

# Hepatocyte 'priming' and increase in transforming growth factor- $\beta$ 1 mRNA expression are delayed in hypothyroid versus euthyroid rats during liver regeneration

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**Abstract.** Hypothyroidism decreases liver weight and delays the compensatory liver growth after partial hepatectomy (PH) as compared with the euthyroid condition. The aim of this study was to investigate, in hypothyroid rats, the mRNA expression of genes modulating these effects, focusing on *c-fos* and *c-myc*, hallmarks of hepatocyte 'priming', and on transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and its receptor, the transforming growth factor- $\beta$ 1 receptor-type II (T $\beta$ R-II), negative regulators of liver growth. Euthyroid and hypothyroid male Wistar rats underwent 70% PH and total RNA was isolated from frozen liver samples removed at basal state and during regeneration, 0-144 h after surgery. In this study, we show for the first time that, in the basal liver state, hypothyroidism increased TGF- $\beta$ 1 and T $\beta$ R-II mRNA levels by 45% and 30%, respectively, as compared with the euthyroid condition and, after PH, resulted in a ~12-h delay in the activation of *c-fos* and *c-myc* mRNA expression. Moreover, the increase in TGF- $\beta$ 1 mRNA levels, detected 24-48 h after PH in euthyroid rats, was delayed by 72 h in hypothyroid rats, occurring when a concomitant reduction in T $\beta$ R-II was measured. These results suggest that, in hypothyroid rats, at the basal liver level, the increase in mRNA expression of genes that negatively regulate liver growth might be involved in the decrease in liver weight and that, after PH, the delay of hepatocyte 'priming' and coordinated changes in mRNA expression of negative regulators of liver regeneration might be involved in delaying the regenerative process.

## Introduction

The regeneration of the rat liver following two-thirds PH is one of the most useful models for studying the cellular and subcellular changes occurring *in vivo* in different phases of normal and pathological cell proliferation because of the

high synchronism of the first cell cycle, the timing of which has been precisely defined in physiological conditions (1). Hepatocyte proliferation following PH begins with the 'priming' of quiescent mature hepatocytes from a resting state (G<sub>0</sub>) to the prereplicative G<sub>1</sub> phase of the cell cycle, which lasts for 12-14 h. The pre-replicative phase is followed by DNA synthesis, which peaks 22-24 h after PH, and by mitosis 6-8 h later (2). Thereafter, the liver mass is gradually recovered, and the regenerative process is complete 96 h after PH (3). The regenerative process involves metabolic and ultrastructural changes (3-5) and, in order to be precisely carried out, multiple hepatic cell types must proliferate in a coordinated fashion (6). A coordinated balance of positive and negative controls, through selective sequence of gene expression, allows the normal regenerative process (7). Following PH, quiescent mature hepatocytes are 'primed' to re-enter the cell cycle. The 'priming' process appears to be triggered by extracellular cytokines (interleukin-6 and tumor necrosis factor- $\alpha$ ) and is characterized by the expression of immediate early genes (8).

The genes induced immediately after PH include some proto-oncogenes, such as *c-fos* and *c-myc*, which are responsible for hepatocyte 'priming' and are believed to act as transcription factors for genes active later in the G<sub>1</sub> phase of the cell cycle (6,7). Negative regulatory influences are induced later by specific growth inhibitors, such as TGF- $\beta$ 1, to stop the regenerative process when the liver mass has been restored (9,10). Signaling by TGF- $\beta$ 1 occurs through T $\beta$ R-II (11) and arrests the cell cycle in G<sub>1</sub> phase by stimulating the production of the cyclin-dependent protein kinase inhibitors and by inhibiting the function or production of essential cell cycle regulators (12).

