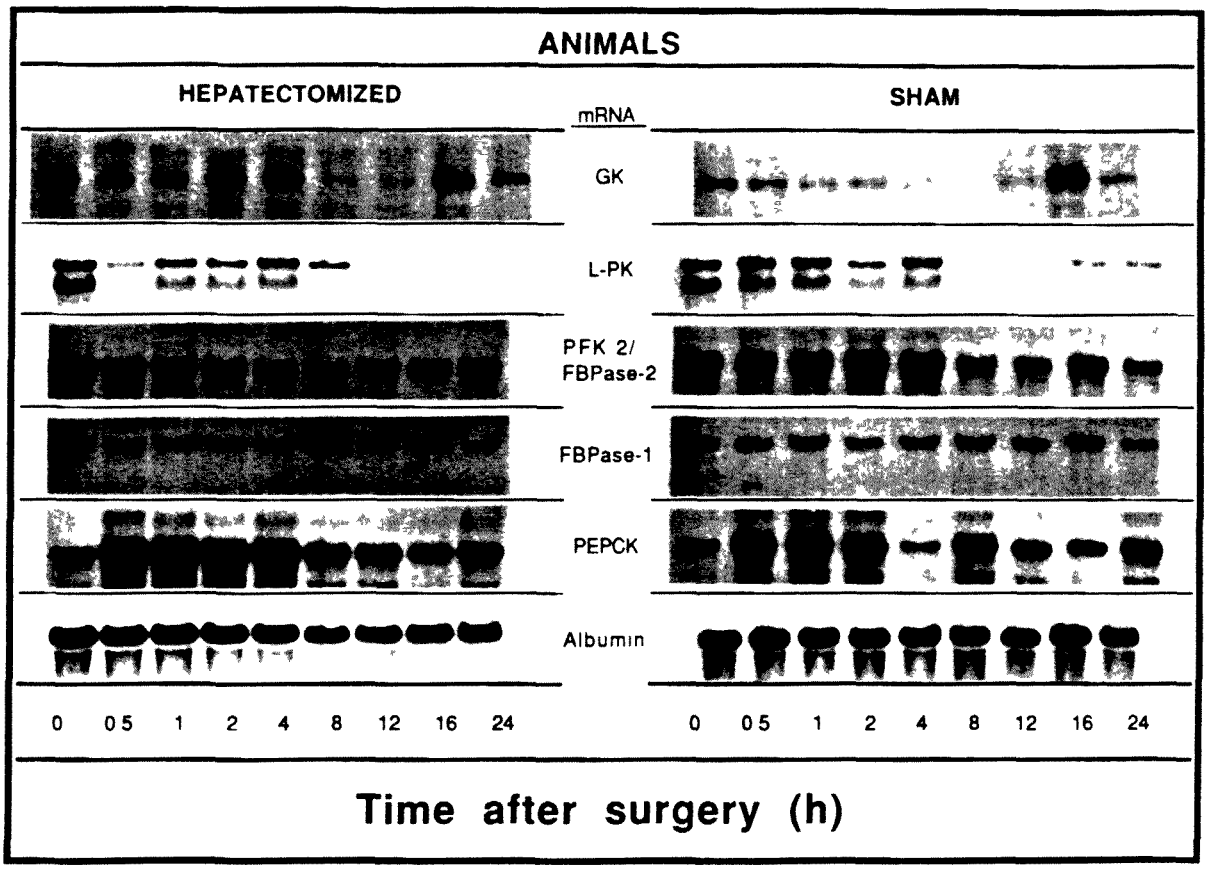

Liver Regeneration
mRNA
(kb)
GK
2.4
L-PK
3.2
2.2
2.0
PFK-2/
FBPase-2
2.2
FBPase-1
1.4
PEPCK
2.8
Albumin
2.2
0 6 12 24 36 48 60 72 96 168
Time (h)

Fig. 1


ANIMALS
HEPATECTOMIZED
SHAM
mRNA
GK
L-PK
PFK 2/
FBPase-2
FBPase-1
PEPCK
Albumin
0 05 1 2 4 8 12 16 24
0 05 1 2 4 8 12 16 24
Time after surgery (h)

Apéndice: Protocolos experimentales

Actividad PFK-2

Referencia: R. Bartrons, L. Hue, E. Van Schaftingen and H.G. Hers (1983) Biochem. J. **214**:829-837.

Principio: Se basa en la formación de Fru-2,6-P_2 a partir de ATP y fructosa 6-fosfato. La actividad PFK-2 *total* y la actividad PFK-2 *activa*, correspondiente a la forma no fosforilada del enzima, fueron medidas a pH 8.5 y 6.6, respectivamente. Basándose en las características cinéticas del enzima, a pH>8 las dos formas tienen la misma actividad mientras que a pH 6.6 la forma fosforilada es prácticamente inactiva en las condiciones de ensayo. A pH intermedios ambas formas tienen actividad.

Reactivos:

1. Tampones de homogenización. Normalmente usamos el primero pero cuando queremos determinar la actividad quinasa y bisfosfatasa de la misma muestra usamos el segundo:
   1) 20 mM KH_2PO_4; 10 mM EDTA; 100 mM KF; 1 mM DTT a pH 7.1.
   2) 50 mM Hepes; 50 mM KCl; 5 mM KH_2PO_4; 0.1 mM EDTA; 1mM DTT; 0.5 mM PMSF a pH 7.5.

