Early Increase in Cyclin-D1 Expression and Accelerated Entry of Mouse Hepatocytes into S Phase after Administration of the Mitogen 1,4-Bis[2-(3,5-Dichloropyridyloxy)] Benzene

Giovanna M. Ledda-Columbano,* Monica Pibiri,* Roberto Loi,* Andrea Perra,* Hisashi Shinozuka,[†] and Amedeo Columbano*

From the Department of Toxicology,* Oncology and Molecular Pathology Unit, University of Cagliari, Cagliari, Italy; and the Department of Pathology,[†] University of Pittsburgh, Pittsburgh, Pennsylvania

We have previously demonstrated that hepatocyte proliferation induced by the mitogen 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) is independent of changes in cytokines, immediate early genes, and transcription factors that are considered to be necessary for regeneration of the liver after partial hepatectomy (PH) or necrosis. To further investigate the differences between mitogen-induced mouse hepatocyte proliferation and liver regeneration after PH, we have measured the expression of cyclin D1, cyclin D3, cyclin E, and cyclin A and of the cyclin-dependent kinases CDK2, CDK4, and CDK6. The involvement of the cyclin-dependent kinase inhibitors *p21* and *p27* and of the oncosuppressor gene *p53* was also examined at different times after stimulation of hepatocyte proliferation. Results showed that a single administration of TCPOBOP caused a very rapid increase in the levels of cyclin D1, a G1 protein, when compared with two thirds PH (8 hours *versus* 30 hours). The early increase in cyclin D1 protein levels was associated with a faster onset of increased expression of S-phase-associated cyclin A (24 hours *versus* 36 hours with PH mice). Accordingly, measurement of bromodeoxyuridine (BrdU) incorporation revealed that, although approximately 8% of hepatocytes were BrdU-positive as early as 24 hours after TCPOBOP, no significant changes in BrdU incorporation were observed at the same time point after two thirds PH. The expression of other proteins involved in cell cycle control, such as cyclin-dependent kinases (CDK4, CDK2, CDK6), was also analyzed. Results showed that expression of CDK2 was induced much more rapidly in TCPOBOP-treated mice (2 hours) than in mice subjected to PH (36 hours). A different pattern of expression in the two models of hepatocyte proliferation, although less dramatic, was also observed for CDK4

and CDK6. Expression of the CDK inhibitors *p21* and *p27* and the oncosuppressor gene *p53* variably increased after two thirds PH, whereas basically no change in protein levels was found in TCPOBOP-treated mice. The results demonstrate that profound differences in many cell cycle-regulatory proteins exist between direct hyperplasia and compensatory regeneration. Cyclin D1 induction is one of the earlier events in hepatocyte proliferation induced by the primary mitogen TCPOBOP and suggests that a direct effect of the mitogen on this cyclin may be responsible for the rapid onset of DNA synthesis observed in TCPOBOP-induced hyperplasia. (*Am J Pathol* 2000, 156:91-97)

Compensatory regeneration after two thirds partial hepatectomy (PH) or after a necrogenic dose of chemicals such as CCl₄ is thought to occur primarily through induction of immediate early genes, which are activated in the absence of *de novo* protein synthesis, through post-translational modifications of intracellular signals of latent pre-existing transcription factors (which initiate liver regeneration) such as activator protein 1 (AP-1), nuclear factor- κ B (NF- κ B), and signal transducers and activators of transcription 3 (STAT3).¹⁻³ These rapid events are followed by activation of delayed early genes whose induction leads to synthesis of various cell cycle-regulatory proteins, namely cyclins and cyclin-dependent kinases (CDKs), which act to form complexes.^{4,5} Activation through phosphorylation of cyclin-CDK complexes leads to progression into the cell cycle. Each cyclin, with its CDK partner, acts at a different step of the cell cycle; CDK4 is considered to play a critical role in G1 phase, and CDK2 is believed to be essential for the transition into S phase.⁶⁻⁷ The best characterized cyclin partner of CDK4 is cyclin D1, whereas CDK2 is associated with the

Supported by grants from the Associazione Italiana Ricerca sul Cancro and Ministero Università e Ricerca Scientifica (MURST 40% and 60%), Italy.

Accepted for publication September 27, 1999.

Address reprint requests to Dr. G.M.Ledda-Columbano, Dipartimento di Tossicologia, Sezione di Oncologia e Patologia Molecolare, Via Porcell 4, 09124 Cagliari, Italy. E-mail: columbano@vaxcal.unica.it.

