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Heparin-binding Epidermal Growth Factor-like Growth Factor Links Hepatocyte Priming with Cell Cycle Progression during Liver Regeneration*

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Claudia Mitchell‡, Mary Nivison‡, Leslie F. Jackson§, Richard Fox‡, David C. Lee§, Jean S. Campbell‡, and Nelson Fausto‡¶

From the ‡Department of Pathology, University of Washington School of Medicine, Seattle, Washington 98195 and §Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

The mechanisms that regulate the transition between the initial priming phase and DNA replication in liver regeneration are poorly understood. To study this transition, we compared events occurring after standard two-thirds partial hepatectomy, which elicits full regeneration, with response to a reduced hepatectomy, one-third partial hepatectomy (1/3PH), which leads to little DNA replication. Although the initial response to partial hepatectomy at the priming phase appeared to be similar between the two procedures, cell cycle progression was significantly blunted in 1/3PH mice. Among the main defects observed in 1/3PH mice were an almost complete deficiency in retinoblastoma phosphorylation and the lack of increase in kinase activity associated with cyclin E. We report that, in two-thirds partial hepatectomy mice, the expression of heparin-binding epidermal growth factor-like growth factor (HB-EGF) preceded the start of DNA replication and was not detectable in 1/3PH animals. Injection of HB-EGF into 1/3PH mice resulted in a >15-fold increase in DNA replication. Moreover, we show that hepatocyte DNA replication was delayed in HB-EGF knock-out mice. In summary, we show that HB-EGF is a key factor for hepatocyte progression through G_1/S transition during liver regeneration.

Adult hepatocytes, while highly differentiated, retain a remarkable ability to proliferate in response to liver injury or reduction in hepatic mass. The surgical removal of ~70% of the liver, two-thirds partial hepatectomy (2/3PH),[1] is the most studied model of liver regeneration. This procedure induces a synchronized growth response involving almost all hepatocytes, leading to the restoration of liver mass in 7–10 days in rodents (1).

Liver regeneration is a complex process involving the activation of multiple pathways. The entry of quiescent hepatocytes into the cell cycle, corresponding to the G_0/G_1 transition, is largely regulated by cytokines. This phase, often referred to as priming, is characterized by the increase in cytokines such as tumor necrosis factor-α and IL-6 and the activation of immediate early genes that include c-*jun*, c-*fos*, and c-*myc* (2). Although this phase is essential for the hepatocyte response to growth factors, the priming stage is reversible. Without the involvement of growth factors, hepatocytes do not progress through cell cycle and they return to quiescence (3).

Hepatocyte growth factor (HGF) and transforming growth factor-α (TGF-α) are two of the most studied growth factors in liver regeneration. It has been demonstrated that the mRNA levels of both growth factors are elevated after 2/3PH (4, 5). HGF stimulates hepatocyte replication by paracrine or endocrine mechanisms (6, 7). Recent studies using conditional knock-out mice for the HGF receptor, c-Met, showed that c-Met signaling is essential for liver regeneration and may primarily affect hepatocyte survival and tissue remodeling (8, 9). By contrast, TGF-α is an autocrine factor whose effects are primarily proliferative (5). However, TGF-α knock-out mice have no defects in liver regeneration, an observation that might be explained by the redundancy of ligands of the EGF family (10). Indeed, heparin-binding EGF-like growth factor (HB-EGF) has been reported to participate in liver regeneration (11), but its role has not been well characterized. Nevertheless, transgenic mice expressing HB-EGF appear to have an accelerated liver growth response after partial hepatectomy (12).

The interaction between the early events of liver regeneration and growth factor availability is crucial for the regenerative process. However, the events linking these two phases have not been characterized. Bucher and Swaffield (13) show that the extent of hepatocyte replication in the regenerating liver of adult rats is proportional to the amount of tissue resected for resections involving 40–70% of the liver (13). Removal of 30% of the liver lies below this threshold and does not elicit a clear wave of DNA replication. Moreover, we have previously demonstrated that continuous infusion of TGF-α or HGF for 24 h into the portal vein of rats with 30% hepatectomies or one-third partial hepatectomy (1/3PH) produced a vigorous replicative response to the growth factors (14). Together, these data indicated that 1/3PH, in contrast to the standard 2/3PH, may induce hepatocyte priming that does not progress to DNA replication. We reasoned that the analysis of events occurring after 1/3PH may uncover linkages between priming

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¶ To whom correspondence should be addressed: Dept. of Pathology, University of Washington, K-078 HSB, Box 357705, Seattle, WA 98195. Tel.: 206-616-4550; Fax: 206-543-3967; E-mail: nfausto@u.washington.edu.

[1] The abbreviations used are: 2/3PH, two-thirds partial hepatectomy; 1/3PH, one-third partial hepatectomy; EGF, epidermal growth factor; HB, heparin-binding; IL, interleukin; HGF, hepatocyte growth factor; TGF-α, transforming growth factor; BrdUrd, bromodeoxyuridine; Rb, retinoblastoma; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; RPA, ribonuclease protection assays Cdk, cyclin-dependent kinase; SOCS, suppressor of cytokine signaling.

and cell cycle progression that are decisive for liver regeneration. Here we show that HB-EGF is a key factor for the progression of hepatocytes through the G_1/S phases.

MATERIALS AND METHODS

Animals and Surgical Procedures—8–10-week-old male wild-type C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were kept on a 12-h light/dark cycle with free access to food and water. HB-EGF-null male mice were from a hybrid C57BL/6 × 129/Sv background (15). In experiments using HB-EGF$^{-/-}$ mice, the wild-type controls were from the same hybrid background. Partial hepatectomies were performed under general anesthesia with inhaled isofluorane. One-third hepatectomy consisted of ligating the pedicle of the left lateral lobe before excising it. Two-thirds hepatectomy was performed as described by Higgins and Anderson (16). Sham surgery consisted of a midline laparotomy with gentle manipulation of the liver. Surgeries were performed between 8 a.m. and 4 p.m., and mice were killed at different time points after surgery as described in the legends of the figures. All of the animal work was done in accordance with the animal care committees of the University of Washington and the University of North Carolina at Chapel Hill.