2. Tampones de incubación PFK-2. Concentraciones en la incubación:

Total: 50 mM Tris HCl; 100 mM KCl; 7 mM $MgCl_2$; 5 mM fructosa 6-fosfato; 17.5 mM glucosa 6-fosfato; 5 mM ATP-Mg; 1 mM KH_2PO_4; ajustado a pH 8.5 a 30°C.

Activa: 50 mM MES; 100 mM KCl; 7 mM $MgCl_2$; 1 mM fructosa 6-fosfato; 3.5 mM glucosa 6-fosfato; 5 mM ATP-Mg; 5 mM KH_2PO_4; ajustado a pH 6.6 a 30°C.

Procedimiento:

1. Las muestras homogenizadas en 10 vol de tampón de homogenización con un Potter, fueron centrifugadas (27.000g x 20 min a 4°C). En algunos experimentos los homogenizados fueron fraccionados con PEG entre el 6-21%.

2. 10 µl de muestra fue incubada a 30°C en un volumen final de 200 µl con el tampón de incubación deseado. Las incubaciones se paraban a 0, 5, 10 y 15 min añadiendo un vol de NaOH 0.1 M y calentando a 80-90°C durante 5-10 min.

3. La Fru-2,6-P_2 formada se mide en el espectrofotómetro utilizando un estándard de Fru-2,6-P_2. Basándose en el poder estimulador de la Fru-2,6-P_2 sobre la PPi:PFK de patata (E. Van Shaftingen *et al.* (1982) Eur. J. Biochem. **129**:191-195).

Actividad FBPasa-2

Referencia: J.L. Rosa, F. Ventura, J. Carreras and R. Bartrons (1990) Biochem. J. **270**:645-649.

Principio: Se sintetiza (2-^{32}P)Fru-2,6-P_2 a partir de fructosa 6-fosfato y (γ-^{32}P)ATP. La actividad FBPasa-2 se sigue mediante la formación de (^{32}P)Pi a partir de (2-^{32}P)Fru-2,6-P_2.

Reactivos:

1. Cóctel de síntesis de (2-^{32}P)Fru-2,6-P_2 (3x):
150mM Hepes; 300 mM KCl; 15 mM $MgCl_2$; 15 mM KH_2PO_4; 30 mM fructosa 6-fosfato; 0.3 mM EDTA; 0.6 mM ATP-Mg ajustado a pH 7.5.

2. Cóctel de actividad FBPasa-2. Concentraciones en la incubación:
50mM Hepes (pH 7.5); 50 mM KCl; 2 mM $MgCl_2$; 5 mM KH_2PO_4; 1 mM DTT; 2 mM EDTA; 0.1 mM NADP; 5 µM (2-^{32}P)Fru-2,6-P_2 (200.000 cpm/ensayo); 9 U/ml de fosfoglucoisomerasa y 4 U/ml de glucosa 6-fosfato deshidrogenasa.

Procedimiento:

1. Síntesis de (2-^{32}P)Fru-2,6-P_2 (M.R. El-Maghrabi *et al.*(1982) J. Biol. Chem. **257**:7603-7607).

- Acetilación de los grupos tiólicos para eliminar la actividad bifosfatasa del enzima. Incubamos 10 µl PFK-2/FBPasa-2 pura de 300 mU/ml con 30 µl iodoacetamida 13.3 mM durante 20' a 30 C. Se para añadiendo 10 µl de DTT 100 mM.
- Añadimos 50 µl de cóctel de síntesis de (2-^{32}P)Fru-2,6-P_2 (3x) y 50 µl (γ-^{32}P)ATP (250 µCi).
- Se incuba 2 h a 30 C y se para con 350 µl KOH 0.1 M. Se calienta 10 min a 90 C.
- Se añade una espátula de carbón activo para retener los nucleótidos.
- 10.000 g x 2'. Se coge el super y el precipitado se lava con 1 ml de 20 mM TEA (trietilamina, pH 8.2). Ambos sobrenadantes se diluyen hasta 9 ml en TEA 20 mM y se aplican a una columna de 1 ml de DEAE Sephadex A25. Se lava con 10 ml del mismo tampón y se aplica un flujo de 20 ml/h. Se eluye la (2-^{32}P)Fru-2,6-P_2 con un gradiente (60 ml) de TEA entre 20 y 700 mM. Se recogen fracciones de 2 ml y se cuenta la radioactividad (10 µl/5 ml líquido de centelleo). Se realiza un pool con las fracciones de (2-^{32}P)Fru-2,6-P_2 (el segundo pico que se detecta; el primero es de (2-^{32}P)Pi). Se añade unas gotas de hidróxido amónico y se rotavapora hasta ≈ 1 ml comprueba que el pH 8-9. Se valora la (2-^{32}P)Fru-2,6-P_2, se alicuota y se guarda a -20 C.

2. 5 µl de las muestras homogenizadas como para la actividad quinasa fueron incubadas a 30C con el cóctel de actividad FBPasa-2, en un volumen total de 40 µl. Se paraba la reacción a 1', 2' y 4' cogiendo 10 µl de la mezcla de incubación y añadiendo 100 µl NaOH 50 mM.

3. Las muestras en NaOH eran diluídas con TEA 20 mM (pH 8.2) hasta un volumen de 5 ml y aplicadas cada una a una columna de DEAE Sephadex A25 (≈0.5 ml de gel). Se lavaba con 5 ml de TEA 20 mM y se eluía el (2-^{32}P)Pi con 10 ml TEA 300 mM. Se recogía en un vial de centelleo y se contaba. De esta formaba podíamos saber el fosfato formado a partir de la (2-^{32}P)Fru-2,6-P_2 y calcular la actividad FBPasa-2.