E- and A-type cyclins during the G1-to-S transition and the S phase, respectively.⁷ The activity of cyclin/CDK complexes is negatively regulated by the cyclin-dependent kinase inhibitors, which are grouped into two structurally related families.⁸ The inhibitors of CDK4 (INH4) family (p15, p16, p18, and p19) inhibits CDK4 and CDK6, whereas the Cip/Kip family (p21, p27, and p57) inhibits numerous CDKs. Of these, p21 and p27 have been extensively studied. In tissue culture systems, p21 is up-regulated in proliferating cells,^{9–10} whereas, in other cell types, it is induced during senescence and terminal differentiation, and it is thought to play a key role in down-regulating CDK activity in these settings.^{11–13} Moreover, p21 inhibits stress activated protein kinase activity *in vitro*,¹⁴ and at low stoichiometric concentrations it may serve as an assembly factor for active cyclin/CDK complexes.^{15,16} Although recent papers using the transgenic mice have suggested that p21 did act as a cell-cycle inhibitor in liver regeneration after PH,^{17–19} the role of p21 *in vivo* is unclear, because the p21 knockout mice did not exhibit developmental abnormalities, increased tumorigenesis, or hyperproliferative disorders.²⁰

We have previously demonstrated that hepatocyte proliferation that is induced by administration of direct mitogens such as TCPOBOP to mice, unlike liver regeneration after two thirds PH, does not require activation of immediate early genes/transcription factors like NF- κ B nor cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6).^{21, 22} Similar results were observed in rats administered two peroxisome proliferators (PPs), Nafenopin and Br 931.^{23,24} These findings suggest that proliferative processes of a different nature, namely compensatory regeneration and direct hyperplasia, the latter occurring in the absence of hepatic cellular loss/death, may be triggered by different mechanisms.²⁵ Because both TCPOBOP and PPs are known to exert their action through activation of nuclear receptors,^{26–28} it is possible that the activated receptors may directly target a critical gene(s) responsible for triggering hepatocyte DNA synthesis.

To further clarify the differences between the two types of proliferative stimuli in the signal activation pathway determining the transition of hepatocyte from a quiescent to a replicative state, in this study we have investigated the timing of activation of G1 and S-phase cyclins and of their CDK partners during compensatory regeneration after two thirds PH and during direct hyperplasia after TCPOBOP administration. Furthermore, the patterns of activation of cyclin-CDK complex inhibitors (p27, p21) and the p21-transactivator p53 were analyzed. The results show a differential expression of several cell cycle-regulatory proteins in TCPOBOP-induced direct hyperplasia compared with liver regeneration after two thirds PH.

Materials and Methods

Animals

Female CD-1 mice (Charles River, Milano, Italy) 10 weeks old were used. Compensatory regeneration was induced

by performing two thirds PH, whereas direct hyperplasia was induced by a single gavage treatment with the primary mitogen 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) (a gift of Dr. B. A. Diwan, Frederick Cancer Center, Frederick, MD), at a dosage of 3 mg/kg body weight, dissolved in oil. Controls received no treatment.

Northern Blot Analysis

For the extraction of total RNA, at each time point, frozen livers from three animals were pooled. RNA extraction was performed by using Ultraspec RNA reagent (Biotecx Laboratories, Houston, TX) by the vendor's instructions. Thirty μ g of heat-denatured total RNA per lane were loaded on 1% agarose/formaldehyde gels, containing ethidium bromide for RNA detection at a UV lamp, and were blotted on Hybond-N⁺ membrane (Amersham, Buckinghamshire, UK). RNA concentration was determined spectrophotometrically at 260 nm. UV-irradiated filters were then hybridized with complementary DNA, labeled as described below, for Histone H3, a 200-bp, *Bam*HI fragment from pCMV vector; p21, a 200-bp *Not*I/*Xho*I fragment from Bluescript SK vector; p53, a 200-bp, *Bam*HI fragment from pCMV vector; and glyceraldehyde-3-phosphate dehydrogenase, a 780-bp, *Pst*I/*Xba*I fragment excised from the pHcGAP clone. For cyclin D1, pBluescript plasmid, containing a 100-bp, *Eco*RI fragment, has been used. DNA probes were labeled with [α -³²P]dCTP by random priming (random priming DNA labeling kit, Boehringer Mannheim, Mannheim, Germany). Membranes were exposed to radiographic film (Eastman Kodak, Rochester, NY).

Western Blot Analysis

Total cell extracts were prepared from frozen livers powdered in liquid nitrogen-cold mortar. Equal amounts of powder (about 50 mg), from three different animals were pooled per each sample point, and resuspended in 1 ml Triton Lysis Buffer (1% Triton X-100, 50 mmol/L HCl, pH 7.4, 140 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol, 1 mmol/L PMSF, 5 mmol/L iodoacetic acid, 10 μ g/ μ l each of aprotinin, pepstatin, leupeptin). Several protease inhibitors were added to the isolation buffer to minimize protein degradation during the isolation protocol. After vortexing, extracts were incubated 30 minutes on ice and centrifuged at 12,000 rpm at 4°C, and the supernatants were recovered. All inhibitors used were purchased from Boehringer Mannheim, Mannheim, Germany) with the exceptions that phenylmethyl sulfonyl fluoride, NaF, and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, MO, and iodoacetic acid was from ICN Biomedicals, Irvine, CA. The protein concentrations of the resulting total extracts were determined by the method of Bradford, using bovine serum albumin as the standard (DC Protein Assay; Bio-Rad Laboratories, Hercules, CA). For immunoblot analysis, equal amounts (from 50 to 200 μ g/lane) of proteins were electrophoresed on sodium dodecyl sulfate-12% or 8% polyacrylamide gels. Acrylamide and bis-acrylamide