Several hormones appear to be essential for liver regeneration, including thyroid hormone (T<sub>3</sub>) (1,2). T<sub>3</sub> is a powerful inducer of hepatocyte proliferation (13,14) and its mitogenic capacity has been used for gene therapy experiments and repopulation of hepatocytes (15,16). T<sub>3</sub> plays a major role in hepatic mitochondrial maturation and metabolism as it regulates the mitochondrial membrane function and the expression of both mitochondrial and nuclear genes (17-19). Additionally, T<sub>3</sub> regulates the expression of nuclear genes involved in the modulation of the cell cycle (20). It has been recently shown that T<sub>3</sub> plays a role in the compensatory liver growth that follows partial surgical removal of the liver (21). On the other hand, either thyroidectomy or chemically-

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induced hypothyroidism reduces the capacity to recover liver mass after PH (21,22) and causes the inhibition of DNA synthesis (23) and mitotic activity (22,23).

We have recently demonstrated that hypothyroidism decreases the liver weight and delays the regenerative process after PH (17,21). However, the molecular mechanisms responsible for these events are largely uncharted. In this study, we investigated whether hypothyroidism affects the mRNA expression of genes that can modulate liver growth and regeneration, focusing on hallmarks of hepatocyte 'priming', such as *c-fos* and *c-myc* (6,7), and on negative regulators of hepatocyte proliferation, such as TGF- $\beta$ 1 and TBR-II (9-11).

## Materials and methods

**Animals.** Male Wistar rats (200-250 g) were housed in a temperature-controlled room (22°C) with food and water *ad libitum*. Three independent series of rats were used. For each series, rats were divided into two groups. The first group (group 1) was given ordinary drinking water (euthyroid); the second group (group 2) in addition was given 0.1% (wt/vol) 6-n-propyl-2-thiouracil (PTU) in drinking water for 21 days (hypothyroid) (21). The transition from euthyroidism to hypothyroidism was evaluated by measuring T<sub>3</sub> serum levels (21). PH was performed with removal of the median and left lateral lobes of the liver of euthyroid and hypothyroid rats, as previously described (21) and the excised tissue was used as non-regenerating liver at basal state. After surgery, rats were kept on a standard diet until they were sacrificed. Blood samples were collected at the time of sacrifice, 0-144 h after PH, for estimation of T<sub>3</sub> serum levels. The livers were removed, weighed, kept in liquid nitrogen and used for RNA extraction. All operations were carried out under sterile conditions. The animals received humane care and the study was approved by the State Commission on Animal Experimentation.

**RNA extraction and Northern blot analysis.** Total cellular RNA was isolated from frozen tissue samples using the guanidinium isothiocyanate-cesium chloride procedure (24). Northern blotting analysis was carried out as described previously (25). Briefly, RNA samples (25  $\mu$ g/lane) were separated on formaldehyde-agarose gels and blotted onto nylon membranes (Hybond N<sup>+</sup>; Amersham, Milan, Italy). The blots were hybridized with cDNA probes, previously labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol; NEN Life Science Products, Boston, MA) by random primer extension (Mega-prime DNA labeling kit; Amersham), for 20 h at 42°C. The filters were washed once with 2X SSPE, 0.1% SDS for 10 min at room temperature; then with 1X SSPE, 0.1% SDS at 42°C; followed by several washes in 0.1X SSPE, 0.1% SDS, at 65°C; and finally exposed, at -80°C overnight or longer, to Kodak X-Omat AR 5 film (Kodak, Rochester, NY). Radiolabeled probes were generated using human *c-fos* DNA obtained from the recombinant plasmid, pBR322-*c-fos* [pcfos (human)-1] (26); the human *c-myc* cDNA cleaved from the recombinant plasmid, pRyc-7.4 (27); the human TGF- $\beta$ 1 cDNA (28) or the human TBR-II cDNA (29). Quantitative analysis was performed by densitometric scanning of the autoradiographs using a GS-700 Imaging densitometer (Bio-Rad, Richmond, CA);

multiple exposures of the same Northern blots in a linear range were performed. Hybridization with a 1.3-kb cDNA fragment for 28S rRNA (30) was used as a control to determine the integrity of RNA and equality of loading in each lane.

**Statistical analysis.** The statistical significance of differences between groups was determined by one-way ANOVA followed by a Student-Newman-Keuls test. Comparison between independent means was performed using the Student's t-test.

