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Induction of Cyclin mRNA and Cyclin-associated Histone H1 Kinase during Liver Regeneration*

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Cyclins and cyclin-associated cdc kinases are key regulators of oocyte maturation (Maller, J. L. (1990) in *The Biology and Medicine of Signal Transduction* (Nishizuka, Y., Endo, M., and Tanaka, C., eds) pp. 323–328, Raven Press, New York), yeast cell cycles (Nurse, P. (1990) *Nature* 344, 503–508), DNA replication in cell-free systems (D'Urso, F., Marraccino, R. L., Marshak, R. R., and Roberts, J. M. (1990) *Science* 250, 786–791), and amphibian cell proliferative transitions (Hunt, T. (1991) *Nature* 350, 462–463). The extent to which these regulatory molecules participate in the growth control of differentiated epithelial cells like hepatocytes is unknown. Therefore, we investigated the expression of "G₁" (E, C, and D) and "G₂/M" (A, B1, and B2) cyclin mRNAs, the relative levels of cyclin A- and B1-associated histone H1-kinase activity, and the appearance of cyclin-associated kinases (p32/p33^{cdc2} and p33/p34^{cdc2}) in regenerating rat liver and in control tissues from sham hepatectomized rats. To do this, we exploited a battery of human cyclin cDNAs and cyclin antisera that recognize rat molecules. The results suggest an apparent sequence of regeneration-specific changes: 1) elevated and induced expression of cyclins E (2.1 kilobases (kb)) and C (4 kb), and D mRNAs (4 kb), within 12 h, respectively; 2) induction of cyclins A (3.4 and 1.8 kb), B1 (2.5 and 1.8 kb), and B2 (1.9 kb) mRNAs at 24 h; 3) induction of cyclin A- and B1-associated nuclear histone H1 kinase at 24 h; and 4) enhanced levels of PSTAIRE-containing proteins of M_r ~32–33 and 33–34 kDa in nuclear extracts from 24-h regenerating liver that co-immunoprecipitate with cyclin A and B1 antisera, respectively. These observations provide an intellectual framework that unifies the biology of hepatocyte mi-

togenesis, proto-oncogene expression, and the machinery of the cell cycle.

Hepatocellular proliferation is controlled by polypeptides and nutrient supply (1–4). Hepatocytes arrest reversibly in three cell cycle states: predominantly in G₀ and occasionally in G₂; or, following manipulation, at the G₁/S boundary (5). In normal growth control models employing primary cultures (6–11) or 70% hepatectomized rats undergoing hepatic regeneration (2, 12–16), different polypeptides generate sequential signals which initiate DNA synthesis (1, 2, 5, 7–11, 13–16). Subsequently, most S phase hepatocytes are committed to divide. The report of hepatitis B virus integration into the cyclin A gene of a hepatocellular carcinoma (17) and the purification of p34^{cdc2}-like histone H1 kinase from cultured hepatomas (18) suggested to us that key cell cycle events (19–22), cyclin mRNA expression and cyclin A- and B1-associated histone H1 kinase activation, might occur in normal proliferating hepatocytes. This report demonstrates that such inductive events unfold sequentially during hepatic regeneration.

