Detection of a Tyrosine-phosphorylated Form of Cyclin A during Liver Regeneration

Jo Ann Spiwak Rinaudo¹ and Snorri S. Thorgeirsson²

Laboratory of Experimental Carcinogenesis, National Cancer Institute, NIH, Bethesda, Maryland 20892-4255

Abstract

Cyclin A functions in both the S and G₂-M phases of the cell cycle. The expression of cyclin A during liver regeneration was compared with that of cyclin B1 and p34cdc2. Liver regeneration was followed at 2-h intervals from 12 to 48 h after partial hepatectomy (PH). Immunohistochemical staining using proliferating cell nuclear antigen revealed DNA synthesis peaks at 18 h after PH. The most intense nuclear staining of hepatocytes with cyclins A and B1 and p34cdc2 antibodies occurred at 26 h post-PH, which corresponds with the onset of mitosis. Quantitative mRNA expression of cyclins A and B1 and p34cdc2 was determined by competitive reverse transcription-PCR. Construction of mRNA internal standards and coamplification during reverse transcription-PCR allowed quantitation of all three cell cycle genes. At 24 h post-PH, cyclin A mRNA levels were approximately 5 fg/100 ng total RNA. In contrast, cyclin B1 and p34cdc2 levels were 20-fold higher, 100 fg/100 ng total RNA. Cyclin B1 and p34cdc2 mRNA levels showed two peaks, at 26 and 38–44 h post-PH, whereas the levels of cyclin A were constant during this interval. Immunoblots revealed the presence of cyclin A in normal liver, and significant amounts were present as early as 12 h post-PH. At 26 h post-PH, tyrosine-phosphorylated forms of cyclin A were detected. Cyclin B1 and p34cdc2 protein were not present until 22–24 h post-PH, and two peaks were observed, at 26 and 38–44 h, coinciding with the mRNA pattern. Histone H1 kinase activity was associated with the two peaks of cyclin B1 and p34cdc2 expression. The unique pattern of cyclin A expression and detection of tyrosine-phosphorylated forms suggest a different mechanism for the regulation of cyclin A during liver regeneration.

Introduction

The adult mammalian liver is characterized by the presence of polyploid hepatocytes (1). These cells arise after birth, during active growth and development of the liver. The majority of hepatocytes proliferate normally, but a fraction become binucleated, indicating altered G₂-M cell-cycle phases and cytokinesis in these cells. Furthermore, these diploid binucleated cells proliferate and generate mononucleated hepatocytes with a tetraploid nucleus. Thus, the binucleated cell serves as the intermediate in the formation of polyploid hepatocytes. This process generates an adult rat liver that contains less than 10% diploid hepatocytes. The majority of cells in the liver are tetraploid with either one nucleus (70%) or two nuclei (10%), and approximately 10% of hepatocytes contain two tetraploid nuclei. The formation of polyploid hepatocytes occurs during the G₂-M and cytokinesis phases of the cell cycle, but the molecular mechanism(s) responsible is unknown. The cell cycle-regulatory proteins, cyclins A and B1 and p34cdc2 kinase, are active during the G₂-M phase of the cell cycle.

Although the adult rat liver is polyploid, the hepatocytes exist in a G₀/G₁ state and are capable of proliferating in response to a PH. After a PH, >90% of hepatocytes undergo DNA synthesis within the first 24 h followed by mitosis, doubling the liver mass by 48 hours (1–3). During liver regeneration, the G₁ phase of the cell cycle spans the first 16 h. DNA synthesis is first detected at 18 h, and the length of the S phase is estimated to be 6 h (1). Mitotic figures appear as early as 24 h post-PH, peak at 34 h, and are found up to 48 h post-PH. Earlier studies have indicated that hepatocytes lose synchrony at the G₂-M transition and a protracted M phase occurs from 26–48 h post-PH (1). There is also evidence to suggest that a second cell cycle follows (4, 5). The presence of an unsynchronized M phase could indicate that different populations of hepatocytes undergo mitosis at various times after PH. We have hypothesized that the polyploid and binucleated cell population in the liver could be generated and maintained through the involvement of cyclins A and B1 and p34cdc2 during liver regeneration.