were purchased from ICN Biomedicals. After gel electro-transfer onto nitrocellulose membranes (Micron Separations) at 300 mA overnight or 800 mA for 2–4 hours, to ensure equivalent protein loading and transfer in all lanes, the membranes and the gels were stained with 0.5% (w/v) Ponceau S red (ICN Biomedicals) in 1% acetic acid for 5 minutes and with Coomassie blue (ICN Biomedicals) in 10% acetic acid for 30 minutes, respectively. Before staining, gels were fixed in 25% (v/v) isopropanol and 10% (v/v) acetic acid (Sigma). After blocking in Tris-buffered saline containing 0.5% Tween 20 (Sigma) and 5% nonfat dry milk for 1 hour at room temperature or overnight at 4°C, membranes were washed in Tris-buffered saline-T and then incubated overnight at 4°C or for 2 hours at room temperature, with 1 to 3 μ g/ml of appropriate primary antibodies diluted in blocking buffer. Whenever possible, the same membrane was used for detection of the expression of different proteins (ie, cyclin D1, cyclin A, p53, p27, and CDK6). Depending on the origin of primary antibody, filters were incubated for 1 hour at room temperature with either anti-mouse or anti-rabbit horse radish peroxidase-conjugated immunoglobulin G (IgG; Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were identified with a chemiluminescence detection system, as described by the manufacturer (Supersignal substrate; Pierce, Rockford, IL). When necessary, antibodies were removed from filters by 30 minutes of incubation at 60°C in stripping buffer (100 mmol/L 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mmol/L Tris-HCl, pH 7.6), and the membranes were reblotted as above.

Antibodies

For immunoblotting experiments, we used mouse monoclonal antibodies directed against: p27 (Anti-Kip1/p27; Transduction Laboratories, Lexington, KY), Cyclin D1 (72–13 G), CDK2 (D-12), and Rb (IF 8) (Santa Cruz Biotechnology); p53 (Ab-1; Calbiochem, La Jolla, CA); p21 (Powerclonal, Upstate Biotech, NY). The following rabbit polyclonal antibodies were from Santa Cruz: CDK4 (H-303), Cyclin A (C-19), CDK6 (C-21), Cyclin D3 (C-16), and Cyclin E (M-20).

Immunohistochemistry

Mice were subjected to two thirds PH or were given a single dose of TCPOBOP. Mice were given BrdU dissolved in drinking water (3 mg/ml, Sigma) and sacrificed 96 hours later, or they received a single intraperitoneal dose of BrdU (100 mg/kg, dissolved in distilled water) and were sacrificed 2 hours after BrdU administration at 10, 18, 22, 28, 34, 40, and 46 hours later. Mouse monoclonal anti-BrdU antibody was obtained from Becton Dickinson, San Jose, CA, and the peroxidase method was used to stain BrdU-positive hepatocytes. Peroxidase goat anti-mouse Ig was obtained from Dako (Dako EnVision⁺ Peroxidase Mouse; Dako Corp., Carpinteria, CA). Sections (4 μ m thick) were deparaffinized and treated with HCl 2 N for 20 minutes and with 0.1% trypsin type II

Figure 1. Representative microphotography that illustrates the presence of BrdU-positive hepatocytes after 4 days of continuous labeling with BrdU (1 mg/ml, dissolved in water) in controls (A) or in TCPOBOP-treated mice (B). Original magnification, $\times 200$; sections were counterstained with hematoxylin.

(crude from porcine pancreas; Sigma, Milano, Italy) for 20 minutes, and then the sections were treated sequentially with normal goat serum (1:10; Dako), mouse anti-BrdU 1:200 for 1 h and 30 minutes, and Dako EnVision⁺ peroxidase mouse ready-to-use. The sites of peroxidase binding were detected by 3,3'-diaminobenzidine.

Results

Studies to estimate the extent of the mitogenic effect of TCPOBOP in mouse liver revealed that approximately 80% of hepatocytes entered S phase within 4 days after treatment, as monitored by BrdU incorporation (Figure 1).

The kinetics of hepatocyte proliferation after a single treatment with TCPOBOP were determined next and compared with that after two thirds PH. As shown in Figure 2, although hepatocytes showed little DNA synthesis at 24 to 30 hours after PH (1% and 2%, respectively), a significant number of hepatocytes were BrdU-positive at the same time period after TCPOBOP administration, with 15% of hepatocytes undergoing division at 30 hours. DNA synthesis peaked in both groups at 36 hours (33% of BrdU-positive hepatocytes in PH mice *versus* 28% of TCPOBOP-treated animals).