MATERIALS AND METHODS

Animal Procedures and Northern Blots—Surgeries, RNA isolation, gel electrophoresis, blotting, prehybridization, and random primer [^α-³²P]dCTP labeling of cloned cDNAs (23–25) were described elsewhere (15). Human cells were cultured by standard procedures. Four blots were prepared: 1) cyclin A; 2) cyclin B1; 3) cyclin D, followed by cyclin E; and 4) cyclin C, followed by cyclin B2. For sequential blots, first or second hybridization probes were removed (15); all blots were ultimately subjected to hybridization with α -tubulin cDNA (15). Blots were washed in 1 × SSC, 0.5% SDS¹ for 15 min (×2) at 21 °C and then as follows: cyclins A and B1 (0.1 × SSC, 1% SDS for 15 min (×2) at 21 °C (autoradiograms were made with intensifying screens at -70 °C and films (Kodak X-Omat) developed after 3 days)); cyclin B2 (0.1 × SSC, 0.5% SDS for 15 min (×4) (developed after 5 days)); cyclin C (0.1 × SSC, 1% SDS for 15 min (×2) at 50 °C (developed after 4 days)); cyclin D (0.1 × SSC, 1% SDS for 15 min (×4) at 60 °C (developed after 4 days)); cyclin E (0.1 × SSC, 0.5% SDS for 15 min (×2) at 50 °C (developed after 5 days)); and α -tubulin (0.1 × SSC, 1% SDS for 15 min (×2) at 60 °C (developed after 1 day)). Relative mRNA levels on autoradiograms were quantified by scanning densitometry (A_{500 nm}) as described elsewhere (8, 15).

Cyclin A- and B1-associated Histone H1 Kinase Assays—Pooled liver lobes (15) (two rats/group) were homogenized at 4 °C using a 5:1 ratio (v/w) of Buffer A (50 mM Tris-HCl, pH 7, 50 mM [NH₄]₂SO₄, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (Sigma), and 1 μ g/ μ l aprotinin, leupeptin, and pepstatin (Boehringer Mannheim) for 10 s at 2000 rpm (10 strokes) with a glass tube and no. A58547 Teflon pestle (Thomas)). Homogenates were centrifuged at 35,000 × g for 30 min at 4 °C. Supernatants ("cytosolic extracts") were aliquoted and stored at -70 °C. Liver nuclear extracts were prepared as described elsewhere (26). Protein concentrations were determined with the Bradford reagent (Pierce Chemical Co.). Kinase assays were performed with HeLa (\approx 400 μ g) or liver (1 mg) proteins, diluted into equal volumes with 2 × lysis buffer (2% deoxycholate, 2% Triton X-100,

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¹ The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylene-nitrilo)]tetraacetic acid; PSTAIRE, single letter amino acid name for conserved 16-amino acid sequence, EGVSTAIRESLLKE; kb, kilobase(s); IGF, insulin-like growth factor; EGF, epidermal growth factor; TGF, transforming growth factor; HBGf, heparin-binding growth factor; HGF, hepatocyte growth factor/scatter factor.