Growth Factor Injections—For the growth factor experiment, male wild-type C57BL/6 mice received a bolus injection of 0.1 mg/kg HB-EGF (R&D Systems, Minneapolis, MN; dissolved in 0.9% NaCl), HGF (a gift from Genentech, San Francisco, CA; dissolved in 0.5 M NaCl and 20 mM Tris, pH 7.6), or TGF-α (R&D Systems; dissolved in 0.9% NaCl) through the tail vein 24 h after a one-third partial hepatectomy. Mice were killed 36 h after hepatectomy and 12 h after growth factor injection.

BrdUrd Immunohistochemistry—Mice received an intraperitoneal injection of BrdUrd (50 mg/kg) (Roche Applied Science) 2 h before killing. Livers were fixed in methacarn (60% methanol, 30% chloroform, and 10% acetic acid) overnight at room temperature and then embedded in paraffin. BrdUrd incorporation was measured using a mouse anti-BrdUrd antibody (Dako, Carpinteria, CA) and the Elite Vectastain ABC kit (Vector laboratories, Burlingame, CA) as described previously (17). For each liver sample, ~3,000 hepatocytes were counted.

Western Blot Analyses—Total liver protein was isolated from snap-frozen tissue by homogenization in immunoprecipitation lysis buffer described previously and briefly sonicated (17). Nuclear extracts were prepared as described previously (18). Proteins (30 μg for total liver protein and 10 μg for nuclear extracts) were separated on 12% SDS-PAGE (29:1 (v/v) acrylamide:Bis) gels with the exception of phospho-retinoblastoma (Rb) blots in which 10% (30:0.4 (v/v) acrylamide:Bis) SDS-PAGE gels were used and TGF-α blot in which Tris-Tricine 10–20% gels (Bio-Rad) were used. Proteins were transferred onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences) and blocked in 5% milk TBST (Tris-buffered saline with 0.1% Tween 20) prior to incubation with primary antibody. The following antibodies were used: cyclin A (sc-596, Santa Cruz Biotechnology, Santa Cruz, CA); p21 (556431, BD Biosciences); phospho-Rb (Ser-807/811, Cell Signaling, Beverly, MA); cyclin D1 (AHF 082, BIOSOURCE, Camarillo, CA); cyclin E (06–459, UBI, Lake Placid, NY); p107 (sc-318, Santa Cruz Biotechnology); TGF-α (clone MF9, LabVision, Fremont, CA); and β-actin (A5316, Sigma). Secondary antibodies were purchased from Amersham Biosciences. Enhanced chemiluminescence (Western Lightning Chemiluminescence reagent, PerkinElmer Life Sciences) was used for detection.

Immunoprecipitation and Kinase Assay—For cyclin-dependent kinase-4 (Cdk-4) immunoprecipitation, total protein lysates (500 μg) were incubated with 1 μg of anti-Cdk-4 antibody (sc-260, Santa Cruz Biotechnology) overnight at 4 °C. Protein A-Sepharose (Sigma) (30 μl of 1:1 (v/v) in lysis buffer) was added for 90 min, and the bound complexes were washed twice in lysis buffer. Immunoprecipitated proteins were subsequently resuspended in 2× sample buffer and resolved by 12% SDS-PAGE. Blotting was performed as described above with cyclin D1 antibody.

For cyclin E/Cdk-2 kinase assay (cyclin E-associated kinase assay), 500 μg of liver lysate were incubated with 1 μg of anti-cyclin E antibody 1 h at 4 °C. Protein A-Sepharose (30 μl of 1:1 (v/v) in lysis buffer) was added for 2 h, and the bound complexes were washed twice in lysis buffer and twice in 1× kinase buffer (3× kinase buffer: 25 mM Tris-HCl, pH 7.5, 70 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol). The beads were resuspended in 10 μl of 3× kinase buffer to which 1.2 μg of histone H1, 10 mM ATP, and 6 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol, PerkinElmer Life Sciences) were added. The samples were subsequently incubated at 30 °C for 25 min, and reactions were terminated by the addition of 2× sample buffer. Samples were subjected to 12% SDS-PAGE, and ^{32}P-labeled proteins were quantified by scintillation counting of excised bands.

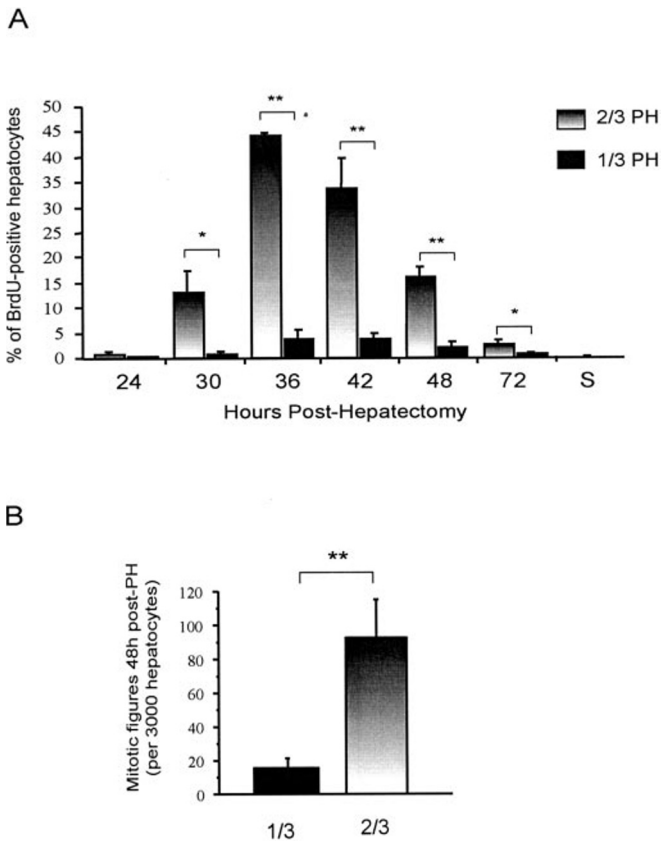

FIG. 1. **Hepatocyte proliferation after one-third or two-thirds partial hepatectomy.** *A*, DNA synthesis was analyzed by BrdUrd incorporation at different times after partial hepatectomy (n = 3 animals/group). Sham animals were killed 36 h post-surgery. *B*, hepatocytes undergoing mitosis at 48 h post-partial hepatectomy. The *graph* reflects the number of mitotic cells per 3,000 hepatocytes counted per animal (n = 4 animals/group). *, $p < 0.05$; **, $p < 0.01$.