Figure 2. Labeling index of mouse hepatocytes after TCPOBOP and PH treatments. Mice treated with a single dose of TCPOBOP (3 mg/kg intragastrically) or subjected to two thirds PH; or controls were given a single intraperitoneal injection of BrdU (100 mg/kg) 2 hours before death at 10, 18, 22, 28, 34, 40, and 48 hours after treatment. At least 5000 hepatocyte nuclei per liver were scored. The labeling index was expressed as number of BrdU-positive hepatocyte nuclei/100 nuclei. Results are expressed as means $\pm$ SE of 5 to 6 mice per group.

Consistent with these findings, that TCPOBOP induced an earlier onset of hepatocyte DNA synthesis, the levels of RNA and protein in two markers of S phase, histone H3 and cyclin A, were increased 24 hours after treatment with TCPOBOP, whereas such increases were seen only after 36–48 hours after two thirds PH (Figure 3, A and B). A similar pattern of a rapid induction of G1 cyclin, namely that of cyclin D1, was observed after TCPOBOP treatment. Indeed, while in agreement with previously reported findings,¹⁷ cyclin D1 protein levels were increased 30 hours after PH, just before DNA synthesis; TCPOBOP caused a rapid elevation of cyclin D1 messenger RNA (mRNA) as early as 2 hours and of the protein 8 hours after the treatment. Cyclin D1 protein levels remained elevated for 36 hours (Figure 4, A and B).

Figure 3. Expression of histone H3 and cyclin A in TCPOBOP-induced mouse hepatocyte proliferation and liver regeneration. **A:** Northern blot analysis of changes in H3 mRNA levels in mouse liver after PH or treatment with TCPOBOP (3 mg/kg). Each lane represents a pool of three livers. Northern blot analysis was done as outlined in Materials and Methods. Filters were then rehybridized with a GAPDH-specific probe; CO, control. **B:** Western blot analysis of cyclin A. Protein extracts (50 μ g/lane) were prepared from the livers, and Western analysis was performed as described in Materials and Methods. Appropriate loading was confirmed by staining the blots with Ponceau S red. Each lane represents a pool of three livers; CO, control.

Figure 4. Expression of cyclin D1 in TCPOBOP-induced mouse hepatocyte proliferation and liver regeneration. **A:** Northern blot analysis of changes in cyclin D1 mRNA levels in mouse liver after PH or treatment with TCPOBOP (3 mg/kg). Each lane represents a pool of three livers. Northern blot analysis was done as outlined in Materials and Methods. Filters were then rehybridized with a GAPDH-specific probe; CO, control. **B:** Western blot analysis of cyclin D1. Protein extracts (50 μ g/lane) were prepared from the livers, and Western blot analysis was performed as described in Materials and Methods. Appropriate loading was confirmed by staining the gel with Coomassie blue, and efficiency of transfer was monitored by staining the membranes with Ponceau S red. Each lane represents a pool of three livers. CO, control.

After PH, activation of CDK2, CDK4, and CDK6 is thought to play a critical role in progression through the G1 restriction point and in the transition into S phase.^{5–7} In agreement with the previously reported findings,¹⁷ CDK2 was up-regulated at 36 to 48 hours after PH (Figure 5). In contrast, the induction of CDK2 expression was observed as early as 2 hours after treatment with TCPOBOP, and it remained elevated until 36 hours. CDK2 expression was inversely correlated with cyclin E that was expressed 12 hours after PH, with a peak corresponding to S phase (36–48 hours), while its expression was only minimally induced in TCPOBOP-treated mice (Figure 5). Increase of CDK4 expression was associated with entry into S phase in both proliferative models (20 hours after TCPOBOP and 36 hours after PH). We also determined CDK6 expression in PH- and TCPOBOP-treated mice. Although a certain degree of variability in the timing of expression of CDK6 was observed in independent experiments in mice subjected to PH, a pattern similar to that of CDK4 was observed; in contrast, a much

Figure 5. Western blot analysis of cell cycle proteins in TCPOBOP- and PH-induced mouse hepatocyte proliferation. Protein extracts (50–100 μ g/lane) were prepared from the livers, and Western analysis was performed as described in Materials and Methods. Appropriate loading was confirmed by staining the gel with Coomassie blue, and efficiency of transfer was monitored by staining the membranes with Ponceau S red. Each lane represents a pool of three livers. CO, control.

Figure 6. Expression of p21, p27 and p53 in TCPOBOP- and two thirds PH-induced mouse hepatocyte proliferation. **A:** Western blot analysis. For p21, 200 μ g of nuclear extracts were used. For p27, two different protein concentrations from total extracts, 100 μ g/lane (**top**) and 50 μ g/lane (**bottom**), and a different dilution of the secondary antibody (1:1000 and 1:2000, respectively) were used. For p53, 50 μ g of total extracts were used. Appropriate loading was confirmed by staining the gel with Coomassie blue, and efficiency of transfer was monitored by staining the membranes with Ponceau S red. **B:** Northern blot analysis was done as outlined in Materials and Methods. Filters were then rehybridized with a GAPDH-specific probe. Each lane represents a pool of three livers. CO, control.

more rapid increase of CDK6, compared with CDK4, was seen in TCPOBOP-treated mice (Figure 5).