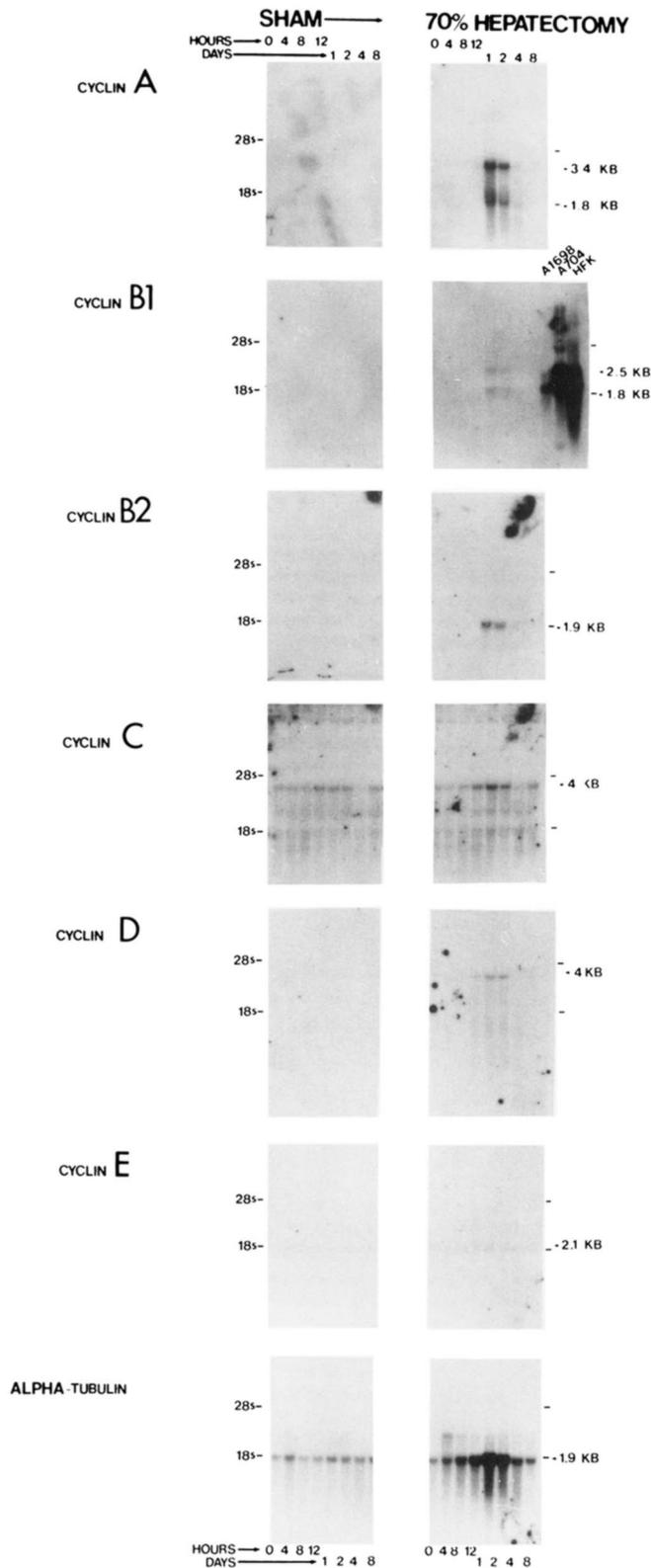


FIG. 1. Expression of cyclin mRNAs during hepatic regeneration in adult rats. Northern blots of total liver RNA (20 μ g/lane) are shown: sham (left-hand panels) and 70% hepatectomized groups (right-hand panels) 0–192 h postsurgery. Hybridization probes were: pGEM4Z 0.86-kb *SphI-PvuII* insert (HeLa cyclin A cDNA (23, 24)); pGEM4Z 0.6-kb *PvuII* insert (HeLa cell cyclin B1 cDNA (23, 24)); pAB24BXN 1.2-kb *BamHI* insert (HepG2 cyclin B2 cDNA (25)); pDB20 1.9-kb *XbaI-SacI* insert (HepG2 cyclin C cDNA (25)); pBluescript KS (Stratagene) 1.3-kb *NotI* insert (human glioblastoma cyclin D cDNA (25)); pDB20 1.1-kb *PstI-HindIII* insert (HeLa cyclin E cDNA (25)); and pTK α 1 1.9-kb *PstI* insert (human α -tubulin cDNA