Ribonuclease Protection Assays (RPA)—Total RNA was extracted from frozen liver tissue using TRIzol reagent (Invitrogen) as described by the manufacturer. Total RNA (10 μg) was then subjected to the RiboQuant Multi-Probe ribonuclease protection assay system (BD Biosciences) according to the manufacturer's specifications. The following sets of Multi-Probe templates (BD Biosciences) were used: c-*myc* (number 556193) and two custom probe sets, A and B. The custom probe set A included the following mouse DNA templates: c-*jun*; HGF; p53; c-*fos*, HB-EGF; TGF-β_1; fatty-acid synthase; L32; and glyceraldehyde-3-phosphate dehydrogenase. The custom probe set B included the following templates: tumor necrosis factor-α; IL-1; SOCS-3; KC (a cytokine-induced neutrophil chemoattractant); ICAM (intercellular adhesion molecule); SOCS-1; IL-6; interferon-γ; IL-12; L32; and glyceraldehyde-3-phosphate dehydrogenase. Samples were loaded on a 6% Tris borate-EDTA/urea gel (Invitrogen) at 150 V for 1.5 h. Gels were subsequently dried, and signals were quantified using a PhosphorImager. To control for equal loading between samples, the level of each mRNA species was normalized against the level of the housekeeping gene transcript, L32.

Real-time Reverse Transcriptase-PCR—One microgram of total RNA from livers was used for the Reverse Transcriptase reaction using Applied Biosystems TaqMan reverse transcription kit, and then one-tenth of the reaction was employed for the real-time PCR analysis. Assay kits for mouse TGF-α and amphiregulin from Applied Biosystems (Foster City, CA) were used for the quantification of the expression of these genes in an Applied Biosystems 7900HT SDS instrument. The housekeeping gene mouse β-glucuronidase (Applied Biosystems) was used to normalize the measurements using the Δ-Δ Ct method. The PCR conditions were the standard when using TaqMan hydrolysis probes.

Statistical Analysis—Statistical calculations were performed using the software Statview 4.5 (SAS Institute, Cary, NC). The Mann-Whitney test and the ANOVA Student's t test were used. p values lower than 0.05 were accepted as significant. *Error bars* indicate the mean ± S.E.

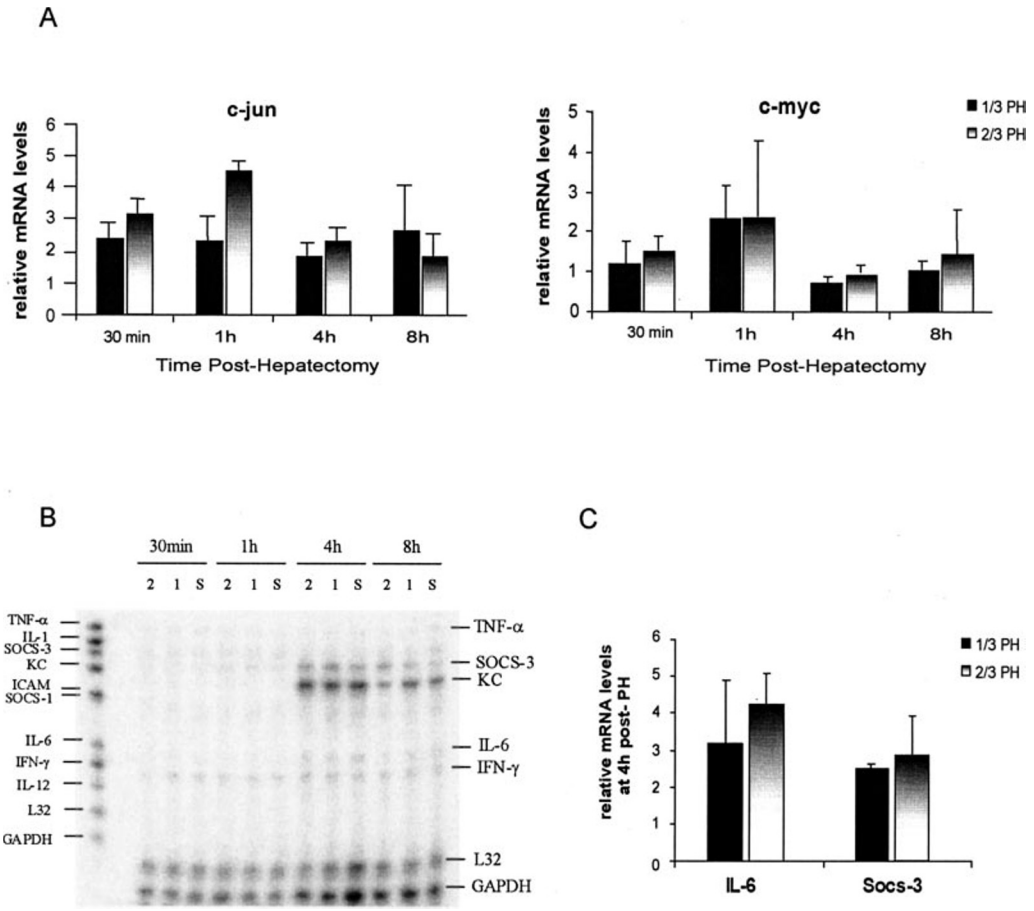

FIG. 2. **Expression levels of immediate early genes.** *A*, mRNA levels of c-*jun* and c-*myc* at different time points post-surgery as analyzed by RPA (c-*myc* probe and custom probe set A). *B*, RPA gel of representative samples from 30 min to 8 h after 2/3PH (*2*), 1/3PH (*1*), or sham (*S*) operations (custom probe set B). *IFN-γ*, interferon-γ; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *TNF-α*, tumor necrosis factor-α. *C*, quantification of mRNA levels of IL-6 and SOCS-3 analyzed by RPA (custom set B) at 4 h post-hepatectomy. The relative mRNA levels were obtained by normalizing each sample against sham-operated mice (three animals were analyzed for each group).