To examine and compare the possible role of the inhibitors of CDKs, p21 and p27, during liver regeneration and mitogen-induced hyperplasia, Western blot experiments were performed on homogenates of liver tissue from mice subjected to PH or injected with TCPOBOP. As shown in Figure 6A, no p21 was detected in quiescent liver tissue, consistent with previous findings.^{17,18} The p21 protein was markedly induced after PH, beginning during G1 phase (24 hours after PH) and peaking during the replicative phase; the increase in p21 protein levels was preceded by an enhancement of hepatic levels of p21 mRNA during the first 24 hours after PH (Figure 6B), with a decline to nearly undetectable levels 36 hours after surgery. On the other hand, Northern and Western analyses did not show any evidence of p21 expression in mouse liver during hepatocyte proliferation induced by TCPOBOP (Figure 6, A and B).

In contrast to p21, p27 protein was expressed in quiescent liver, and only minimal changes could be observed during liver regeneration and TCPOBOP-induced hyperplasia (Figure 6A, top). The possibility that the signal observed by using 100 μ g of protein could be strong enough to mask any difference possibly existing between the experimental groups was examined. By modifying conditions so that no protein could be detected in control

liver (Figure 6A, bottom), an up-regulation of p27 was evident in PH-treated mice with a peak between 12 and 30 hours (G1 phase) and a return to control values in correspondence with S phase (36–48 hours). In contrast, no up-regulation of p27 was observed during cell proliferation induced by TCPOBOP. A striking difference between TCPOBOP-induced hepatocyte proliferation and liver regeneration after PH was also noticed when the expression of the oncosuppressor gene p53 was examined. Indeed, although some variability was observed in the timing of p53 expression in PH-subjected mice, the levels of this protein were induced only in regenerating liver, while little if any change in p53 protein content occurred in mitogen-treated mouse liver (See Figure 6A).

Discussion

From the present study it is clear that administration of the mitogen TCPOBOP to mice causes 1) an extremely rapid induction of the G1 protein cyclin D1; 2) a shortening of the G1 phase of cell cycle, accounting for approximately 12 to 16 hours.

It has long been known that mouse hepatocytes, after two thirds PH, enter DNA synthesis much later than rat hepatocytes.^{29,30} Indeed, although the time of S phase can be modulated by factors such as sex, age and strain,^{3–29} mouse hepatocytes usually enter S phase between 30 and 42 hours after PH, approximately 12 to 18 hours later than rat hepatocytes.^{31–33} The finding that changes in immediate early genes/transcription factors such as *c-fos*, *c-jun* and *c-myc* occur almost concomitantly in the two species^{21,34–38} suggests that more stringent check points may exist in mouse liver, that prolong G1 phase of the cell cycle. Our current study showing that the direct mitogen TCPOBOP is able to induce entry of mouse hepatocytes into the cell cycle in only 20 to 24 hours provides clear evidence that the duration of the G1 phase of the cell cycle is dependent on the nature of the proliferative stimulus.

Our study also shows that signaling pathways involved in TCPOBOP-induced mitogenesis are different from those evoked during liver regeneration and may escape some of the checkpoints acting in the G1 phase of cell cycle. The notion that liver regeneration and direct hyperplasia induced by certain direct mitogens may occur through different signal-transducing pathways is supported by our previous findings^{21–24} and has subsequently been confirmed by other groups,^{39,40} who show that the latter type of proliferation, unlike the former, does not require activation of transcription factors, ie, AP-1, NF- κ B, STAT3, and CCAT/enhancer binding protein- α and - β , or induction of immediate early genes such as *c-fos*, *c-jun*, *LRF-1*, *egr-1*, and *c-myc*, and it is independent of the presence of TNF- α and IL-6. In this study we have demonstrated that the pattern of proteins related to the progression of cell cycle (cyclins, CDKs, and CDK inhibitors) is also different in the two proliferative models. One of the most significant differences concerns cyclin D1. Indeed, although the content of cyclin D1 protein increased only at 30 hours after PH, just before DNA

synthesis, an extremely rapid induction of both cyclin D1 mRNA and protein levels was observed after TCPOBOP treatment. Recently, it was shown that cyclin D1 promoter contains an estrogen-responsive region⁴¹ that could be involved also in the regulation of this gene activity by other nuclear receptor ligands (retinoids, thyroid hormones, PPs). The finding that TCPOBOP is able to bind and activate a nuclear orphan receptor (CAR) of the superfamily of steroid/thyroid receptors²⁶ would support the notion that cyclin D1 may be the common target of different members of nuclear receptors. Moreover, the observation that a rapid increase of cyclin D1 also occurs in the rat liver after administration of PPs (manuscript in preparation) and in other cell types after a mitogenic dose of estrogens^{42–45} suggests that the cyclin D1 gene may be a target of mitogens that are ligands for nuclear receptors. This may be a possible explanation for the absence of changes in early parameters such as immediate early genes and/or transcription factors in cell proliferation induced by direct mitogens.