The overexpression of cyclins A and B1 or increased activity of the p34cdc2/cyclin kinase complex can lead to cell cycle arrest at the G₂-M boundary (6). There is also evidence that overexpression of cyclins can also result in the formation of multinucleated cells. In cells transfected with a cyclin B mutant lacking the destruction cyclin box, multinucleated cells appear (7). Furthermore, premature destruction of p34cdc2, as a temperature-sensitive mutant, results in cells returning to the G₀/G₁ point without completing mitosis (8).

¹ Present address: Division of Molecular Virology and Immunology, Georgetown University School of Medicine, Rockville, MD 20852.
² To whom requests for reprints should be addressed, at National Cancer Institute, Building 37, Room 3C28, 37 Convent Drive MSC4255, Bethesda, MD 20892-4255. Phone: (301) 496-5688; Fax: (301) 496-0734; E-mail: snorti_thorgeirsson@nih.gov.
The preceding are all examples using mutant cell cycle proteins, while the formation and maintenance of polyploid cells is an inherent property of the liver.

Cyclin A can function in the S phase of the cell cycle by association with p33cdc2, whereas, during the G2-M phase, it forms a complex with p34cdc2 (9). This is a unique role of cyclin A in two important transition steps during the cell cycle, and any mutations or alterations could affect the S or G2-M phases of the cell cycle differently. Cyclin A is implicated in the development of hepatocellular carcinoma in hepatitis B virus-infected livers (10, 11). Studies on liver regeneration indicate that during the G2-M phase of the cell cycle, the majority of p34cdc2 forms a complex with cyclin B1 and only a small fraction with cyclin A (12). Other studies show a correlation between cyclin B1 and p34cdc2 expression, kinase activity, and the peak of cell proliferation after PH (12-14). Therefore, during liver regeneration, the expression and function of cyclin A during the G2-M phase of the cell cycle may differ from cyclin B1.

Phosphorylation reactions are key mechanisms for regulating the activity of cyclin/cdk complexes. For example, activation of the cyclin/p34cdc2 kinase requires both dephosphorylation of Thr-14 and Tyr-15 by cdc25 phosphatase and phosphorylation of Thr-161 by a cyclin-activating kinase (15). The cyclin proteins are also capable of being phosphorylated (16-18). Recently, cyclin B1 was reported to be phosphorylated on five serine residues during oocyte maturation (19). But these phosphorylations were not determined to be essential for kinase activity of the cyclin B1/cdc2 complex. In contrast, phosphorylation of cyclin A on tyrosine residues in an osteosarcoma cell line was associated with increased kinase activity (20, 21). These studies suggest that phosphorylations of cyclins could be important determinants of cdk activation and possibly of substrate specificity.

RT-PCR is a sensitive technique for detection of low-abundance mRNA and requires only small amounts of total RNA for analysis. Gene expression can be quantitated by designing a shorter mRNA internal standard that utilizes the same primers as the endogenous mRNA (22-24). Using competitive RT-PCR, a known amount of internal standard mRNA is coamplified with endogenous mRNA, and the levels of endogenous mRNA can be quantitated. This technique is both sensitive and specific to allow detection of differences in levels of mRNA expression between cyclins A and B1 and p34cdc2 during liver regeneration.

In this study, a unique pattern of cyclin A expression was observed after PH. Cyclins A and B1 and p34cdc2 mRNA levels were increased at 26 h posthepatectomy, which correlated with the G2-M transition. The peak of cyclin B1 protein expression correlated with p34cdc2 phosphorylation and kinase activity during liver regeneration. In addition, at 26 h post-PH, three distinct tyrosine-phosphorylated forms of cyclin A were observed. The detection of phosphorylated forms of cyclin A during G2-M transition may impart a different function for cyclin A during mitosis compared to its role in S phase.

Results

Cell Cycle Phases during Liver Regeneration. Immunohistochemistry was used to study the temporal and spatial localization of the cyclins and p34cdc2 and to correlate them with the kinetics of liver regeneration. PCNA, an accessory protein to DNA polymerase δ, is an indicator of DNA synthesis (4, 25). During liver regeneration, no PCNA staining was detectable prior to 18 h post-PH (not shown). Between 18 and 24 h, a significant number of hepatic nuclei were positive, indicating a 6-h S phase. The greatest number of positive hepatocytes were observed at 18 h (>50%), and the staining was distributed randomly within the hepatic acinus (Fig. 1A). At all other subsequent time points examined, only a very few positive cells were detected (not shown). This
correlates with earlier studies that found that >90% hepatocytes undergo DNA synthesis within the first 24 h after PH (26).