RESULTS

A Lack of Synchronized DNA Replication after 1/3PH in Mice—In rats, the removal of 30% of the liver results in little DNA replication (13, 19). To determine whether 1/3PH also fails to elicit a wave of hepatocyte replication in mice, we analyzed DNA replication of hepatocytes after 1/3PH at different times after surgery and compared with DNA replication after 2/3PH. We observed a striking difference in the proportion of hepatocytes that incorporated BrdUrd from 30 to 72 h after partial hepatectomy between these procedures (Fig. 1*A*). At 36 h, which corresponds to the peak of DNA replication after 2/3PH, the livers of mice with 2/3PH had almost 10-fold higher levels of DNA replication compared with animals with 1/3PH. The mitotic index determined 48 h after hepatectomy was ~5 times higher in 2/3PH mice compared with 1/3PH animals (Fig. 1*B*). These data demonstrated that, after a 1/3PH, there is lack of a robust synchronous wave of DNA replication and that this procedure can serve as a model for the analysis of incomplete regeneration.

Priming Phase Present after 1/3PH—To identify factors that may be responsible for the incomplete regenerative response after 1/3PH, we first examined the expression of a few representative genes that are known to be highly expressed during the priming phase that occurs during the first few hours after partial hepatectomy. It is well established that c-*jun*, c-*fos*, and c-*myc* are transiently expressed between 1 and 4 h after 2/3PH as part of the immediate early gene response (20–22). RPA analysis revealed that these three genes were similarly expressed in 1/3PH and 2/3PH from 30 min to 8 h post-surgery (Fig. 2*A* and data not shown). We also found that the expression of genes that participate in the acute phase response, such as IL-6 and SOCS-3, are similarly expressed after 1/3PH and 2/3PH (Fig. 2, *B* and *C*). These findings are in agreement with and greatly extend a previous report that suggested that 1/3PH can trigger a priming response (14), and it is consistent with data from DNA microarray gene expression analyses in progress in the laboratory.[2]

Deficient G_1/S Progression after 1/3PH—Cell cycle progression is regulated by the activity of complexes consisting of cyclins, Cdks, and cyclin-dependent kinase inhibitors (23). It is well established that Cdk-2 complexed with either cyclin E or A cooperates with cyclin D-Cdk-4/6 to phosphorylate the Rb and related p107 and p130 proteins. The phosphorylated proteins release bound E2F transcription factor, which stimulates the expression of genes required for S-phase (24). Therefore, we analyzed the expression and activity of key regulators of the G_1/S transition from 24 to 48 h after 1/3PH compared with 2/3PH mice. First, we analyzed the expression of the Rb and of p107, another member of the Rb family. In 2/3PH livers, the phosphorylation of Rb was detected at 30 h after hepatectomy and remained throughout the period of time studied (Fig. 3*A*). In marked contrast, we could not detect phosphorylated Rb after 1/3PH during the same period of time. The expression of p107 was detectable but greatly diminished in 1/3PH (Fig. 3*A*). We also analyzed the expression of p21, cyclin A, and cyclin D1

[2] J. Li, C. Mitchell, J. Campbell, N. Fausto, and R. Bumgarner, manuscript in preparation.

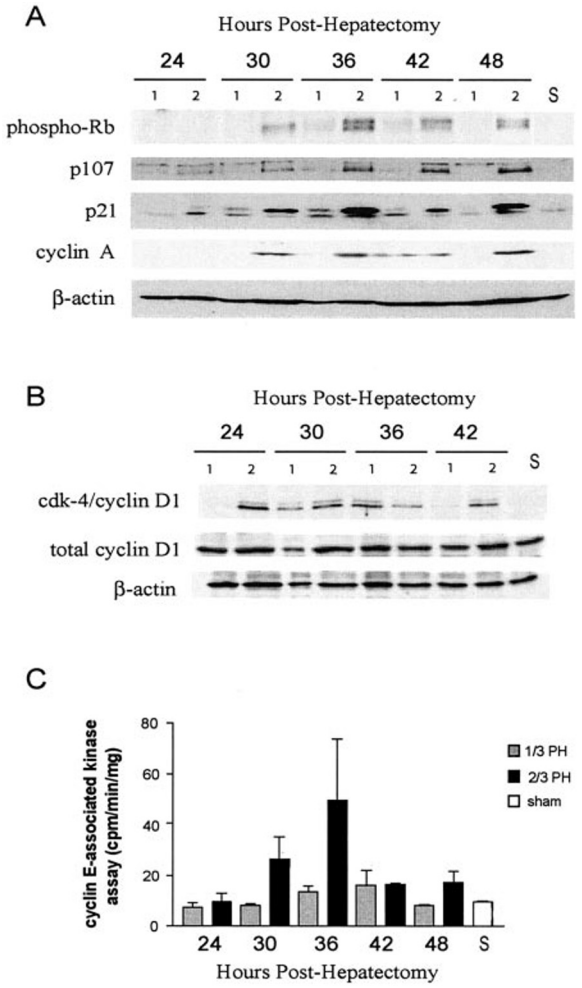

FIG. 3. **Analysis of cell cycle proteins.** *A*, Western blot analysis of phospho-RB, p107, cyclin A, and p21 at different time points after one-third (*1*) or two-thirds (*2*) partial hepatectomies. *B*, Western blot analysis of cyclin D1 in samples previously immunoprecipitated with Cdk-4 antibody or in total liver extracts at different time points after one-third (*1*) or two-thirds (*2*) partial hepatectomies. β-actin was used as loading control for *A* and *B*. Sham animals (*S*) were killed 36 h post-surgery. Each lane corresponds to a representative sample from each group (n = 3/group). *C*, densitometric quantification of cyclin E-associated kinase activity at different time points post-hepatectomy (n = 3 animals/group).