Another finding of considerable interest was a rapid induction and elevation of CDK2 in TCPOBOP-treated liver. It is commonly known that D-type cyclins form complexes with and activate CDK4 and CDK6, and cyclins E and A form complexes with CDK2.⁷ Thus, it is somewhat surprising that the rapid induction of cyclin D1 was not accompanied by elevated levels of CDK4 and -6, and was accompanied instead by a striking elevation of CDK2. Furthermore, no cyclin E was present in TCPOBOP-treated mice at a time when CDK2 was already very highly expressed. On the other hand, no CDK2 was detected in the liver after PH at a time when cyclin E was highly induced. Expression of cyclins and CDKs varies in different cell types,^{46–48} and it is greatly influenced by the environment in which cells are stimulated to grow. It is clear that two different proliferative stimuli in the mouse liver, TCPOBOP and PH, induce different patterns of expression of CDKs.

Other striking differences between TCPOBOP and PH involved CDK inhibitory proteins p21 and p27 and the tumor suppressor protein p53. On one hand, in agreement with the literature, increased levels of p21 mRNA were found 8 hours after PH and were still elevated 24 hours after surgery.⁴⁹ The quantities of p21 protein were increased concomitantly to DNA synthesis (36–48 hours) and paralleled the increase in the expression of cyclin A and histone H3 and in the incorporation of BrdU, all markers of S phase. On the other hand, no evidence of increased p21 mRNA or protein levels could be found in TCPOBOP-treated mice. These findings are of some interest, because p21 has been shown to play a role in G1-S progression of hepatocytes after PH in the transgenic mice, either overexpressing or lacking p21.^{17,18} It is not clear why, in the model of TCPOBOP-induced accelerated hepatocyte DNA synthesis, p21 appears to have no apparent role in the process. p27 also showed a different pattern of expression in two proliferative models, being up-regulated in G1 phase of the cell cycle in liver of PH-subjected mice and unaltered in TCPOBOP-treated mice at all times examined. Similarly, expression of p53 was found to be high immediately after surgery and just

before S phase in PH animals, with little, if any, increase in TCPOBOP-treated mice.

Altogether, the results suggest that the accelerated onset of DNA synthesis observed after treatment with TCPOBOP is the result of a combination of two different factors: 1) a very rapid induction of G1 protein such as cyclin D1, possibly through activation of the cyclin D1 gene by the activated nuclear receptor, and 2) overriding of cell-cycle checkpoints, which may allow for premature DNA synthesis and mitotic entry.

In conclusion, even though in the current study no efforts have been made to characterize the pattern of formation and activation of cyclin/CDK/inhibitor complexes in the two models, this paper demonstrates that hepatocyte proliferation induced by the primary mitogen TCPOBOP occurs through a signal transduction pathway that is different from that observed during compensatory regeneration after PH.

These findings may hopefully stimulate future studies aimed at determining whether present knowledge on the molecular mechanisms associated with the progression of the cell cycle is uniformly applicable to all conditions of cell proliferation or dependent on the nature of the proliferative stimulus. Moreover, in view of the carcinogenic potency of TCPOBOP and other mitogens that are ligands of nuclear receptors (ie, PPs),^{50–53} studies aimed at elucidating the full pattern of possible hepatocyte response to mitogenic stimuli might improve our knowledge of the mechanism of action of nongenotoxic carcinogens.

References

1. Fausto N, Laird AD, Webber EM: Role of growth factors and cytokines in hepatic regeneration. *FASEB J* 1995, 9:1527–1536
2. Taub R: Transcriptional control of liver regeneration. *FASEB J* 1996, 10:413–427
3. Michalopoulos GK, DeFrances MC: Liver regeneration. *Science* 1997, 276:60–66
4. Morgan DO: Cyclin dependent kinase: engines, clocks, and microprocessors. *Annu Rev Cell Dev Biol* 1997, 13:261–291
5. Grana X, Reddy PE: Cell cycle control in mammalian cells: role of cyclins, cyclin-dependent kinases (CDKs), growth suppressor genes and cyclin dependent kinase inhibitors (CKIs). *Oncogene* 1995, 11: 211–219
6. Pines J: Cyclins and cyclin dependent kinases: a biochemical view. *Biochem J* 1995, 308:697–711
7. Sherr CJ: Cancer cell cycles. *Science* 1996, 274:1672–1677
8. Sherr CJ, Roberts JM: Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev* 1995, 9:1149–1163
9. Nakanishi M, Adami GR, Robetoye RS, Noda A, Venable SF, Dimitrov D, Pereira-Smith OM, Smith JR: Exit from G0 and entry into the cell cycle of cells expressing p21Sdi1 antisense RNA. *Proc Natl Acad Sci USA* 1995, 92:4352–4356
10. MacLeod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, Vogelstein B, Jacks T: p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes Dev* 1995, 9:935–944
11. Noda A, Ning Y, Venable SF, Pereira-Smith OM, Smith JR: Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp Cell Res* 1994, 211:90–98
12. Guo K, Wang J, Andres V, Smith RC, Walsh K: MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. *Mol Cell Biol* 1995, 15:3823–3829
13. Marx J: Cell biology: cell cycle inhibitors may help brake growth as cells develop. *Science* 1995, 267:963–964
14. Shim J, Lee H, Park J, Kim H, Choi EJ: A non-enzymatic p21 protein