4. La (2-^{32}P)Fru-2,6-P_2 que no había reaccionado se eluía de la columna con 10 ml de TEA 700 mM y servía para comprobar que la cantidad de radioactividad colocada en cada incubación era similar.

5. Es necesario realizar blancos, normalmente no excedieron de 0.1 % de la radioactividad aplicada.

Electroforesis de proteínas en geles de poliacrilamida con SDS

Referencia: Laemmli, U.K. (1970) Nature (London) **227**:680-685.

Principio: Las proteínas son desnaturalizadas y reducidas por calentamiento en presencia de SDS y DTT, y separadas electroforéticamente en geles de poliacrilamida con SDS (PAGE/SDS).

Reactivos:

1. Tampón separador 4x (1.5 M Tris (181.65 g Tris base/L), 0.4 % SDS (20 ml de 20 % /L), ajustar a pH 8.8 con HCl).

2. Tampón concentrador 4x (0.5 M Tris (60.55 g Tris base/L), 0.4 % SDS, (20 ml de 20 % /L), ajustar a pH 6.8 con HCl).

3. Stock de Acrilamida (40 % acrilamida (40 g/100 ml), 1,07 % bis-acrilamida (1.07 g/100 ml), una vez preparada filtrarla (0.45 µm) y almacenarla en una botella oscura a 4 g). Se estropea con el tiempo.

4. SDS 20 % (200 g SDS en 800 ml de agua).

5. Tampón de electroforesis (25 mM Tris (3.03 g Tris base/L), 192 mM Glicina (14.4 g/L), 0.1 % SDS (5 ml de 20 %/L), pH≈8.3 sin ajustar).

6. Tampón de muestra 5x (50 % sacarosa (5 g/10 ml), 50 mM Tris (0.06 g Tris base/10 ml), 200 mM DTT (0.31 g/10 ml), 5 mM EDTA (0.1 ml de 0.5 M/10 ml), 5 % SDS (2.5 ml de 20 %/10 ml), y 0.005 % Azul de bromofenol (o de Pyronin Y) (0.5 mg/10 ml), ajustar a pH 8 y congelar en alicuotas de 100 µl a -20°C).

7. Coomassie Blue 1% (1 g Coomassie Brilliant Blue R250/100 ml agua).

8. Solución colorante (Metanol 45 %, Ac. Acético 10 % y Coomassie Blue 0.1 %).

9. Solución decolorante y fijadora (Metanol 45 %, Ac. Acético 10 %).

Procedimiento:

1. Limpia los vidrios con etanol y realiza el montaje como está descrito por el manufacturador.

2. Mezcla los siguientes componentes:

	Gel separador (Volúmenes ml)			Gel concentrador (Stacking)
	8 %	10 %	12.5 %	3 %
Tampón	3.75	3.75	3.75	2.00
Acrilamida (40 %)	2.98	3.75	4.65	0.60
Agua	8.27	7.50	6.60	5.40

Desgasar

3. Añadir:

	Gel separador	Gel concentrador
TEMED	25 µl	10 µl
Persulfato 13%	75 µl	40 µl

Mezclar rápidamente y aplicar : 6 ml/gel 2 ml/gel

Primero se realiza el separador y se añade ≈0.5 ml de agua. Cuando esté gelificado se elimina el agua con un papel de filtro y se realiza el concentrador.

4. Las muestras y stándards se calientan en el tampón de muestra 1x durante 5-10 min a 90°C. Y se aplican con una jeringa Hamilton. Si tienes un volumen de muestra muy grande puedes concentrarla precipitándola con tricloroacético (TCA) al 10 % final (≈20' a 4°C, 13000 rpm x 15'). Después de centrifugar lava el precipitado con acetona/1 % HCl (- 20°C) y centrifuga de nuevo durante un par de minutos. Se redisuelve con NH_4HCO_3 0.1 M y se añade el tampón de muestra. Si la muestra adquiere un color amarillo significa que el pH no es 8. Añade en este caso Tris-HCl 1M (pH 8) hasta que cambie a un color azul. Hasta 100 µg de proteína se pueden cargar por carril.

5. Una vez aplicada la muestra se aplica un voltaje de ≈70 V (I≈ 35 mA cuando realizamos dos geles) x 2-3 h.

6. Una vez finalizada la electroforesis se puede realizar diversas técnicas con el gel:

6.1. Tinción con Coomassie. Se coloca el gel en ≈100 ml de solución colorante durante más de 30 min mezclándolo suavemente. Se destiñe con la solución decolorante (añadir un trozo de esponja). Se secan con el secador de geles (≈ 1 h). Si los geles son

superiores al 12 % de acrilamida sumergirlos durante un par de horas en glicerol al 2 % antes de secarlos.

6.2. Tinción con plata. Se sumergen en:

2x15' en 40 % metanol y 10 % ac. acético.

15' en 10 % etanol y 5 % ac. acético.

10' en solución oxidante (10x (2.5 g $K_2Cr_2O_7$ + 380 µl H_3PO_4 en 250 ml de agua))

3x5' lavados con agua.

15' tinción con plata (10x (5.1 g $AgNO_3$ en 250 ml de agua)).

1' lavado con agua.

1' revelador (19.8 g Na_2CO_3+ 0.5 ml formaldehido 37 % en 1 litro de agua).