Cyclin A-positive nuclei were seen as early as 16–18 h post-PH (not shown), which confirms its function in late G, and early S phase (27–29). In contrast, cyclin B1 and p34cdc2-positive cells were not observed until 20 h post-PH when cytoplasmic staining was detected (not shown). For all three cell cycle proteins, the greatest number of positive nuclei (>50%) were present at 26 h post-PH, and the staining pattern was again random throughout the liver acinus (see Fig. 1, B–D). This correlates with the first appearance of mitotic figures in the liver and represents the G2-M transition during liver regeneration. After 26 h, the number of positive cells diminished but randomly scattered positively stained cells persisted up to 48 h post-PH. The cyclin A-positive cells remained low, but a slight increase in cyclin B1- and p34cdc2-positive hepatocytes was observed at 40 h post-PH (not shown). The immunohistochemical data showed the majority of hepatocytes entering S phase (>50%) at 18 h, but by the G2-M transition, the hepatocyte population was no longer synchronized. The hepatocytes appeared to progress through the G2-M phase of the cell cycle at various intervals within the first 48 h after PH. To further ascertain any differences in cyclin A versus cyclin B1 and p34cdc2 during liver regeneration, mRNA and protein expression was examined.

Construction of the mRNA Internal Standards and Concentration Curves. For quantification of mRNA levels, standards were constructed, which had two important properties: (a) the internal standard had to have the ability to be coamplified with the endogenous cellular mRNA using the same primers, and (b) the PCR products generated by the internal standards had to be distinguishable on gels from those amplified from the cellular mRNA.

Using sequence-specific primers (see "Materials and Methods") and total RNA from liver 24 h post-PH, expression of cyclins A and B1 and p34cdc2 was detected by RT-PCR. The resultant PCR products were purified, sequenced, and determined to be identical with the corresponding cDNA sequences (data not shown). The sizes of the PCR products were 153, 300, and 285 bp for cyclins A and B1, and p34cdc2, respectively. This established that the selected primers were able to detect the mRNA for the respective cell cycle genes. The 5’ and 3’ oligonucleotide sequences used to generate the PCR products are named primers A and D, respectively, for each cell cycle gene.

Sequence overlap extension (30) was used to make 30–50-bp deletions in the PCR products for each of the three cell cycle genes (Fig. 2). Two new oligonucleotide primers were synthesized. Primer B was the reverse complement of the sequence that was 5’ of the deleted region, and primer C was 3’ of the deleted region (see "Materials and Methods" for sequences). Furthermore, the 5’ region of each primer contained an additional 18 bp complementary with the other primer. Using the full-length PCR product as a template, two separate PCR reactions were performed using the combination of primers A and B in one reaction and primer set C and D (Fig. 2a) in the second reaction. The resulting PCR products from these two reactions had overlapping sequences. A third PCR reaction, using these two DNA fragments as the template and primers A and D, resulted in a DNA internal standard that utilized the same primers as the full-length PCR product but was of a shorter length. This process was used to generate DNA internal standards for all three cell cycle genes. The DNA standards for cyclins A and B1 and p34cdc2 were 114, 235, and 205 bp, respectively.

To make the mRNA internal standard, the DNA internal standard was the template for another PCR reaction using primers D and A, modified by the addition of the T7 promoter sequence to the 5’ region (Fig. 2b, T7A). The internal standard containing this additional sequence allowed transcription with the T7 polymerase to generate a mRNA internal standard. Large quantities of mRNA internal standard could be produced, and the DNA template could easily be removed by digestion with DNase.