- inhibitor of stress-activated protein kinases. *Nature* 1996, 381:804–806
15. Zhang H, Hannon GJ, Beach D: p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev* 1994, 8:1750–1758
16. LaBaer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow E: New functional activities for the p21 family of CDK inhibitors. *Genes Dev* 1997, 11:847–862
17. Albrecht JH, Poon RY, Ahonen CL, Rieland BM, Deng C, Cray GS: Involvement of p21 and p27 in the regulation of CDK activity and cell cycle progression in the regenerating liver. *Oncogene* 1998, 16: 2141–2150
18. Wu H, Wade M, Krall L, Grisham J, Xiong X, Van Dyke T: Targeted in vivo expression of the cyclin-dependent kinase inhibitor p21 halts hepatocyte cell-cycle progression, postnatal liver development, and regeneration. *Genes Dev* 1996, 10:245–260
19. Timchenko NA, Wilde M, Nakanishi M, Smith JR, Darlington GJ: CCAAT/enhancer-binding protein α (C/EBP α) inhibits cell proliferation through the p21 (WAF-1/Cip-1/SDI-1) protein. *Genes Dev* 1996, 10:804–815
20. Deng C, Zhang P, Harper JW, Elledge SJ, Leder P: Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* 1995, 82:675–684
21. Columbano A, Ledda-Columbano GM, Pibiri M, Piga R, Shinozuka H, DeLuca V, Cerignoli F, Tripodi M: Increased expression of c-fos, c-jun and LRF-1 is not required for in vivo priming of hepatocytes by the mitogen TCPOBOP. *Oncogene* 1997, 14:857–863
22. Ledda-Columbano GM, Curto M, Piga R, Zedda AI, Menegazzi M, Sartori C, Shinozuka H, Bluethmann H, Poli V, Ciliberto G, Columbano A: In vivo hepatocyte proliferation is inducible through a TNF, and IL-6-independent pathway. *Oncogene* 1998, 17:1039–1044
23. Menegazzi M, Carcereri A, Suzuki H, Shinozuka H, Pibiri M, Piga R, Columbano A, Ledda-Columbano GM: Liver cell proliferation induced by nafenopin and cyproterone acetate is not associated with increases in activation of transcription factors NF- κ B and AP-1 or expression of tumor necrosis factor- α . *Hepatology* 1997, 25:585–592
24. Ohmura T, Ledda-Columbano GM, Piga R, Columbano A, Glemba J, Katyal SL, Locker J, Shinozuka H: Hepatocyte proliferation induced by a single dose of a peroxisome proliferator. *Am J Pathol* 1996, 148:815–824
25. Columbano A, Shinozuka H: Liver regeneration versus direct hyperplasia. *FASEB J* 1996, 10:1118–1128
26. Honkakoski P, Zelko I, Sueyoshi T, Negishi M: The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* 1998, 18:5652–5658
27. Tugwood JD, Issemann I, Anderson RG, Bundell KR, McPheat WL, Green S: The mouse peroxisome proliferator activated receptor recognizes a response element in the 5' flanking sequence of the rat acyl CoA oxidase gene. *EMBO J* 1992, 11:433–439
28. Peters JM, Cattley RC, Gonzalez FJ: Role of PPAR α in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator Wy-14,643. *Carcinogenesis* 1997, 18:2029–2033
29. Bucher NLR: Regeneration of mammalian liver. *Int Rev Cytol* 1963, 15:245–300
30. Hoffman AL, Rosen HR, Ljubimova JU, Sher L, Podesta LG, Demetriou AA, Makowka L: Hepatic regeneration: current concepts and clinical implications. *Semin Liver Dis* 1994, 14:190–210
31. Brodsky WY, Uryvaeva IV: Cell polyploidy: its relation to tissue growth and function. *Int Rev Cytol* 1973, 50:275–332
32. Paulsen JE: Variations in regenerative growth of mouse liver following partial hepatectomy. *In Vivo* 1990, 4:235–238
33. Lorup C: An autoradiographic study of the 3 H-uridine and 3 H-thymidine incorporation in the regenerating mouse liver. *Cell Tissue Kinet* 1977, 10:477–485
34. Morello D, Fitzgerald MJ, Babinet C, Fausto N: C-myc, c-fos, and c-jun regulation in the regenerating livers of normal, and H-2K/c c-myc transgenic mice. *Mol Cell Biol* 1990, 10:3185–3193
35. Cressman DE, Greenbaum LE, DeAngelis RA, Ciliberto G, Furth EE, Poli V, Taub R: Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science* 1996, 274:1379–1383