## Results

**Hypothyroidism increases TGF- $\beta$ 1 and TBR-II mRNA expression in rat liver.** The administration of PTU to euthyroid rats for 21 days induced hypothyroidism, with T<sub>3</sub> serum levels of 86 $\pm$ 5 ng/dl versus 182 $\pm$ 11 ng/dl of euthyroid controls (P<0.03), and resulted in a reduced liver weight (7.5 $\pm$ 0.4 g) as compared with euthyroid controls (12.1 $\pm$ 0.8 g) (P<0.01), as previously reported (17). The mRNA expression levels of genes regulating liver growth and proliferation, i.e. *c-fos*, *c-myc*, TGF- $\beta$ 1 and TBR-II, were measured in the liver of hypothyroid rats at the basal state and compared with euthyroid controls by Northern blotting (Fig. 1A). *c-fos* and *c-myc* mRNAs were undetectable both in hypothyroid and euthyroid rats, at variance with TGF- $\beta$ 1 and TBR-II. The values of TGF- $\beta$ 1 and TBR-II mRNA levels were related to 28S RNA, accounting for RNA loading in each sample. Both TGF- $\beta$ 1 and TBR-II levels were higher (P<0.01) in hypothyroid than in euthyroid animals, by 45 $\pm$ 4% and 30 $\pm$ 5%, respectively (Fig. 1B).

**Hypothyroidism delays the mRNA expression of immediate early genes during liver regeneration.** 'Priming' characterizes the initiation of hepatocyte proliferation after PH (31). To investigate whether the 'priming' of hepatocytes was altered as a result of hypothyroidism, we measured the pattern of expression of the immediate early response genes, *c-fos* and *c-myc*, during the regenerative process. First we confirmed that, during liver regeneration, T<sub>3</sub> serum levels in hypothyroid rats remained lower than in euthyroid controls, as previously reported (21). Then, we measured steady-state levels of *c-fos* and *c-myc* mRNAs by Northern blot analysis of total RNA isolated from livers at different times after PH (Fig. 2A). *c-fos* and *c-myc* mRNA levels in all samples were related to the 28S RNA levels, accounting for RNA loading in each sample, and calculated as a percentage of the maximal levels of expression detected in euthyroid rats during the time course experiment. Fig. 2B shows the *c-fos* and *c-myc* mRNA expression profiles in the livers of euthyroid and hypothyroid rats. The mRNA expression of the two genes at time of surgery, i.e. in non-regenerating liver, was undetectable. After surgery, the mRNA expression of these genes was activated both in euthyroid and hypothyroid rats at different times. In fact, in euthyroid rats, *c-fos* and *c-myc* were expressed within 0.5 h following PH, and reached peaks of expression at 0.5-3 h and at 48 h, consistent with previous reports (31,32). On the contrary, in hypothyroid rats, *c-fos* mRNA was detected at 12 h, and its expression was prolonged for up to 72 h, at which point it reached levels comparable to those detected in euthyroid rats at 0.5 h. Another peak of *c-fos* mRNA expression