El revelador se prepara cada vez que se necesite.

2x5' revelador.

Stop con ac. acético al 1 % y se seca como está descrito en 6.1.

6.3. Si hemos realizado un fosfoenzima marcado radioactivamente o una fosforilación con ATP marcado, antes de poner el gel a contactar se fijan las proteínas con la solución decolorante (más de 30'). Se seca con el secador de geles (≈ 1h) y entonces se pone a contactar.

6.4. Transferencia a una membrana de nitrocelulosa para incubar las proteínas con anticuerpos. Detallado en la siguiente página.

Transferencia de proteínas a una membrana de nitrocelulosa y detección con anticuerpos ("Western Blot")

Referencias: Burnette, W.N. (1981) Anal. Biochem. **112**: 195-203.

Principio: Después de la electroforesis en PAGE/SDS las proteínas son transferidas mediante electroforesis a una membrana de nitrocelulosa. La membrana se bloquea con BSA y se incuba con el primer anticuerpo. La membrana se lava y se incuba con la proteína A (^{125}I) que se une a las cadenas pesadas de las inmunoglobulinas. Las membranas son lavadas y la localización de la proteína (antígeno)-anticuerpo1-proteína A (^{125}I) se visualiza radiográficamente.

Reactivos:

1. Tampón de transferencia (25 mM Tris (12.12 g/4 L), 192 mM Glicina (57.6 g/4 L), 20 % metanol (800 ml/4 L), pH≈8.3 sin ajustar). Para proteínas de PM>60000 se puede disminuir el % de metanol para mejorar la eficiencia de la transferencia.

2. Tampón de inmunodetección (20 mM Tris (9.69 g/4 L), 0.5 M NaCl (116.87 g/4 L) ajustado a pH 7.5 con NaOH). Para anticuerpos pocos específicos usar 0.15 M de NaCl.

3. Anticuerpos: - Anticuerpo realizado contra el decapéptido sintético GELTQTRLQK correspondiente al extremo amino de la PFK-2/FBPasa-2 de hígado (Crepin *et al.* (1989) Biochem. J. **264**:151-160). Utilizado con una dilución 1:200. Denominado: hígado específico (Ab_L).

- Anticuerpo realizado contra la PFK-2/FBPasa-2 de hígado (El-Maghrabi *et al.* (1986) Proc. Natl. Acad. Sci. USA **83**:5005-5009). Utilizado con una dilución 1:1000. Denominado: Abp.

4. Proteína A etiquetada con ^{125}I se emplea a concentracciones saturantes y a una radioactividad específica de 0.5-1x 10^6 cpm/ml.

5. Marcadores de peso molecular (Boehringer o Amersham).

Procedimiento:

1. Se corta una membrana de nitrocelulosa del tamaño del gel y cuatro hojas de papel Whatman 3MM del mismo tamaño. Recuerda marcar el frente y el punto de aplicación inicial en la membrana de nitrocelulosa. Se moja todo en el tampón de transferencia y se realiza el siguiente sandwich: dos hojas de papel Whatman 3MM, el gel, la membrana de nitrocelulosa y dos hojas de papel Whatman 3MM. Evita que queden burbujas entre el gel y la membrana. Cierra el sandwich y colócalo en el aparato de transferencia ("Transblot"). Recuerda que las proteínas migran hacia el ánodo (rojo).

2. Aplica una diferencia de potencial de 60 V durante 3 h a 4ºC. Agita el tampón con un agitador magnético. También lo puedes dejar toda la noche a 30 V. Si la proteína es >60 kDa aumenta los tiempos. Se puede controlar muy bien la transferencia usando un estándard de proteínas coloreadas (Rainbow markers, Amersham). También puedes teñir el gel para observar si las proteínas se han transferido.

3. Extrae con cuidado la membrana de nitrocelulosa del sandwich y lávala durante 5 minutos en el tampón de inmunodetección.

4. Coloca la membrana en el tampón anterior + 3% BSA (10 ml/membrana) en una bolsa y agita suavemente durante 1 h para bloquear la membrana.

5. Añade el anticuerpo con la dilucción apropiada en la bolsa. Incuba a T ambiente durante ≈90 min. Si está más tiempo añade 0.02 % de azida sódica para prevenir el crecimiento bacteriano.

6. Lava la membrana con ≈100 ml de tampón de inmunodetección durante ≈6 min. Repite hasta un total de 5 veces (≈30 min).

7. Incuba la membrana con prteína A (^{125}I) durante 30 min a T ambiente en el mismo tampón + 3% BSA. Dilucción de la proteína A (^{125}I) 1:1000 a una radioactividad final ≈ 0.2-1 10^6 cpm/ml (10 µl\10 ml).

8. Lava la membrana con ≈100 ml de tampón de inmunodetección durante ≈6 min. Repite hasta un total de 5 veces (≈30 min) y autoradiografía.

Aislamiento de ARN

Referencia: Auffray, C. and Rougeon, F. (1980) Eur. J. Biochem. 107:303-314.

Principio: El tejido es homogenizado en urea y SDS, desnaturalizándose las proteínas. El ARN es separado del ADN por precipitación con LiCl y la contaminación proteica es eliminada mediante extracciones con fenol.