36. Goyette M, Petropoulos GJ, Shank PR, Fausto N: Expression of cellular oncogene during liver regeneration. *Science* 1983, 219:510–512
37. Makino R, Hayashi K, Sugimura TC: C-myc transcript is induced in rat liver at a very early stage of regeneration or by cycloheximide treatment. *Nature* 1984, 319:694–698
38. Coni P, Simbula G, Carcereri DePrati A, Menegazzi M, Suzuki H, Sarma DSR, Ledda-Columbano GM, Columbano A: Differences in the steady-state levels of c-fos, c-jun and c-myc-messenger RNA during mitogen-induced liver growth and compensatory regeneration. *Hepatology* 1993, 17:1109–1116
39. Goldsworthy TL, Goldsworthy SM, Sprinkle CS, Butterworth BE: Expression of myc, fos and H-ras associated with chemically induced cell proliferation in the rat liver. *Cell Prolif* 1994, 27:269–278
40. Skrtic S, Ekberg S, Wallenius V, Enerbak S, Hedin L, Jansson JO: Changes in expression of CCAT/enhancer binding protein α (C/EBP α) and C/EBP β in rat liver after partial hepatectomy but not after treatment with cyproterone acetate. *J Hepatol* 1997, 27:903–911
41. Altucci L, Addeo R, Cicatiello L, Dauvois S, Parker MG, Beato M, Truss M, Sica V, Bresciani F, Weisz A: 17 β -estradiol induces cyclin D1 gene transcription, p36D1–p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G1-arrested human breast cancer cells. *Oncogene* 1996, 12:2315–2324
42. Altucci L, Addeo R, Cicatiello L, Germano D, Pacilio C, Battista T, Canciani M, Petrizzi VB, Bresciani F, Weitz A: Estrogen induces early and timed activation of cyclin-dependent kinases 4, 5 and 6 and increases cyclin mRNA expression in rat uterus. *Endocrinology* 1997, 138:978–984
43. Neuman E, Ladha MH, Lin N, Upton TM, Miller SJ, DiRenzo J, Pestell RG, Hinds PW, Dowdy SF, Brown M, Ewen ME: Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4: *Mol Cell Biol* 1997, 17:5338–5347
44. Planas-Silva MD, Weinberg RA: Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution. *Mol Cell Biol* 1997, 17:4059–4069
45. Geum D, Sun W, Paik SK, Lee CC, Kim K: Estrogen-induced cyclin D1 and D3 gene expressions during mouse uterine cell proliferation in vivo: differential induction mechanism of cyclin D1 and D3. *Mol Reprod Dev* 1997, 46:450–458
46. Motokura T, Keyomarsi K, Kronenberg HM, Arnold A: Cloning and characterization of human cyclin D3, a cDNA closely related in sequence to the PRAD1/cyclin D1 proto-oncogene. *J Biol Chem* 1992, 267:20412–20415
47. Matsushima H, Roussel MF, Ashum RA, Sherr CJ: Colony-stimulation factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* 1995, 65:701–713
48. Sun W, Lee DK, Lee CC, Kim K: Differential expression of D-type G1 cyclins during mouse development and liver regeneration in vivo. *Mol Reprod Dev* 1996, 43:414–420
49. Albrecht JH, Meyer AH, Hu MY: Regulation of cyclin-dependent kinase inhibitor p21(WAF1/Cip1/SDI1) gene expression in hepatic regeneration. *Hepatology* 1997, 25:557–563
50. Diwan BA, Lubet RA, Ward JM, Hrabie JA, Rice JM: Tumor-promoting and hepatocarcinogenic effects of 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) in DBA/2Ncr and C57BL/6Ncr mice and an apparent promoting effect on nasal cavity tumors but not on hepatocellular tumors in F344/Ncr rats initiated with N-nitrosodiethylamine. *Carcinogenesis* 1992, 13:1893–1901
51. Reddy JK, Lalwani ND: Carcinogenesis by hepatic peroxisome proliferators: evaluation of the risk of hypolipidemic drugs and industrial plasticizers to humans. *Crit Rev Toxicol* 1983, 12:1–58
52. Kraupp-Grasl B, Huber W, Putz B, Gerbracht U, Schulte-Hermann R: Tumor promotion by the peroxisome proliferator nafenopin involving a specific subtype of altered foci in rat liver. *Cancer Res* 1990, 50: 3701–3708
53. Marsman DS, Popp JA: Biological potential of basophilic hepatocellular foci and hepatic adenoma induced by the peroxisome proliferator WY 14,643. *Carcinogenesis* 1994, 15:111–117