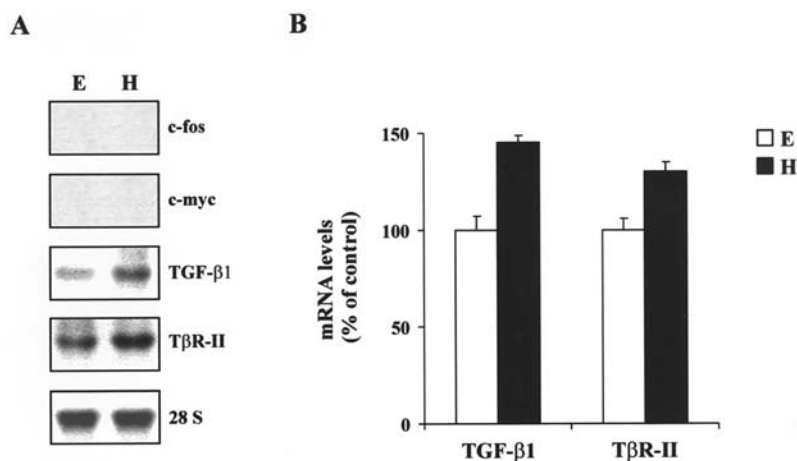


Figure 1. c-fos, c-myc, TGF-β1 and TβR-II mRNA expression in the liver of euthyroid (E) and hypothyroid (H) rats. (A) Northern blot analysis for c-fos, c-myc, TGF-β1 and TβR-II mRNA expression. Hybridization with a 28S rRNA probe was used as control to determine the equality of loading in each lane. (B) A quantification of TGF-β1 and TβR-II mRNA levels in hypothyroid rat liver is reported as the percentage of mRNA levels in euthyroid rat liver (control), set at 100. The mean values ± SE of three different experiments performed on samples obtained from three different animals for each experimental group are reported.