The mRNA internal standards were coamplified with the endogenous mRNA in the RT-PCR reaction, using one set of primers. The inclusion of a standard in the RT-PCR reaction served as an internal control, ensuring that the PCR amplification reaction occurred efficiently and reproducibly in all
Fig. 3. Concentration curves for cyclins A and B1 and p34cdc2. RT-PCR was performed using RNA from liver 24 h post-PH at a concentration of 100 ng/reaction and increasing amounts of internal standard mRNA, for each of the respective cell cycle genes. Scanned images are shown (top), and the pixel values obtained are graphed (bottom). On each graph, the pixel values of the RNA for the cell cycle genes [■] and the internal standard values [□] are shown. Concentration curves are shown for cyclin A (A), cyclin B (B), and p34cdc2 (C). ng RNA, ng of total RNA added from regenerating liver 24 h post-PH; fg IS, fg of internal standard mRNA added.

tubes. This allowed comparisons between tubes within an experiment. The ratio of the endogenous mRNA:internal standard was used for quantitation of cyclins A and B1 and p34cdc2 mRNA during liver regeneration.

For quantitation, [³²P]dCTP is added to the PCR reaction and incorporated into the PCR products generated from both the endogenous mRNA and the internal standard. The full-length PCR products for cyclins A and B1 and p34cdc2 have approximately 1.25-fold more cytosines than the corresponding internal standard PCR products. To compensate for this, the internal standards were designed 25\% shorter so that 1 fg of the shorter internal standard contains 1.25-fold more fmol DNA than the full-length PCR product. Therefore, when the band intensities of the endogenous mRNA and the internal standard mRNA are equal, this represents the actual quantity (fg) of mRNA for that cell cycle gene.

A concentration curve was established to determine the mRNA internal standard range to use in the RT-PCR reactions. Total RNA from 24 h post-PH (100 ng/tube) was coamplified with varying amounts of internal standard mRNA (1–200 fg/reaction). The concentration curves for cyclins A and B1 and p34cdc2 are shown in Fig. 3A, B, and C, respectively. As the internal standard mRNA concentration increases, the endogenous mRNA band intensity gradually decreases, and the point at which the two lines intersect represents the mRNA concentration in regenerating liver for that specific cell cycle gene. Approximately 5 fg of cyclin A internal standard mRNA resulted in a band intensity equal to 100 ng of total RNA from regenerating liver 24 h post-PH (Fig. 3A). In contrast, 100 fg of cyclin B1 internal standard mRNA was needed to produce a band of similar intensity to 100 ng of total RNA (Fig. 3B). A similar value of 100 fg was obtained for p34cdc2 (Fig. 3C). These concentration curves indicate that the level of cyclin A mRNA was 20-fold lower than cyclin B1 or p34cdc2 in regenerating liver 24 h post-PH.

**Cyclins A and B1 and p34cdc2 mRNA Expression during Liver Regeneration.** The expressions of cyclins A and B1 and p34cdc2 mRNA during liver regeneration are shown by representative gels and bar graphs (Fig. 4, A–C). The internal standard mRNA band intensity did not vary between reaction tubes, indicating consistent amplification of the PCR products in all tubes. The histograms showing the fg quantities of mRNA were obtained by determining the ratio of the endogenous mRNA:internal standard. Above the gels (Fig. 4), the + denotes the presence of reverse transcriptase, and the − indicates no addition of reverse transcriptase. Omission of the enzyme resulted in no detection of bands on the gel, indicating that the bands produced in the presence of reverse transcriptase originated from RNA and not any DNA. This was seen in all gels.

From 12–22 h post-PH, the cyclin A mRNA levels began rising. This initial rise in cyclin A mRNA levels corresponds to the entry of hepatocytes into the G1-S phase of the cell cycle. By 24–26 h, at the G2-M transition, the levels peaked at 2–3 fg/100 ng total RNA (Fig. 4A). This represented a 10-fold increase in mRNA expression. Between 24 and 48 h, cyclin A mRNA levels remained elevated. At 72 h post-PH, the levels were declining. The control, sham hepatectomy had low levels of cyclin A mRNA (<0.5 fg/100 ng total RNA).