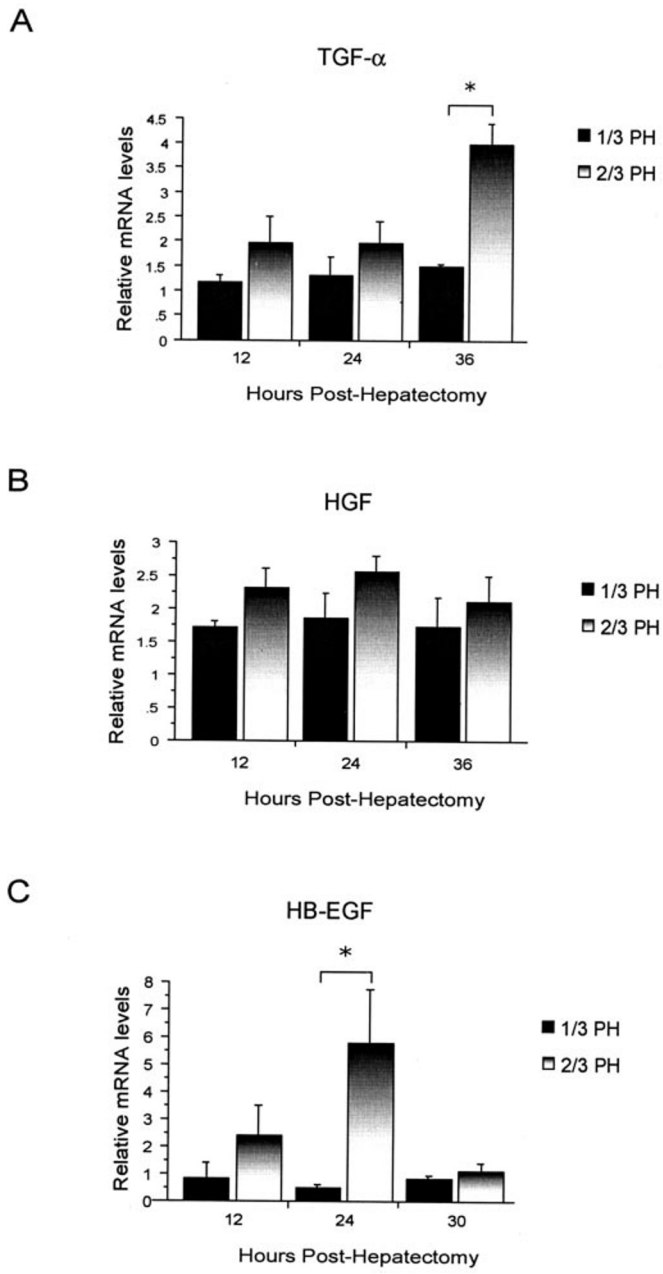

FIG. 4. **Growth factors expression levels after hepatectomy.** The mRNA levels of TGF-α (*A*), HGF (*B*), and HB-EGF (*C*) were analyzed at different time points post-surgery. The HB-EGF and HGF levels were assessed by RPA, and the levels of TGF-α were assessed by real-time PCR as described under "Material and Methods." The relative mRNA levels were obtained by normalizing each sample against sham-operated mice (n = 3 animals/group). *, $p < 0.05$.

and cyclin E complexes, proteins associated with the G_1/S transition. In 2/3PH, we detected cyclin A and p21 expression from 24/30 to 48 h (the last time point sampled in Fig. 3*A*). However, in 1/3PH mice, the expression of these proteins was delayed, diminished, and of shorter duration (Fig. 3*A*). The expression of the Cdk-4/cyclin D1 complex was more robust and longer lasting in 2/3PH than in 1/3PH, even though the total levels of cyclin D1 did not vary after either procedure (Fig. 3*B*). Finally, the kinase activity associated with cyclin E increased only in 2/3PH mice (Fig. 3*C*). Altogether, these results showed that there are well defined differences between 1/3PH and 2/3PH regarding the expression of various cell cycle proteins. Whereas 2/3PH hepatocytes are clearly committed to proliferation between 24 and 48 h after the operation, at identical time points, 1/3PH hepatocytes do not exhibit protein expression patterns characteristic of the G_1/S transition.

HB-EGF Expression Increases prior to the G_1-S Transition in 2/3PH but is Not Changed in 1/3PH—Growth factors drive cell cycle progression from early to late G_1, at which time replication becomes growth factor-independent (25). Therefore, we examined the expression of growth factors from 12 to 36 h after 1/3PH and 2/3PH. The 12–24-h time period corresponds to a phase that precedes the G_1/S transition (see Fig. 1). We first examined, by RPA and real-time PCR, the liver mRNA levels of the two main growth factors implicated in liver regeneration, TGF-α and HGF. TGF-α mRNA levels increased at 36 h only in 2/3PH mice (Fig. 4*A*), but there were no obvious differences in HGF mRNA levels between 1/3PH and 2/3PH at the time points examined (Fig. 4*B*). Given that the expression of HGF and TGF-α mRNA at 12–24 h after hepatectomy (a period of time that precedes the G_1/S transition) was similar in 1/3PH and 2/3PH, we determined whether HB-EGF might be differentially expressed in these two procedures. In 2/3PH, HB-EGF mRNA expression approximately doubled between 12 and 24 h after 2/3PH and