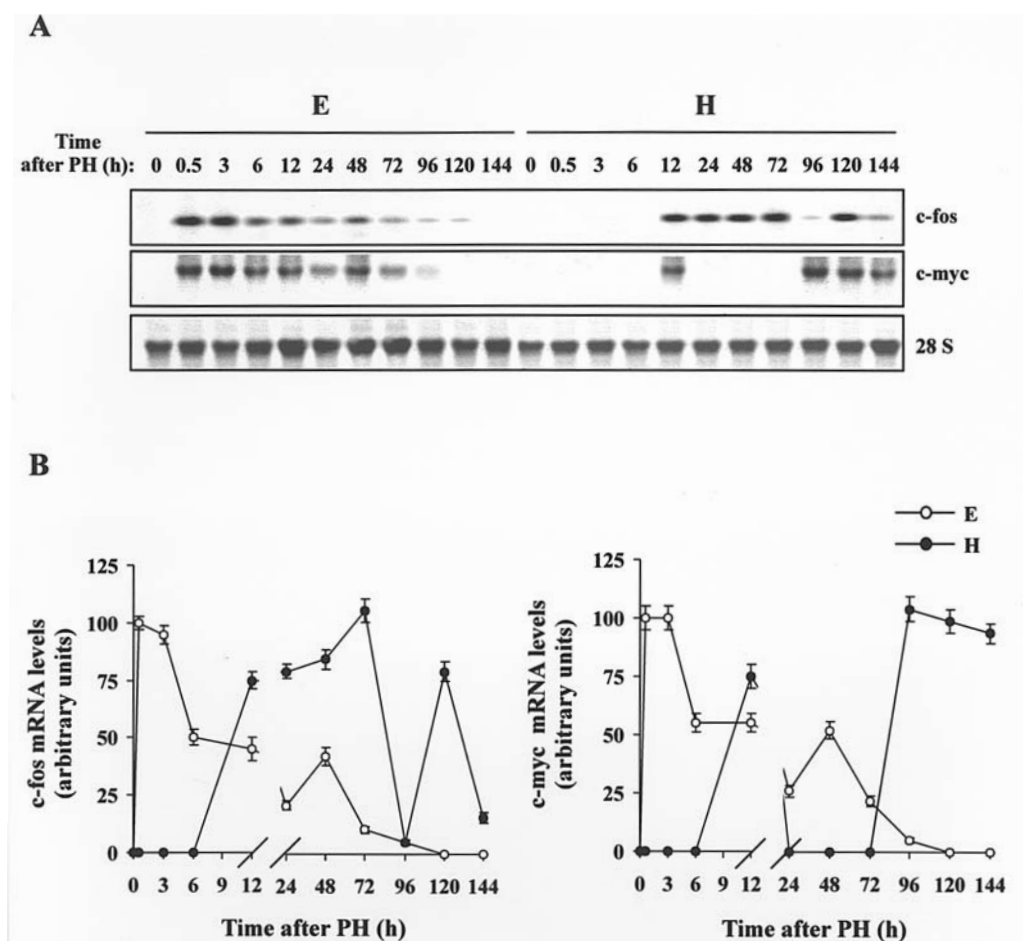


Figure 2. c-fos and c-myc mRNA expression in the liver of euthyroid (E) and hypothyroid (H) rats after PH. (A) Northern blot analysis for c-fos and c-myc expression at 0-144 h of liver regeneration. Hybridization with a 28S rRNA probe was used as control to determine the equality of loading in each lane. (B) c-fos and c-myc mRNA expression levels in the livers of euthyroid or hypothyroid rats after PH were reported as the percentage of maximal levels detected during liver regeneration in euthyroid rats, set at 100. The mean values ± SE of three different experiments performed on samples obtained from three different animals for each experimental group are reported.

was detected at 120 h that reached 75±4% of the control. Similar to c-fos, a biphasic expression profile was found for c-myc mRNA with peaks at 12 h (74±5% of the control;

P<0.001) and at 96 h, at which point c-myc mRNA levels reached values comparable to those detected at 0.5-3 h in euthyroid rats.