Reactivos:

1. M. H., medio homogenización: 6 M Urea (72.1 g/200 ml)
   3 M LiCl (25.4 g/200 ml)
   0.2 % SDS (0.4 g/200 ml)
2. TE 5% SDS: 10 mM Tris-HCl, pH=8 (autoclavado)
   1 mM EDTA (autoclavado)
   5 % SDS

3. Fenol saturado de Tris-base: se añade a una botella de fenol de 250 g (Merck-206) 62.5 ml de agua estéril y 30 ml de Tris-base 1 M. Se mezcla en un baño a 50°C y se deja que se disuelva completamente. Se añade 0.3 g (0.1 % final) de 8-hidroxi-quinoleina (Merck-7098). Mezclar bien, alicuotar, cubrir con papel de plata y guardar a -20°C. Una vez descongelado se puede guardar a 4°C durante un mes. La 8-hidroxi-quinoleina es un antioxidante e inhibidor parcial de ribonucleasas que da el color amarillo a la solución de fenol.

4. Chisam: Cloroformo:alcohol isoamílico (50:2).

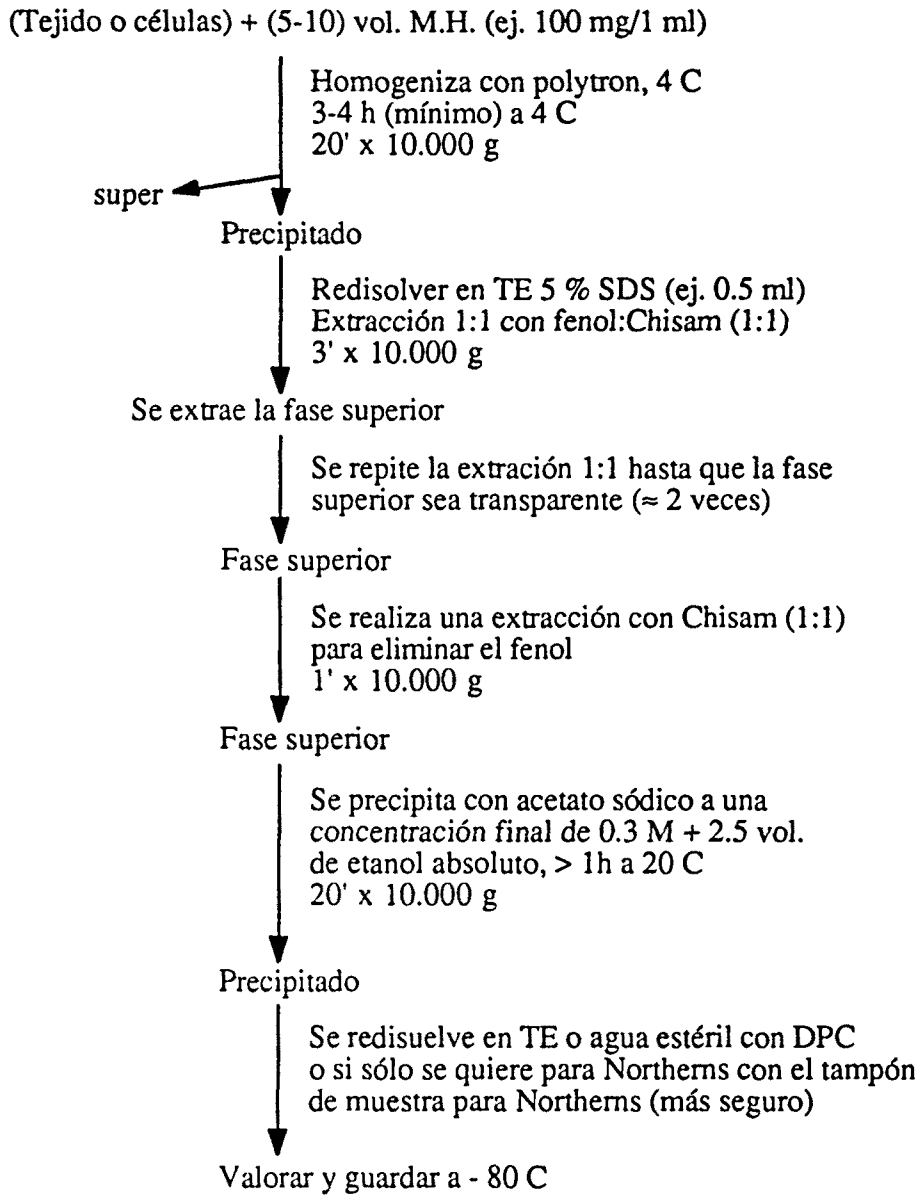
5. Acetato sódico 3 M para la precipitación con etanol.

6. Etanol 100 % absoluto y 70 %.

7. Agua + DPC (dietilpirocarbonato): añadir a un litro de agua mili Q 1 ml (0.1 %) de DPC. Dejar a T ambiente ≈1 h y autoclavar.

Procedimiento:

1. Esquema:



2. Valoración: A_{260}=40 µg/ml

   Pureza: $A_{260}/A_{280} > 1.6$

Electroforesis de ARN. Transferencia a una membrana de nylon e hibridación con sondas.

Referencia: Sambrook, J., Fritsch, E.F. and Maniatis T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor laboratory Press, N.Y. 2ond edition.

Principio: El ARN es separado en una electroforesis de agarosa después de desnaturalizarlo con formamida y formaldehido. Se transfiere a una membrana de nylon y se hibrida con sondas de ADN.