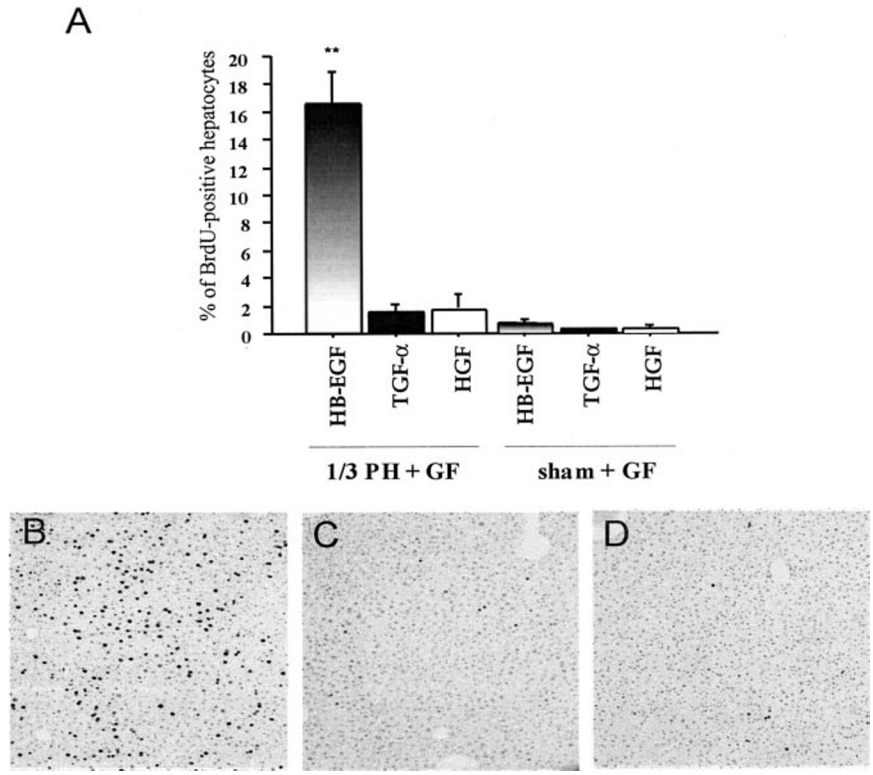

FIG. 5. **Effect of growth factor (*GF*) injection in DNA replication.** Mice were either submitted to 1/3PH or to sham operation. 24 h later, they received a bolus injection of 0.1 mg/kg HB-EGF, TGF-α, or HGF. The animals were killed 36 h post-surgery, and the livers were analyzed by BrdUrd immunohistochemistry. *A*, quantification of BrdUrd incorporation in hepatocytes (n = 3 animals/group). **, $p < 0.01$. The pictures show BrdUrd immunostaining of animals having received HB-EGF (*B*), TGF-α (*C*), or HGF (*D*) 24 h after 1/3PH (original magnification, ×100).

returned to basal levels at 30 h (Fig. 4*C*). In marked contrast, there was no increase in HB-EGF after 1/3PH. At 24 h after partial hepatectomy, HB-EGF levels were ~10-fold higher in 2/3PH compared with 1/3PH. Thus, the elevated HB-EGF levels are correlated with liver regeneration and precede the activation of cell cycle proteins. We conclude from these experiments that HB-EGF might be a key factor in determining cell cycle progression in regenerating liver and that its deficiency could be associated with incomplete regeneration.

HB-EGF at a Low Dosage Stimulates DNA Replication in 1/3PH Mice—We compared the effects of small dosages of HB-EGF, TGF-α, and HGF in stimulating DNA synthesis in animals subjected to 30% hepatectomies. We gave a single injection of 0.1 mg/kg of each of these growth factors to 1/3PH mice 24 h after the surgery, and the animals were killed 12 h after growth factor treatment. HB-EGF injection increased DNA replication by >15-fold in 1/3PH mice but had little or no effect in sham-operated animals (Fig. 5*A*). Moreover, DNA replication in 1/3PH mice injected with HB-EGF was >8-fold higher than that of 1/3PH animals injected with either HGF or TGF-α (Fig. 5, *A–D*). These data demonstrate that HB-EGF triggers DNA replication in 1/3PH mice at a dose in which HGF and TGF-α are without significant effects. Injections of any of the growth factors into sham-operated mice had little effect on hepatocyte DNA replication.

Liver Regeneration Is Delayed in HB-EGF Knock-out Mice—Because of the large differential sensitivity to HB-EGF and TGF-α effects demonstrated in 1/3PH mice (Fig. 5) and the earlier timing of the expression of HB-EGF during liver regeneration (Fig. 4*C*), we investigated whether HB-EGF knock-out mice would have defects in liver regeneration. These knock-out mice are viable and do not present abnormalities during liver development or in non-injured livers. We performed 2/3PH in HB-EGF knock-out mice and found that hepatocyte DNA replication in these animals was delayed and significantly lower at 30 and 36 h after partial hepatectomy compared with wild-type mice (Fig. 6*A*). Despite its delayed onset, the levels of DNA hepatocyte DNA replication in HB-EGF knock-out mice at 42 and 48 h after partial hepatectomy did not differ from the levels in wild-type mice. To investigate whether other ligands of the EGF family may compensate in part for the lack of HB-EGF in the knock-out animals, we analyzed the expression of TGF-α and amphiregulin mRNAs in these animals. TGF-α mRNA was significantly increased at 24 h after 2/3PH in the knock-out mice compared with wild-type animals (Fig. 6*B*), but there was no difference in the expression of amphiregulin between wild-type and knock-out animals after 2/3PH (data not shown). In agreement with these findings, we detected a higher TGF-α protein expression at 36 h post-hepatectomy in the HB-EGF knockouts compared with the wild-type mice (Fig. 6*C*). These data indicate that the lack of HB-EGF does affect the kinetics of liver regeneration and that increases in TGF-α expression may compensate for the absence of HB-EGF.

DISCUSSION

In the regeneration of the liver after partial hepatectomy, hepatocytes that are normally quiescent cells enter the cell cycle, replicate once or twice, and then return to quiescence. Information obtained primarily in studies using genetically modified mice support the view that regeneration is initiated by a priming phase largely regulated by cytokines in which hepatocytes undergo the G_0/G_1 cell cycle transition. During this phase, which lasts approximately 4–6 h in regenerating mouse liver, multiple genes are activated including several proto-oncogenes. It is also well established that hepatocyte DNA replication in the regenerating mouse liver does not start until ~30 h after partial hepatectomy. Cell-cycle progression through the G_1 phase is driven primarily by two growth factors, HGF and TGF-α. In addition to its proliferative effects, HGF is important for cell survival and motility, whereas TGF-α is an autocrine growth factor for hepatocytes and has mostly proliferative effects. Despite the vast amount of information on the role of cytokines and growth factors in liver regeneration, the mechanisms that connect hepatocyte priming with cell cycle progression are largely unknown.