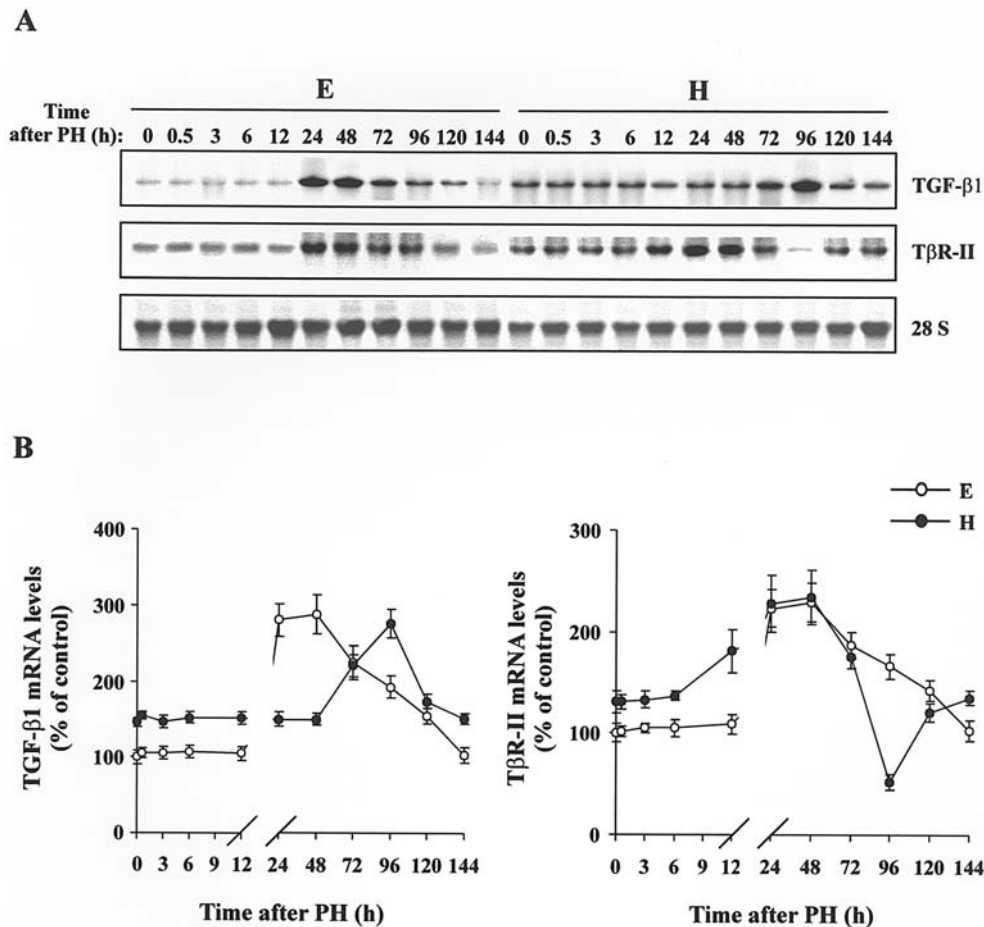


Figure 3. TGF- $\beta$ 1 and T $\beta$ R-II mRNA expression in the liver of euthyroid (E) and hypothyroid (H) rats after PH. (A) Northern blot analysis for TGF- $\beta$ 1 and T $\beta$ R-II mRNA expression. Hybridization with a 28S rRNA probe was used as control to determine the equality of loading in each lane. (B) TGF- $\beta$ 1 and T $\beta$ R-II mRNA expression levels in the livers of euthyroid and hypothyroid rats after PH were reported as a percentage of the mRNA levels in euthyroid rats at 0 h (control), set at 100. The mean values  $\pm$  SE of three different experiments performed on samples obtained from three different animals for each experimental group are reported.

*Hypothyroidism affects TGF- $\beta$ 1 and T $\beta$ R-II mRNA expression during liver regeneration.* The regenerative response after PH stops abruptly when the liver mass has been restored (1,2,6). To investigate the effect of hypothyroidism on the signals involved in the arrest of hepatocyte proliferation, the pattern of expression of TGF- $\beta$ 1 and T $\beta$ R-II mRNAs during liver regeneration was measured by Northern blot analysis on the same blots used to evaluate *c-fos* and *c-myc* mRNA expression (Fig. 3A). TGF- $\beta$ 1 and T $\beta$ R-II mRNA expression levels were calculated as a percentage of the levels of expression detected in the excised tissue at surgery time. Fig. 3B shows the TGF- $\beta$ 1 and T $\beta$ R-II mRNA expression profiles in euthyroid and hypothyroid rat liver during the regenerative process. In euthyroid rats, the expression of TGF- $\beta$ 1 and T $\beta$ R-II remained constant for up to 12 h after PH, increased at 24–48 h by  $288 \pm 26\%$  and  $229 \pm 19\%$  ( $P < 0.001$ ), respectively, and thereafter slowly returned to basal levels. In hypothyroid rats, TGF- $\beta$ 1 mRNA levels did not change for the first 48 h, increased between 72 and 120 h with a peak of  $277 \pm 19\%$  at 96 h, and returned to basal levels at 144 h, whereas T $\beta$ R-II mRNA levels remained constant for up to 6 h after PH, increased  $>2$ -folds at 24–48 h, reaching levels similar to those detected in the euthyroid group, and returned to basal levels after a decrease to  $52 \pm 8\%$  at 96 h.

## Discussion

Liver regeneration after PH provides an excellent *in vivo* model for studying the role of factors involved in the transition of hepatocytes from a quiescent to a proliferative state and *vice versa*, when the liver mass has been restored. We have previously reported that hypothyroidism impairs the liver's regenerative capacity after PH (17,21). The results reported here reveal that this effect involves a delay in hepatocyte 'priming' as well as in stopping proliferative signals.

Activation of immediate early genes characterizes the initiation of hepatocyte proliferation after PH. We show that *c-fos* and *c-myc* are expressed in the liver within a few hours following PH, consistently with previous reports (31,32). Expression of these genes is widely assumed to be integral to the hepatic regenerative response as it would lead to the formation of transcription factors that are supposed to play an important role in liver regeneration (33). Previous studies on different liver regenerative models have shown that hepatocyte 'priming' can be variably affected by treatments/conditions that delay or abrogate hepatic cell replication. Dexamethasone-treatment and hypophysectomy, which delay DNA synthesis, (34) or acute hepatic failure, which completely blocks DNA synthesis and hepatocyte proliferation (9), do not cause any



change in expression of immediate early genes. On the contrary, inhibition of DNA synthesis by retinoic acid in regenerating liver is associated with repression of c-fos and c-jun expression (35). T<sub>3</sub> modulates liver growth (36) by regulating the gene expression for a diverse range of cellular pathways, including glycogenolysis, gluconeogenesis, lipogenesis, cell proliferation and apoptosis (37). In this context, it has been reported that hypothyroidism causes a reduction in DNA synthesis, PCNA (proliferating cell nuclear antigen) expression and mitotic activity in the liver after hepatic resection (20,22,23,38). We show here that hypothyroidism significantly delays c-fos and c-myc mRNA expression after PH, suggesting that it might partly exert its antiproliferative activity on liver cells during the 'priming' stage.