0.2% SDS, 100 mM Tris-HCl, pH 7.5, 2 mM Na[PO₄]₂, containing 600 mM NaCl, and brought up to 500 μ l with RIPA buffer in sterile Eppendorf tubes. Protein A-Sepharose beads (Pharmacia LKB Biotechnology Inc.; 1:10 (beads:1 \times lysis buffer)) were added (30 μ l), mixed, and incubated for 30 min at 4 $^{\circ}$ C. Following centrifugation at 4 $^{\circ}$ C, the supernatants were transferred to tubes containing 1 μ l of monospecific rabbit anti-cyclin A or B1 antisera (23, 24) and incubated for 1 h at 4 $^{\circ}$ C with occasional mixing. Then, fresh 30- μ l aliquots of Protein A beads were added, and incubations continued for 1 h at 4 $^{\circ}$ C with occasional mixing. A series of washing/centrifugation steps was performed as follows: RIPA buffer (1 \times lysis buffer containing 300 mM NaCl) \times 3; RIPA buffer (minus SDS, Na(PO₄)₂, and NaCl) \times 1; and reaction buffer (20 mM Tris-HCl, pH 7.5, 7.5 mM MgCl₂, 1.4 mM ZnSO₄) \times 2. Supernatants were aspirated and, to the undisturbed beads, 5 μ l of reaction mixture was added (2.5 μ l of 2 \times reaction buffer, 1 μ l of histone H1 (1 mg/ml solution, Boehringer Mannheim), 0.5 μ l of H₂O, and 1 μ l of [γ -³²P]ATP (4500 mCi/mM, ICN)). Reaction tubes were incubated for 30 min at 37 $^{\circ}$ C with gentle vortexing. Twenty μ l of SDS-PAGE gel sample buffer was added (8), the tubes vortexed and boiled 5 min, and the contents subjected to 15% SDS-PAGE (8) (stacking and separating gels, 100 and 150 V for 1 and 3–4 h, respectively) at 21 $^{\circ}$ C. The gel was fixed for 1 h in H₂O:methanol:acetic acid (5:5:1), boiled for 20 min in 5% trichloroacetic acid, washed 2 times for 15 min in H₂O, dried (1 h at 80 $^{\circ}$ C), and subjected to autoradiography using an intensifying screen.

Detection of cdc Kinase-like Proteins by Western Blotting—Liver cytosolic and nuclear extracts were prepared from sham (S) and 70% hepatectomized (H) rats, and from cyclin A and B1 immunoprecipitates formed as described above. Extracted proteins were suspended in 2 \times SDS-PAGE sample buffer, subjected to 12% SDS-PAGE, and blotted onto nitrocellulose paper (Schleicher and Schuell) by electrotransfer at 25 V overnight at 21 $^{\circ}$ C. Nitrocellulose strips were subjected to blocking solutions for 1 h, using 150 mM NaCl, 10 mM Tris-HCl, pH 8, 2% bovine serum albumin (Promega), 0.2% Tween 20 (Promega), and 5% nonfat milk (Carnation). Anti-PSTAIRE antibodies (27, 28) were added at 1:5000 at 21 $^{\circ}$ C for 1 h. Detection of antibody-specific bands was performed with a Promega Western Blot AP System, following the supplier's recommended procedures.

RESULTS AND DISCUSSION

In Fig. 1, cDNA probes to cyclins A and B1 (Refs. 23 and 24) and B2, C, D, and E (Ref. 25) were hybridized to liver RNAs from rats subjected to sham or 70% hepatectomy. Hepatocyte DNA replication, G₂ transitions, and mitoses start between 13–14, 19–25, and 23–27 h following partial hepatectomy, respectively; regeneration ends within 8 days (12–15). No proliferation is induced by sham hepatectomy. The relative mRNA levels during the cell cycle are diagrammed in Fig. 2.

Two principal patterns of cyclin mRNA expression were observed: 1) "induction" of A, B1, B2, and D, following 70% hepatectomy, with no detectable hybridization signals after sham surgery; and 2) constitutive, and possibly oscillating, expression of cyclins C and E, their mRNA levels rising relatively more after 70% than after sham hepatectomy. These changes occurred within and peaked around 24 h postsurgery, before the onset of non-parenchymal cell proliferation (12); presurgical mRNA levels were restored by 192 h. RNA gel-loading differences could not explain these results, as the α -tubulin hybridization signals showed invariant and biphasic patterns of 1.9-kb mRNA expression in sham and 70% hepatectomized rats, respectively (15). These expression patterns are indirect evidence of their occurrence in hepatocytes, the dominant proliferating pool through 30 h postsurgery (2, 5, 9, 12).

(15)). Ribosomal RNA markers and putative cyclin mRNA positions are indicated (left and right panel margins, respectively). Blots to RNAs from human lines are shown, right-hand cyclin B1 panel (A1698 (gall bladder carcinoma); A704 (kidney adenocarcinoma); and, HFK 1321 (foreskin keratinocytes)). The topmost bands on the cyclin C autoradiograms are washing artifacts; this region of the nitrocellulose paper was placed inadvertently above the wash solution levels.