Reactivos:

1. MOPS 10x, tampón de electroforesis: 0.2 MOPS (41.8 g/L)
   50 mM Acetato de sodio (2.05 g/L)
   5 mM EDTA (10 ml de 0.5 M/L)
   ajustar pH 7.4 y autoclavar

2. Formamida desionizada: Mezcla 100 ml de formamida (Fluka-47670; Carlo Erba-452286) con 10 g de resina de intercambio iónico (Bio-Rad AG 501-X8, 20-50 mesh) y agitar durante 30 min a T ambiente. Filtrar dos veces con papel Whatman No.1, dispensar en alicuotas ≈50 ml y almacenar a -20°C.

3. Formaldehido 37 % (mira que el pH > 4, filtrar (0.22 μm)).

4. Tampón de aplicación: 15 % Ficoll (ó 50 % de glicerol), 0.4 % Azul de bromofenol, 0.4 % Xylene cyanol.

5. Tampón de muestra: MOPS 1x
   Formaldehido 6 %
   Formamida 50 %
   Bromuro de etidio (≈ 20 μg/ml)

6. PSE 10x, tampón de hibridación (Gene Screen). Ajustar a pH 6.4 con NaOH.
   0.1 M Pipes (30.2 g/L)
   4 M NaCl (233.7 g/L)
   10 mM EDTA (20 ml de EDTA 0.5 M/L)

7. SSPE 20 x, tampón de hibridación (N-Hybond). Ajustar a pH 7.4 y autoclavar.

0.2 M NaH_2PO_4 (27.6 g de $NaH_2PO_4.H_2O$/L)
3 M NaCl (175.3 g/L)
20 mM EDTA (40 ml de EDTA 0.5 M/L)

8. SSC 20x, tampón de transferencia. Ajustar pH 7.4 con HCl y autoclavar.
3 M NaCl (175.3 g/L)
0.3 M Citrato sódico (88.2 g/L)

9. Solución Denhardt's 100x: Ficoll/polivinilpirrolidona/BSA (1g:1g:1g/50 ml). Filtrar (0.22 µm). Alicuotar y guardar a -20ºC.

10. ADN de esperma de arenge, 10 mg/ml. Calentar para disolver. Alicuotar y guardar a -20ºC.

11. Random Primer Kit (Boehringer).

12. α-(^{32}P)dCTP 250 µCi (3000 Ci/mmol). (Amersham PB10205).

Procedimiento:

1. Prepara el gel:

	/50 ml	/100 ml	/200ml	Final
Agua	36	72	144	
MOPS 10x	5	10	20	1x
Agarosa	0.75 g	1.5 g	3 g	1.5 %

Se calienta hasta que la agarosa se disuelva completamente. Se enfría a 55-65ºC y se añade:

Formaldehido 37% (≈12 M)	9	18	36	6 % (≈2 M)

2. Se pipetea 20 µg ARN en un eppendorf. Si el volumen es muy grande o el ARN está disuelto en otro tampón que no sea el de muestra se puede concentrar o eliminar el tampón con el speed-vac y luego disolver con el tampón de muestra. Se calienta a 55-65ºC 5-10 min y se añade 2 µl de tampón de aplicación por cada 20 µl. A continuación se aplica a los pocillos del gel y se realiza la electroforesis (Voltaje: 3-4 V/cm).

3. Se realiza la transferencia por capilaridad con SSC 10 x a una membrana de nylon (N-Hybong o Gene Screen) durante >12 h.

4. Se seca la membrana con papeles de filtro y secador. Se fija el ARN por calor o con luz UV (mira las indicaciones del manufacturador; ej. Para N-Hybond 10 min a 80ºC o 5 min a 312 nm).

5. Prehibrida en un baño con agitación durante 6 h a 42ºC. Solución de prehibridar:

	/20 ml	/50 ml	Final
S. Denhardt´s 100x	1	2.5	5x
SSPE 20x	5	12.5	5x
SDS 20 %	0.5	1.25	0.5 %
Formamida	10	25	50 %
ADN 10 mg/ml	1	2.5	0.5 mg/ml
Agua	2.5	6.25	

Calentar a 65ºC durante 15 min antes de añadir al filtro.

6. Prepara la sonda marcada siguiendo el protocolo del kit de random primer. Brevemente:

- Calienta la sonda (ADN) 10' a 95-100ºC y enfríalo en hielo rápidamente.
- Toma ≈25 ng y colócalos en un eppendorf.
- Añade 3 µl de dATP:dGTP:dTTP (1µl:1µl:1µl).
- Añade 2 µl de hexanucleótidos (reaction mixture).
- Añade 2 µl de α-(^{32}P)dCTP (20 µCi).
- Añade agua autoclavada hasta un volumen de 19 µl.
- Añade la Klenow polimerasa 1 µl.
- Incuba ≈37ºC durante 30 min.
- Stop: 2 µl EDTA 0.5 M.
- Añade agua hasta un V=101 µl. Toma 1 µl y cuenta la radioactividad (R. Total).
- Elimina el exceso de radioactividad pasando la muestra a través de una columna Sephadex G50 medium. Se puede preparar una columna utilizando un eppendorf agujereado al que se le coloca lana de vidrio autoclavada. Se añade 1.5 ml de gel y se centrifuga 5' x 1500 g. Se aplican los 100 µl, se centrifuga 5'x1500 g recogiéndose el líquido en otro eppendorf. Se toma 1 µl y se cuenta la radioactividad (R. Incorporada). El cociente R. Incorporada/R. Total nos dará la eficiencia del marcaje normalmente del 60-70 %.

7. Una vez preparada la muestra Se calienta 2-5' min a 95-100ºC. Se enfría en hielo y se añade al tampón de prehibridación. Hibridándose durante >16 h en un baño con agitación a 42ºC.