To investigate these mechanisms, we compared some aspects

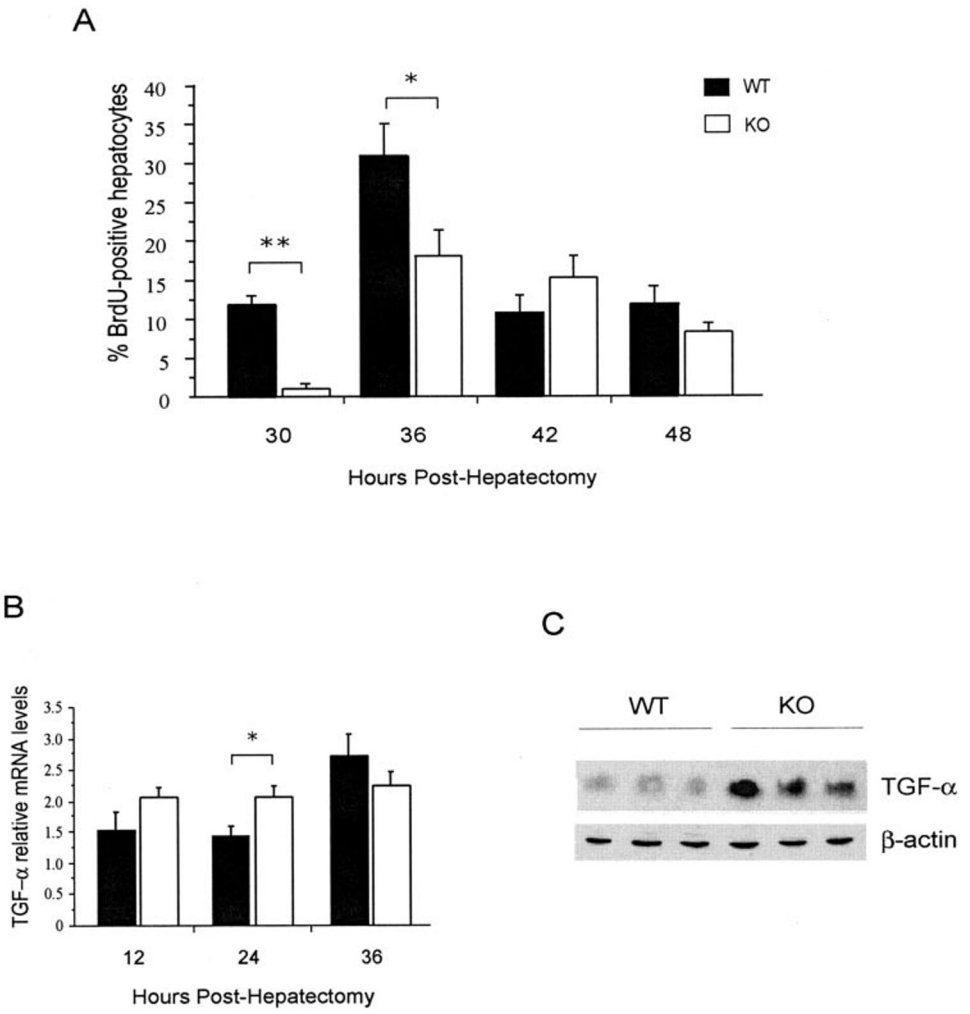

FIG. 6. **Liver regeneration after 2/3PH in HB-EGF knock-out mice.** *A*, DNA synthesis was analyzed by BrdUrd incorporation at different times after two-thirds partial hepatectomy in HB-EGF knock-out (*KO*) or wild-type (*WT*) mice (4–7 animals/group were analyzed). *B*, the mRNA levels of TGF-α were analyzed at different time points post-surgery in these animals, and the relative mRNA levels were obtained by normalizing each sample against non-operated control mice. *, $p < 0.05$; **, $p < 0.01$. *C*, Western blot analysis of TGF-α (6-kDa form) and β-actin at 36 h after 2/3PH in wild-type and HB-EGF knock-out animals.

of liver regeneration after 2/3PH, the standard procedure used to elicit full synchronized liver regeneration, with 1/3PH, which causes a delayed and asynchronous regenerative process (13, 19, 26). Using this approach, we established that the expression of HB-EGF, which occurs between 12 and 30 h after 2/3PH, is critical for cell cycle progression and the G_1/S transition in the regenerating liver. Compared with 2/3PH mice, 1/3PH animals had a very small replicative and mitotic response and had defects in the expression of Rb and p107 and deficiencies in the formation of cyclin/Cdk complexes. In these animals, the expression of HB-EGF between 12 and 24 h after partial hepatectomy was ~10-fold lower than that of 2/3PH mice.

Previous reports have shown that HB-EGF is expressed in the regenerating liver before the hepatic expression of HGF and TGF-α, but it is not known whether HB-EGF is a crucial player for the G_1/S transition (11, 12). Injection of HB-EGF into 1/3PH mice 24 h post-PH resulted in a >15-fold increase in DNA replication measured 12 h after the injection. In contrast, the injection of HGF or TGF-α at the same dosages failed to elicit significant hepatocyte DNA replication. We have previously demonstrated that the continuous infusion of HGF and TGF-α for 24 h increases DNA replication in 1/3PH rats (14). However, in this study, we gave a single injection of growth factors at a specific time during liver regeneration and used a lower dose of growth factors. The results indicate that, at 24 h after partial hepatectomy, a period that precedes the G_1/S transition in 2/3PH mice, hepatocytes of 1/3PH mice are highly sensitive to the proliferative effects of HB-EGF in comparison with TGF-α or HGF. The fact that an injection of TGF-α did not stimulate proliferation suggests that the mechanisms of induction of cell proliferation and the times at which hepatocytes are particularly sensitive to a specific factor are not necessarily the same for various ligands of the EGF family, even though HB-EGF and TGF-α share the same receptor (27). The differential activity of these ligands may reflect differences in affinity or prolonged binding for each ligand or be a consequence of changes in the constitution of the EGF receptor dimer. However, no difference in the expression of EGF receptor 1, ErbB2, and ErbB4 could be seen between 1/3PH and 2/3PH animals at 24 h after surgery (data not shown).