Cyclin B1 and p34cdc2 mRNA levels and expression patterns were similar during liver regeneration (Fig. 4, B and C).
The levels were initially low between 12 and 18 h (<10 fg/100 ng total RNA) and began to increase at 20 h. Two peaks were detected at 26 and 38–44 h post-PH (>150 fg/100 ng total RNA). Between the peaks, at 34 h, the mRNA levels declined slightly but significantly (50 fg/100 ng total RNA), and by 48 h, the levels were declining. The sham heptectomy had low levels (<10 fg/100 ng total RNA) of both cyclin B1 and p34cdc2 expression. These two peaks in cyclin B1 and p34cdc2 expression represent two waves of mitoses that occur within the initial 48 h after PH.

**Immunoblotting.** Cyclin A protein was immunoprecipitated from equal amounts of liver extracts (500 μg) obtained from regenerating liver and unstimulated, control liver. Cyclin A was detected as a Mr 58,000 band on immunoblots in regenerating liver from 12–48 h post-PH (Fig. 5A). Unstimulated, control liver also showed the presence of cyclin A. Although immunoprecipitation is not quantitative, the most intense bands appeared between 12 and 34 h post-PH. The period from 12–22 h represents the G1-S phase of the cell cycle, and the G2-M phase occurs from 26 to 34 h post-PH. During this period, more than 90% of the hepatocytes undergo DNA synthesis and mitosis, completing the first cell cycle of liver regeneration (26). The phosphorylation state of cyclin A was also examined. The samples immunoprecipitated with the cyclin A Ab were probed with a phosphotyrosine-specific Ab (Fig. 5B). At only one time point, 26 h post-PH, three distinct bands were observed in the Mr 58,000 range. Furthermore, when the immunoprecipitate
The phosphorylation of cyclin A at the G2-M transition during liver regeneration could alter its binding to p34cdc2 and affect kinase activity. The protein expression patterns of cyclin B1 and cdc2 were very similar (Fig. 6). No cyclin B1 protein was detectable in normal liver and in regenerating liver until 24 h post-PH. Very high levels of cyclin B1 were present between 24 and 30 h, diminished between 32 and 36 h, increased again from 38 to 42 h, and dropped to barely detectable levels at 48 h. The p34cdc2 protein was first observed faintly at 18 h post-PH but peaked at 24 h and remained high up to 48 h post-PH (Fig. 6). The p34cdc2 protein was observed as a distinct doublet, which was prominent at both 24–28 h and at 38–42 h post-PH. This doublet coincided with the peak levels of the cyclin B1 protein expression. The protein expression pattern again indicates that the two mitotic waves occurred during the initial 48 h after PH.

**Histone H1 Kinase Activity.** The activity of the cyclin/cdc2 kinase complex during liver regeneration was determined by assessing histone H1 kinase activity after isolating the complex by p13-agarose beads. The p13 protein is capable of binding to the cyclin B1 (or A)/cdc2 complex present during G2-M or the cyclin A/cdk2 complex active during S phase. Therefore, the kinase activity detected would correspond to the cyclin/cdk complex predominantly present during that phase of the cell cycle. The kinase assay was repeated twice, and a representative histogram of the relative amount of histone H1 phosphorylation that was obtained from scanning the bands on the gel is shown in Fig. 7. There was no kinase activity detected in control liver, but some kinase activity was always detectable in regenerating liver at the time points examined. During the G1-S phase of the cell cycle (12–24 h post-PH), a peak of kinase activity was detected at 16 h. This peak represents the cyclin A/cdk activity during the S phase of the cell cycle; during this early time, both the cyclin B1 and p34cdc2 proteins were barely detectable. The second peak of kinase activity was at 26 h post-PH, during the G2-M phase of the cell cycle, when cyclins A and B1 and p34cdc2 protein levels were elevated. At 30–34 h, a third slightly lower peak of activity was observed. This coincides with decreasing cyclin B1 levels and dephosphorylation of p34cdc2 protein, and therefore the kinase activity would be associated with G1-S phase of the cell cycle, implicating cyclin A. The fourth peak of kinase activity, at 40 h, is associated with a protein pattern similar to 26 h. Taken together, the protein expression pattern for cyclins A and B1, cdc2, and the four peaks of histone H1 kinase activity suggests that the hepatocytes are undergoing two cell cycles during the 48 h post-PH.