8. Lavados. Se realizan 4 lavados (≈ 200 ml/lavado) de 30 min cada uno a 50°C en SSC 0.1x y SDS 0.1 % (10 ml SSC 20x/2 L y 10 ml SDS 20 %/2 L).

9. Contactar con pantallas intensificadoras a -80°C.

10. Deshibridar. Mira instrucciones de la membrana. Para N-Hybond: hierve una solución de 0.1 % de SDS y añade la membrana. Deja enfriar a T ambiente. Chequea la membrana para comprobar que la sonda radioactiva ha sido trasladada.

Purificación de núcleos

Referencia: J.L. Rosa, A. Tauler, A.J. Lange, S.J. Pilkis and R. Bartrons (1991) Proc. Natl. Acad. Sci. USA (in the press). Modificación del método de Laitinen *et al.* (1990) J. Cell. Biol. **111**:9-17.

Principio: Este método permite una rápida purificación de núcleos a partir de tejido o células. Se basa en sucesivas homogenizaciones y centrifugaciones en un medio hipotónico (RSB) y utilizando el detergente no-iónico Nonidet P40. En estas condiciones la membrana nuclear está preservada, lo que permite la purificación de los núcleos.

Reactivos:

1. Tampón STM, ajustado pH 7.4 con HCl. Medio isotónico.
   250 mM sacarosa (171.15 g sacarosa/2 L)
   50 mM Tris-HCl (12.11 g Tris-base/2 L)
   5 mM $MgSO_4$ (2.47 g $MgSO_4$ heptahidratado/2 L).

2. Tampón RSB (Reticulocyte standard buffer), ajustado pH 7.4. Medio hipotónico.
   10 mM Tris-HCl (5 ml Tris-HCl pH 7.4 1M/ 500 ml)
   10 mM NaCl (5 ml NaCl 1M/ 500 ml)
   3 mM $MgCl_2$ (1.5 ml $MgCl_2$ 1M/ 500 ml)

3. PMSF. Se prepara un stock 100 mM en isopropanol anhidrido (1.74 g/100 ml) y se usa a una concentración 1 mM. Se añade inmediatamente antes de usarlo. Es un inhibidor de serin-esterasas. Se almacena a 4°C.

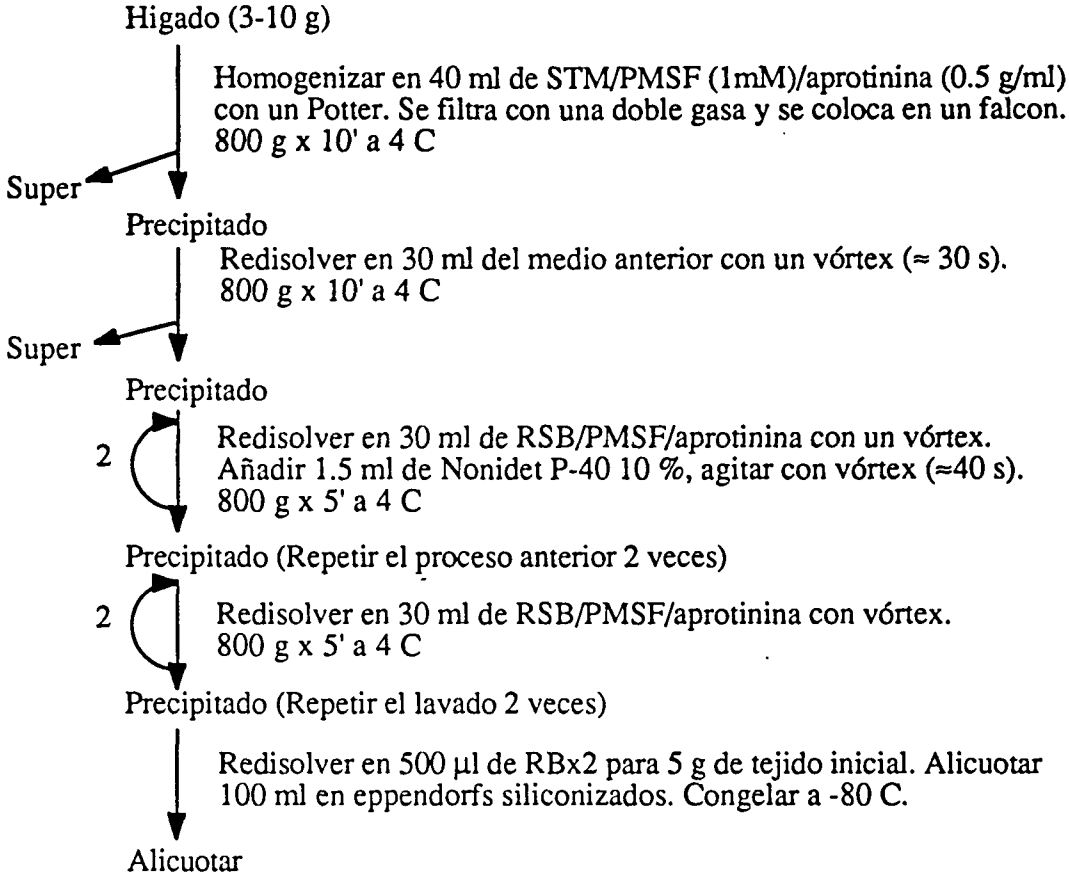
4. Nonidet P-40 10 %

5. Aprotinina. Se prepara un stock de 0.5 mg/ml y se usa a una concentración de 0.5 µg/ml.

6. Tampón RB 2x, ajustar a pH 8.0 con HCl y autoclavar.
   40 mM Tris-HCl (0.48 g Tris-base/100 ml)
   0.1 mM EDTA (20 µl EDTA 0.5 M/100 ml)
   10 mM $MgCl_2$ (1 ml $MgCl_2$ 1M/100 ml)
   40 % Glicerol (40 ml/100 ml)

Procedimiento:

1. Esquema:



Se puede seguir la purificación tomando alicuotas y tiñiendo los núcleos con hematoxilina.