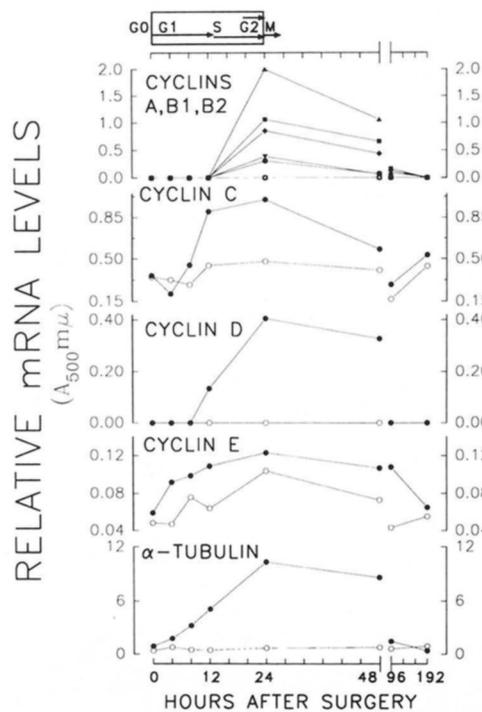


FIG. 2. Quantification of cyclin mRNAs during hepatic regeneration in adult rats. Sham (open) or 70% hepatectomy (closed symbols) is shown. Curves for cyclins A, B1, and B2 are shown in the top panel (triangles, diamonds, circles, inverted triangles, and squares are A (3.4 kb), A (1.8 kb), B1 (2.5 kb), B1 (1.8 kb), and B2 (1.9 kb), respectively). Curves for cyclins C (4 kb), D (4 kb), E (2.1 kb), and α -tubulin (1.9 kb) are labeled in each panel (upper left). Times postsurgery are indicated on the bottom and top abscissae. The box (upper left) indicates cell cycle landmarks (G_0 (quiescent state), G_1 (prereplicative state), S (DNA replication), G_2 (interval between DNA replication and mitosis), M (mitosis)) denoting temporal phases, approximately, by the lengths of arrows (\rightarrow). The vertical line breaks (abscissae) are at 48.1 and 95.9 h, respectively.

Additional features of cyclin mRNA expression were notable during regeneration. First, a "cascade-like" expression pattern was observed: $E \rightarrow C \rightarrow D \rightarrow (A, B1, B2)$. Thus, elevated levels of cyclin E, C, and D mRNA were detected early and late in G_1 (4, 12, and 12 h, respectively), prior to S phase entry, while levels of cyclin A, B1, and B2 mRNA, showing no detectable pre-S phase change, peaked at later times, by 24 h, before or during G_2/M transitions. These and other findings (23–25, 27–30) suggest that cyclins E, C, and D play hepatocyte G_1 regulatory roles. Cyclins E and C were tentatively assigned a G_1 role in HeLa cells (using CLN⁻rescue assays in *Saccharomyces cerevisiae*) although HeLa cyclin D levels were constitutive and invariant (25). In contrast, as expected (21–25, 28) from their delayed expression, cyclins A, B1, and B2 mRNAs might play S and/or G_2/M regulatory roles.

Second, except for cyclin E mRNA (2.1 kb), several unexpected cyclin mRNA size classes were observed. Two A and B1 classes were found. For cyclin A (3.4 kb (major) and 1.8 kb (minor)), only the minor size species was similar to one of two cyclin A mRNAs (a 1.8-kb species) in normal human liver (17); neither of the regenerating liver bands was similar in size to HeLa cyclin A (2.2 kb) (23). For cyclin B1, one mRNA was similar in size to that in HeLa cells (1.8 kb) but not the other (a 2.5-kb size class), which was absent from HeLa cells (23, 24). Regenerating liver cyclin B2 mRNA (1.9 kb) probably differs from the 1.7-kb HeLa mRNA as the B2 cDNA probe (25), unlike its B1 counterpart, hybridized to only one mRNA