The importance of HB-EGF for liver regeneration is further highlighted by the delay in DNA replication observed in HB-EGF knock-out mice after 2/3PH. This result is particularly significant because TGF-α knock-out mice have no defects in liver regeneration, an observation that may be due to redundancy between the ligands of the EGF family. In HB-EGF knock-out mice, there is an early increase in the expression of TGF-α, presumably as a compensation for the lack of HB-EGF. Nevertheless, the early expression of TGF-α is not sufficient to overcome the defect caused by the lack of HB-EGF.

In summary, to uncover mechanisms that link the priming phase of liver regeneration with hepatocyte DNA replication, we used the model of 1/3PH, which differs from the standard 2/3PH by the lack of a clear wave of replication. These animals have a defect in the expression of cell cycle genes that may be attributed to the lack of expression of HB-EGF, a growth factor that appears to play a key role in the induction of the G_1/S transition in the regenerating liver. The differential expression of HB-EGF between 2/3PH and 1/3PH is consistent with data showing that HB-EGF is not detectable in the serum of patients who underwent minor hepatectomies (subsegmentectomy) but is present in the serum of patients with larger hepatectomies (lobectomy or segmentectomy) (28).

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REFERENCES

1. Grisham, J. W. (1962) *Cancer Res.* **22,** 842–849
2. Fausto, N. (2000) *J. Hepatol.* **32,** 19–31
3. Webber, E. M., Bruix, J., Pierce, R. H., and Fausto, N. (1998) *Hepatology* **28,** 1226–1234
4. Zarnegar, R., DeFrances, M. C., Kost, D. P., Lindroos, P., and Michalopoulos, G. K. (1991) *Biochem. Biophys. Res. Commun.* **177,** 559–565
5. Mead, J. E., and Fausto, N. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1558–1562
6. Noji, S., Tashiro, K., Koyama, E., Nohno, T., Ohyama, K., Taniguchi, S., and Nakamura, T. (1990) *Biochem. Biophys. Res. Commun.* **173,** 42–47
7. Kinoshita, T., Tashiro, K., and Nakamura, T. (1989) *Biochem. Biophys. Res. Commun.* **165,** 1229–1234
8. Huh, C. G., Factor, V. M., Sanchez, A., Uchida, K., Conner, E. A., and Thorgeirsson, S. S. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 4477–4482
9. Borowiak, M., Garratt, A. N., Wustefeld, T., Strehle, M., Trautwein, C., and Birchmeier, C. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 10608–10613
10. Russell, W. E., Kaufmann, W. K., Sitaric, S., Luetteke, N. C., and Lee, D. C. (1996) *Mol. Carcinog.* **15,** 183–189
11. Kiso, S., Kawata, S., Tamura, S., Higashiyama, S., Ito, N., Tsushima, H., Taniguchi, N., and Matsuzawa, Y. (1995) *Hepatology* **22,** 1584–1590
12. Kiso, S., Kawata, S., Tamura, S., Inui, Y., Yoshida, Y., Sawai, Y., Umeki, S., Ito, N., Yamada, A., Miyagawa, J., Higashiyama, S., Iwawaki, T., Saito, M., Taniguchi, N., Matsuzawa, Y., and Kohno, K. (2003) *Gastroenterology* **124,** 701–707
13. Bucher, N. L., and Swaffield, M. N. (1964) *Cancer Res.* **24,** 1611–1625
14. Webber, E. M., Godowski, P. J., and Fausto, N. (1994) *Hepatology* **19,** 489–497
15. Jackson, L. F., Qiu, T. H., Sunnarborg, S. W., Chang, A., Zhang, C., Patterson, C., and Lee, D. C. (2003) *EMBO J.* **22,** 2704–2716
16. Higgins, G., and Anderson, R. (1931) *Arch. Pathol.* **12,** 186–212
17. Vail, M. E., Chaisson, M. L., Thompson, J., and Fausto, N. (2002) *Oncogene* **21,** 1548–1555
18. Yamada, Y., Kirillova, I., Peschon, J. J., and Fausto, N. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 1441–1446
19. Yusuf, A., Laconi, E., Rao, P. M., Rajalakshmi, S., and Sarma, D. S. (1999) *Carcinogenesis* **20,** 1143–1145
20. Morello, D., Fitzgerald, M. J., Babinet, C., and Fausto, N. (1990) *Mol. Cell. Biol.* **10,** 3185–3193
21. Alcorn, J. A., Feitelberg, S. P., and Brenner, D. A. (1990) *Hepatology* **11,** 909–915
22. Thompson, N. L., Mead, J. E., Braun, L., Goyette, M., Shank, P. R., and Fausto, N. (1986) *Cancer Res.* **46,** 3111–3117
23. Sherr, C. J., and Roberts, J. M. (1999) *Genes Dev.* **13,** 1501–1512
24. Costa, R. H., Kalinichenko, V. V., Holterman, A. X., and Wang, X. (2003) *Hepatology* **38,** 1331–1347
25. Nelsen, C. J., Rickheim, D. G., Timchenko, N. A., Stanley, M. W., and Albrecht, J. H. (2001) *Cancer Res.* **61,** 8564–8568
26. Scotte, M., Laquerriere, A., Masson, S., Hiron, M., Teniere, P., Hemet, J., Lebreton, J. P., and Daveau, M. (1997) *Liver* **17,** 171–176
27. Jones, J. T., Akita, R. W., and Sliwkowski, M. X. (1999) *FEBS Lett.* **447,** 227–231
28. Yamada, A., Kawata, S., Tamura, S., Kiso, S., Higashiyama, S., Umeshita, K., Sakon, M., Taniguchi, N., Monden, M., and Matsuzawa, Y. (1998) *Biochem. Biophys. Res. Commun.* **246,** 783–787