We have previously reported that, after chemical induction of hypothyroidism, the regenerative process starts with a liver mass lower than the euthyroid controls (17,21). TGF- $\beta$ 1 can be involved in the control of liver growth, acting as a growth inhibitor of hepatocytes in culture (39), and low levels of TGF- $\beta$ 1 mRNA are supposed to maintain the quiescent state of hepatocytes (40). Consistently, we found basal expression of TGF- $\beta$ 1 mRNA in the euthyroid liver. The TGF- $\beta$ 1 action on liver growth is mediated by T $\beta$ R-II (11,41) and an increase in T $\beta$ R-II mRNA expression has been associated with increased sensitivity of hepatocytes to TGF- $\beta$ 1-related growth inhibition (42). We show here that hypothyroidism increases liver TGF- $\beta$ 1 and T $\beta$ R-II mRNA expression, suggesting that coordinated changes in the expression of TGF- $\beta$ 1 as well as of its receptor, T $\beta$ R-II, could be responsible, at least in part, for the negative regulation of liver growth by a hypothyroid state.

After PH, the liver 'knows' exactly when to stop growing and various studies have been focused on the mechanisms involved in the arrest of liver regeneration (1,2,6). Besides its role as a negative regulator of liver growth, TGF- $\beta$ 1 has been proposed as a major candidate regulating the termination of constitutive DNA synthesis in hepatocytes after PH once the functional liver mass has been regained (43). In fact, suppression of TGF- $\beta$ 1 expression results in up-regulation of transcripts for regeneration factors and inhibitors of apoptosis (44). Consistent with previous findings (40,42,45,46), we show that, in euthyroid rats, TGF- $\beta$ 1 mRNA expression reaches a peak 48 h after PH, when the liver weight is ~60% of the initial mass (21). Interestingly, hypothyroidism delays by 72 h the increase in TGF- $\beta$ 1 mRNA expression, which occurs 96 h after PH, at which point the liver weight is still ~50% of the initial mass (21). The rising levels of TGF- $\beta$ 1 mRNA during liver regeneration can act to limit the proliferative response of the hepatocytes through restriction of G1 to S phase cell cycle transition (47). However, the sensitivity to TGF- $\beta$ 1 signaling is dependent on T $\beta$ R-II expression. We show that, in euthyroid animals, the increase in TGF- $\beta$ 1 mRNA expression is accompanied by an increase in T $\beta$ R-II levels, which is recognized as being a physiologically important mechanism underlying the termination of liver regeneration (42). In hypothyroid rats, the initial increase in T $\beta$ R-II mRNA expression is followed by a decrease 96 h after PH. Because the TGF- $\beta$ 1 protein can suppress the expression of T $\beta$ R-II in hepatocytes (42), it is possible that this decrease in T $\beta$ R-II mRNA expression may be in part mediated by the increase in

hepatic TGF- $\beta$ 1 protein. These orchestrated changes in TGF- $\beta$ 1 and T $\beta$ R-II expression could allow the hepatocytes of hypothyroid animals to continue proliferating until the functional liver mass is gained. Indeed, hypothyroid rats are able to regenerate their liver mass completely but the process is significantly delayed, with ~50% recovery at 96 h after PH, at which point regeneration is complete in the euthyroid group (21).

In conclusion, our results suggest that an impaired liver regenerative capacity in hypothyroidism after PH might be due to the delayed expression of genes involved in the control of G0-G1 progression and growth arrest. Since these changes could affect the normal course of liver regeneration and, thus, the liver specific functions in transplantation or following viral infections, future genetic approaches to examine either deleted or enhanced signals of specific gene expression might demonstrate the causal relationship between changes in the expression of positive and negative proliferative regulators and liver diseases in a hypothyroid state. Based on this knowledge, potential therapeutic strategies could be developed.

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