species (Fig. 1); therefore, the 1.8- and 1.9-kb liver mRNAs might share identity. Liver cyclin C and D mRNA sizes (both 4 kb (major)) also differed from HeLa analogs (2–2.5 and 4.5 kb, respectively) (25). Although three cyclin D-like mRNAs were cloned recently from a cultured mouse macrophage cell line (29), the minor bands (<4 kb) on the autoradiograms of liver C and D mRNAs (Fig. 1) might instead be cross-hybridization artifacts, as they were not seen at higher stringency hybridization conditions (60 and 65 °C, respectively). Recent observations with cultured cell lines suggest marked differences between the expression of human and rodent G_2 cyclins (31). Our results support this conclusion, but overlap might still exist (see, for example, the human RNA Northern blots using cyclin B1 cDNA (Fig. 1)). The cellular and molecular origin of these and, possibly, other cryptic rat cyclin mRNAs remain to be determined.

H1 kinase activity also was induced 24 h after partial hepatectomy but not after sham surgery (Fig. 3). Kinase activity was recoverable preferentially in nuclear extracts with anti-cyclin A- and B1-monospecific antibodies, but not with anti-PSTAIR antibodies (24). No activation was seen early in G_1 (4 h) or at the time (14 h) when hepatocyte DNA replication has started (3, 5, 12, 14). The different G_2/M nuclear levels of cyclin-associated kinase activities ($A > B1$) parallel the graded differences observed in control HeLa cell extracts (Fig. 3) and in synchronized HeLa cells observed *in vitro* by light microscopy (28). The basis for this disparity in regenerating liver cells is unknown, though periodic cell cycle

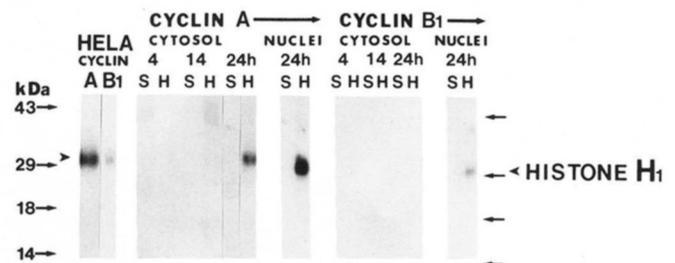


FIG. 3. Induction of cyclin A- and B1-associated histone H1 kinase activity during hepatic regeneration in adult rats. Liver cytosolic extracts were from sham (S) or 70% hepatectomized rats (H) 4, 14, or 24 h postsurgery. Liver nuclear extracts were prepared as described under "Materials and Methods." HeLa extracts were from nocodazole-synchronized cells (25). Histone H1 kinase activity was determined in anti-cyclin A and anti-cyclin B1 co-immunoprecipitates by SDS-PAGE and autoradiography. Films were exposed 30 min for HeLa cyclin A at 21 °C; or 2 h for HeLa cyclin B1 and nuclei cyclin A, 24 h for cytosol cyclin A, and 48 h for nuclei cyclin B1 at -70 °C. Prestained low M_r markers (Bethesda Research Laboratories) were subjected to silver staining (8) in separate tracks to authenticate accuracy of their migration on the gel (not shown).