**Discussion**

The novel finding in this study was the identification of a tyrosine-phosphorylated form of cyclin A during liver regeneration. This corresponds to the G2-M transition phase during liver regeneration. Phosphorylated forms of cyclin A have been observed during embryonic development in Xenopus (31). In an osteosarcoma cell line, the appearance of a tyrosine-phosphorylated form of cyclin A corresponded with increased kinase activity (20, 21). The function of this phosphorylated form of cyclin A is currently unknown, but these modifications could affect the activity of cyclin A during G2-M. There is evidence accumulating that cyclin A functions primarily during the G1 and S phases of the cell cycle. Studies on liver regeneration indicate that during the G2-M transition, the majority of p34cdc2 is bound to cyclin B1, and less than 25% is associated with cyclin A (12). Furthermore, cyclin A-associated kinase activity is the rate-limiting factor for entry into S phase (32). Recent studies demonstrated the presence of cyclin A in quiescent fibroblasts as a complex bound to cdk2 and the cdk inhibitor, p27 (33). Therefore, the phosphorylation of cyclin A could serve as another level of controlling cyclin A activity during G2-M or other stages of the cell cycle.

In this study, during liver regeneration both cyclin A mRNA and protein levels were lower than those of cyclin B1 and p34cdc2. In the regenerating liver, this low, sustained expression may potentiate the function of cyclin A during mitosis. In other cell types, such as HeLa cells, the peak level of cyclin A mRNA is about 2-fold lower than cyclin B1 (34). Cyclin A activation of p34cdc2 kinase in Xenopus egg extracts occurs at a 5-fold lower concentration than does cyclin B1 (35). Furthermore, during Xenopus development, the cyclin A/cdc2 kinase complex forms prior to the cyclin B1/cdc2 complex, escapes the inhibitory mechanisms that affect the cyclin B1/cdc2 complex, and induces the conversion of cyclin B1/cdc2 kinase from an inactive to an active form (36).
Recent studies have determined that the half-life of the cyclin A/cdc2 complex is 4 h compared to 15 h for cyclin B1/cdc2 (37). The presence of cyclin A could furthermore be controlled by binding to p27. Recent work indicates that cyclin A is present in quiescent fibroblasts as a cyclin A/cdk2 complex that is bound to the inhibitor p27 (33). In a fibroblast cell line, an inducible plasmid-containing cyclin A can be expressed continuously throughout the cell cycle. Under those conditions, cyclin A-associated kinase activity is negatively regulated in G1 by the presence of p27 (32, 38). Therefore, during liver regeneration, the presence of low sustained levels of cyclin A could be negatively regulated by proteins such as p27 in G1-S phase.

The absence of cyclin B1 and p34cdc2 kinase prior to 20 h post-PH and in sham-hepatectomized rats indicates that these genes are not expressed in quiescent liver and must be induced as cells enter the cell cycle. This is in contrast to p34cdc2 kinase, which is expressed continuously during oocyte maturation, in rapidly dividing embryos, and in vitro in cell lines (39, 40). Both the cyclin B1 and p34cdc2 mRNA levels and protein expression were similar during liver regeneration. Furthermore, cyclin B1 showed the classic oscillatory pattern, which correlated with the phosphorylation state of p34cdc2 and the corresponding kinase activity. Earlier studies on liver regeneration focused on the first 36 h post-PH (12, 13, 41). These studies demonstrated that cyclin B1 expression peaked at 24 h, and only two peaks of kinase activity were observed at 24 and 30 h post-PH. In this study, the second peak of cyclin B1 expression at 40 h signifies another cell cycle and agrees with the work of Grisham, who observed second peak of mitosis starting at 38–40 h post-PH (42).

In this study, the absolute levels of cyclins A and B1 and p34cdc2 mRNA during liver regeneration were determined using RT-PCR. The advantages of competitive RT-PCR to detect mRNA expression are an increased sensitivity, the use of only ng quantities of mRNA template, and the ability to quantify mRNA levels. In this assay, using only ng quantities of total RNA, fg amounts of cyclins and p34cdc2 mRNA were detectable. Other studies confirm the use of an internal standard in quantifying RNA levels (27, 28, 43–45). In this study, addition of an internal standard eliminates a problem encountered in Northern blots, using another mRNA as a control, the expression of which may also oscillate during liver regeneration. In addition, the mRNA internal standards were made from the full-length PCR products generated from the endogenous mRNA. This eliminated any differences in the efficiency of amplification due to differences in primer pairs or secondary structure of the template RNA. The advantage of the current method is the synthesis of the mRNA internal standard directly from the DNA internal standard without prior cloning into a plasmid.