2. Valorar. - Se puede realizar un contaje (10 $\mu l_{núcleos}$ + 190 μl_{RSB}).
   - Se puede leer la A_{260} (1 U.A.= 50 µg ADN/ml): 10 $\mu l_{núcleos}$ + 1 $ml_{SDS\ 1\%}$ vórtex y leer.
   - Rendimiento ≈ 10^8 núcleos/ 2-3 g tejido.

Medida de la velocidad de transcripción (Run-on)

Referencia: G.S. McKnight and R.D. Palmiter (1979) J. Biol. Chem. **254**:9050--9058.

Principio: Se incuban los núcleos aislados con (α-^{32}P)UTP. Los transcritos de ARN nuclear de las células eucariotas que se han iniciado *in vivo* pueden incorporar (α-^{32}P)UTP *in vitro* extendiendo la cadena de ARN naciente hasta 300-500 nucleótidos. Se asume que todas las cadenas de ARN iniciadas *in vivo* y conteniendo ARN polimerasa II son elongadas *in vitro* a la misma velocidad y con la misma extensión. Los ARN marcados se aislan y se identifican con una sonda de ADN complementaria de un ARNm específico.

Reactivos:

1. Núcleos aislados en RBx2 (>5 millones). Mira el protocolo anterior.

2. Mezcla de nucleótidos trifosfato (NTP) 20x (10 mM de cada): ATP:GTP:CTP.

3. (α-^{32}P)UTP (400-3000 Ci/mmol) 1mCi (Amersham PB-10203).

4. KCl 2.5 M, autoclavado.

5. DTT 100 mM.

6. Solución de lisis: 0.5 M NaCl; 10 mM Tris-HCl pH 7.4; 50 mM $MgCl_2$; 2 mM $CaCl_2$.

7. DNasa (RNasa free) 35 U/µl (Boehringer).

8. Proteinasa K 10 mg/ml (Boehringer).

9. Glucógeno 20 mg/ml (Boehringer).

10. Reactivos de extración de ARN.

11. Material que aguante cloroformo.

Procedimiento:

1. Incubar :	Concentraciones en el ensayo
100 µl núcleos en RBx2	Núcleos (> 5 millones)
	20 mM Tris-HCl pH 8.0
	5 mM $MgCl_2$
	0.05 mM EDTA
	20 % glicerol
10 µl NTP 10mM	0.5 mM ATP
	0.5 mM GTP
	0.5 mM CTP
10-20 µl (α-^{32}P)UTP (3000 Ci/mmol)	0.16-0.32 µM (100-200 µCi)
12 µl KCl 2.5 M	0.15 M KCl
4 µl DTT 100 mM	2 mM DTT
64-54 µl agua estéril	
Vol. = 200 µl	T= 37°C, 30 min.

2. Stop:
- 1ml Solución de lisis.
- 6 µl DNasa (RNasa free) de 35 U/µl. 5 min a T ambiente.
- 12 µl Proteinasa K de 10 mg/ml. 5 min a T ambiente.
- 1.5 ml de 10 mM Tris-HCl pH 8.0; 10 mM EDTA; 0.5 % SDS.

3. Extracción:
- 2.5 µl de glucógeno 20 mg/ml (transportador).
- 5 ml fenol:cloroformo:isoamílico (25:24:1). 5 min x 2500 rpm. Extraer con Pasteur *cogiendo la emulsión blanca de la interfase*. Repetir otra vez.
- 5 ml cloroformo:isoamílico (24:1).
- Precipitar en córex con acetato sódico y etanol. >2 h a -20°C.
- Centrifugar 30 min a 9000 rpm (Sorvall SS-34).
- Resuspender en TE (100-150 µl).

4. Eliminar el exceso de radioactividad mediante una columna Sephadex G-50 medium.

5. Ajustar con el medio de hibridar de los Northerns las mismas cpm/ml y el mismo volumen para cada eppendorf. Volumen aconsejable para hibridar ≈ 1ml. Radioactividad >600.000 cpm/ml en contaje directo del eppendorf (real ≈ 2.000.000 cpm/ml).

6. Hibridar con las membranas de nylon en las que habíamos fijado previamente las sondas (> 4 µg ADN) que queremos estudiar. Para aplicar las sonda usamos un *slot-blot*. Estas membranas habían sido previamente prehibridadas como en el protocolo de los Northerns. Antes de añadir los ARN marcados en la bolsa de hibridación caliéntalos 5 min a 65ºC. Hibrida a 42ºC durante ≈72 h.

7. Lavados:	SSC	SDS
- 20 min T ambiente	2x	1%
- 20 min a 65ºC	2x	1%
- 20 min a 65ºC	0.1x	1%
- 20 min a 65ºC	0.1x	1%
- 20 min a 65ºC	0.1x	0.1%

8. Contactar.