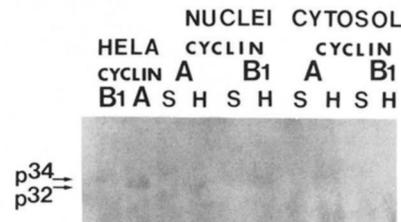


FIG. 4. Detection of p32 and p34 and other putative cdc-like "kinases" in liver extracts by Western blots. Anti-cyclin A or B1 co-immunoprecipitates were obtained (as in Fig. 3), Western-blotted, and probed with anti-PSTAIR monoclonal antibody. Specific proteins containing the PSTAIR motif were localized by a secondary immunodetection system as described under "Materials and Methods." The band positions of putative "cdc"-like proteins are marked with arrows.

expression and compartmentation of cyclin-cdc kinase complexes might underlie it (28). Co-immunoprecipitation experiments of this kind suggest that one or more kinase-binding rat liver proteins, the structures of which must contain epitopes displayed by both human cyclins, were synthesized concomitantly with the abrupt appearance of cyclin A and B1 mRNA at 24 h (Fig. 1) or accessed from preformed but inactive pools.

Results in Fig. 4 show that cyclin A and B1 antisera co-immunoprecipitate PSTAIRE-containing HeLa cell proteins of M_r ~32–33 and 33–34 kDa, respectively. The molecular sizes and the presence in these proteins of the cdc kinase-specific motif (27) support previous observations identifying these proteins as p32/p33^{cdc2} and p33/p34^{cdc2}-kinase, respectively (23, 24, 32). When Western blots of liver cytosolic and nuclear extracts were probed with anti-PSTAIRE antibody, three bands (~32–35 kDa) were observed in 24-h samples from both sham and regenerating tissue; these bands were visualized more intensely in nuclear extracts from regenerating tissue (data not shown). When similar blotting experiments were performed by probing liver cyclin A and B1 immunoprecipitates with anti-PSTAIRE antibody, nuclear extracts from 24-h regenerating liver showed evidence of PSTAIRE-containing proteins of M_r ~32–33 and 33–34 kDa, respectively (Fig. 4). These proteins were not easily visualized in parallel cytosolic extracts (although a PSTAIRE-containing protein of M_r ~34–35 was co-immunoprecipitated by cyclin A antisera (Fig. 4), a finding consistent with the relatively lower levels of cyclin-associated H1 kinase activities of cytosolic in comparison with nuclear extracts (Fig. 3).

These observations together provide an intellectual bridge between current understanding of hepatocyte growth factors and their receptor-mediated actions *in vivo* and *in vitro* (1–11), and the increasingly apparent universality of the eucaryotic cell cycle machinery (19–25, 27–33). Currently, eight identified polypeptides stimulate rat hepatocyte proliferation (4). Except for glucagon, the mitogenic actions of which are linked to cyclic AMP-dependent protein kinase A (3–5, 10, 14), all known receptors to these polypeptides contain intrinsic tyrosine kinase activity (4, 34). Two hepatic mitogens, insulin and glucagon, are obligatory for regeneration late in the prereplicative phase ("Signal 2" generators) (7–10); two others, IGF1 and IGF2, might play insulin-like roles at this time (2, 3). Of the other four growth factors, EGF, TGF α , HBGF-I, and HGF, EGF has been shown to act mitogenically together with insulin and glucagon when infused into intact rats (16). Moreover, EGF and TGF α initiate hepatocyte DNA synthesis in quiescent primary cultures (7, 8–11). Because the effects of EGF or TGF α must precede those of insulin and glucagon, EGF-like peptides have been termed "Signal 1" generators (1–4, 7–11). It seems plausible, then, that sequen-

tial effects of hepatocyte growth factors are linked to the expression of different cell cycle genes and gene products. Protein phosphorylation might play several critical roles. Since a cascade of proto-oncogene expression (cFOS/cJUN \rightarrow cMYC \rightarrow p53 \rightarrow pRIG \rightarrow cRAS) is induced following 70% hepatectomy (3, 4, 8), and following treatment of primary quiescent adult hepatocytes with EGF or TGF α , insulin, and glucagon (3, 4, 8, 11), it would appear reasonable to expect that regulatory networks involving proto-oncogene and cyclin gene expression, linked to periodic p32/p33^{cdc2} and p33/p34^{cdc2} activation (21, 24, 27–33) are required for liver regeneration.

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