The model of liver regeneration that emerges from these data is as follows. During G1 and S phases, 12–20 h post-PH, cyclin A levels increase. The majority of hepatocytes undergo DNA synthesis around 18 h as evidenced by PCNA staining. The two waves of mitosis, at 24–26 h and 40 h post-PH, are accompanied by elevated levels of cyclin B1, phosphorylated forms of p34cdc2, and increased kinase activity. Concurrent with this process is the presence of low levels of cyclin A and the detection of a phosphorylated form at the G2-M transition. Furthermore, after liver regeneration is complete, the hepatocytes acquire the next higher ploidy class (1). The formation of these polyploid cells could be mediated by the continued presence of cyclin A. In normal adult mouse liver, there is evidence for the presence of a population of hepatocytes with an extended G2 phase (46). If the functions of cyclin A were modulated by phosphorylation, DNA synthesis could be stimulated and mitosis inhibited, with eventual development of polyploid cells.

Hepatic carcinogens in rat and mouse livers act initially on binucleated cells, decreasing this cell population (29). Furthermore, when preneoplastic foci first develop, they initially
consistent mainly of diploid hepatocytes (47). The cell cycle genes have also been implicated in the development of hepatocellular carcinoma (10). In hepatitis B-infected cells, the virus was found integrated within the first intron of cyclin A, resulting in the potential for producing a mutant protein (11). The identification and further study of the phosphorylated forms of cyclin A during hepatic regeneration could lead to an understanding of how cyclin A functions in both S and G2-M phases, how it contributes to the formation of polyploid hepatocytes, and how perturbations in cyclin A may contribute to the development of carcinogenesis.

Materials and Methods

Treatment of Animals and Preparation of RNA. Male Fischer 344 rats weighing 120–150 g were used for all experiments. All animals were treated according to the NIH guidelines for animal handling. Two-thirds partial hepatectomies were performed between 8:00 and 10:00 a.m., and animals were sacrificed every 2 h between 12 and 48 h after surgery and at 72 h. Control rats received a laparotomy only (sham hepatectomy) and were sacrificed after 24 h. Livers were excised and rinsed in 1× PBS (pH 7.4). RNA was prepared using RINazol (Tel-Test, Friendswood, TX), a modification of the guanidinium thiocyanate method (48). The 260/280 absorbance ratio for all RNA preparations was 1.8 or higher. All experiments were performed in duplicate.

RT-PCR Conditions. For cyclin A, primers were selected based on the mouse sequence. The 5’ oligonucleotide sequence was GACCTGGCGTCAGCCCAACCTGAAT, and the 3’ oligonucleotide was AATGAAGTGAAAGCAGGCTAAGGAGTAC. For cyclin B, the 5’ sequence was ACCTACTCGAGGTGCTGAATGCTCACTGAAA, and the 3’ sequence was TCAGAACTTCTACTATGATGCTGAT. The 5’ and 3’ oligonucleotide primers used to detect p34cdc2 kinase mRNA were GAGAAAATCTGGAGAGGACTAT and AGAGATCCATGAGCCGCGAGGAGG, respectively. The internal oligonucleotide primer sequences used to generate the mRNA internal standards were CCCCCCCTGGAGAAAAATGTTGG (primer B) and CTTTGCCGTTTGGGGAGAGTT (primer C) for cyclin A, TTGCTTTGCCACCCCTGCTAAATGGA (primer B) and ATGGATTGGCTGACTGAGCAATACAGGA (primer C) for cyclin B1, and AATGACATCCGGCTCCTTGAAGTCT (primer B) and GAAAGCGGAGGTAGCACTTCGGA (primer C) for p34cdc2 (see “Results” and Fig. 2 for description). Internal standards were constructed using sequence overlap extension (30). Transcription from the T7 promoter of the internal standard DNA was used to generate the mRNA internal standards. The mRNA concentration was determined by absorbance at 260 nm, and the appropriate dilutions were made. All PCR products were verified by sequencing.


