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PRIMARY RAT HEPATOCYTES EXPRESS CYCLIN D1 MESSENGER RNA DURING THEIR GROWTH CYCLE AND DURING MITOGENIC TRANSITIONS INDUCED BY TRANSFORMING GROWTH FACTOR-ALPHA

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SUMMARY: Cyclin D1 messenger RNA expression was investigated in differentiated proliferation-competent primary cultures of adult rat hepatocytes. Periodic expression was observed during the 12-day growth cycle (≈two population doublings). Upon reaching a quiescent G_0-state, 4-kb cyclin D1 mRNA levels were undetectable. When G_0 cultures were shifted into defined media without or with 0.2-0.8 nM TGF-α, conditions that reinitiate full proliferative transitions synchronously, cyclin D1 mRNA levels were elevated 1.2-4.6-fold, respectively. These findings support the hypothesis that hepatic mitogens stimulate periodic cyclin mRNA expression directly.

Hepatocyte proliferation during rat liver regeneration is an excellent model of animal cell growth control (for reviews, see 1-3). Much of what is known about hepatocellular mitogens like TGFα (4,5) , EGF (6,7), HBGF-I (8,9), HGF/SF (10-12), insulin and glucagon (4,6,7,13,14), including their modes of interaction and their properties of signal transduction, has been obtained from experiments with primary hepatocyte cultures (4-13) coupled with physiological studies *in vivo* (14-18). Defined by assays of growth reinitiation using quiescent "G_0" systems (4,7,19-23), these studies have led to a "two-signal hypothesis" of animal cell growth control (1-3, 19). According to this view, mitogens like EGF or TGFα ("Type I") promote $G_0 \rightarrow G_1$ transitions; mitogens like insulin and glucagon ("Type II") potentiate $G_1 \rightarrow S$ transitions. Complete cell cycle transitions ($G_1 \rightarrow S \rightarrow G_2 \rightarrow M$) are stimulated by the sequential and synergistic effects of both types

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Abbreviations are: TGFα, transforming growth factor-alpha; EGF, epidermal growth factor; HBGF-I, heparin binding growth factor-I; HGF/SF, hepatocyte growth factor/scatter factor; PCNA, proliferating cell nuclear antigen; and, SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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and additional classes of mitogens, and by the attenuated effects of several autocrine and paracrine intrahepatic growth inhibitors (1-5, 23-26).

Recent reports suggest that hepatic proliferation during liver regeneration is governed by the synchronized expression of a group of cyclins (E→C→D1→A→B1/B2) and their associated cell division kinases ("CDKs"; 27-30). Because diurnal variables are not easily eliminated, the *in vivo* observations predict but do not prove that mitogens stimulate cascades of cyclin/CDK expression (27). Since the induction of eukaryotic DNA synthesis and mitosis require the sequential and periodic activation of CDKs by their cyclin partners (31,32), we decided to see if cyclin mRNA expression fluctuates and responds to physiological mitogens when normal hepatocytes are free from *in vivo* diurnal influence. We report here direct evidence of such fluctuations and responses by examining primary cultures of adult rat hepatocytes for cyclin D1 mRNA expression *in vitro*.

METHODS

Animals and surgery. Livers were obtained from Fisher 344 ♂ rats (180-200g). Sham or 67% hepatectomies were performed as described elsewhere (13,22,27).

Long-term primary hepatocyte culture. Freshly isolated hepatocytes were plated and cultured up to 13 days *in vitro* according to standard procedures (7,33). Quantitative measurements of hepatocyte proliferation and DNA replication during the lag, log and stationary phases of constitutive growth cycles, and during synchronized proliferative transitions in quiescent cultures ($G_0 \rightarrow G_1 \rightarrow S \rightarrow G_2 \rightarrow M$) were performed as described elsewhere (7,19-22,33). All measurements were made in triplicate (with errors ±10%).

RNA isolation and Northern analyses. Total and poly(A)$^+$-RNAs were isolated from livers (1-2g) or from pooled cultures (5-8 x 10^6 cells) using standard procedures (4,21,22,27). Experimental or control Northern blot analyses of 4-kb cyclin D1 or 1.9-kb α-tubulin mRNAs, respectively, were performed with random-primer [α-^{32}P]dCTP-labelled cDNA probes and quantified by densitometric or radioisotopic scanning as described elsewhere (22,27).

RESULTS

Mid-G_1 cyclin D1 mRNA expression during rat liver regeneration. Cyclin D1 mRNAs were not observed in quiescent liver (27) or 6h after sham hepatectomy (**Fig.1** ["**IN VIVO**"]). When poly(A)$^+$-RNAs were analyzed, however, small elevations were detected 6h after 67% hepatectomy; and, marked elevations were apparent between 12-24h, as reported previously (27,28). Prereplicative changes were not artifacts caused by sample loading or gel transfers as determined by internal control hybridizations with an α-tubulin probe (data not shown).

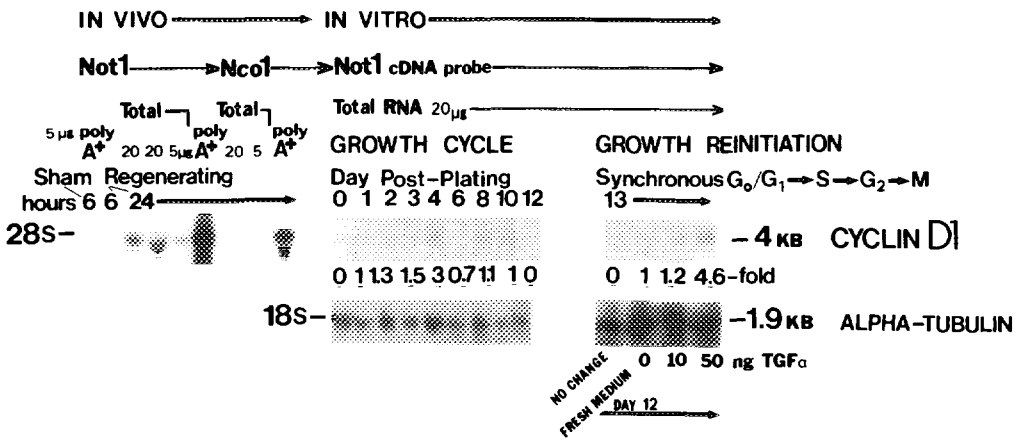

FIG.1. Northern blot analyses of rat cyclin D1 mRNA expression during hepatocellular regeneration *in vivo* and proliferative transitions *in vitro*. Complementary DNA hybridization probes consisted of either a 1245 bp *Not*I insert from pBSKS containing the complete cyclin D1 open reading frame or its derivative *Nco*I cyclin box-specific 604 bp fragment (experimental); or a 1.9-kb *Pst*I insert from pTKα1 containing the coding region of human α-tubulin (internal control). "IN VIVO" RNA samples were obtained from the caudate lobes of animals subjected to sham (6h [1st track at left]) or regenerating (6h [track 2] or 24h post-67% hepatectomy [tracks 3-9]) treatment: both cyclin probes were used (*Not*I: tracks 1-6; *Nco*I: tracks 7-9). Sample quantities loaded per track were either 5 μg poly(A)+ RNA (tracks 1,2,6 and 9) or, as indicated, 5 or 20 μg total RNA (tracks 3,4,5,7 and 8). "IN VITRO" samples (20 μg total RNA per track) were analyzed with *Not*I and α-tubulin probes. Samples were obtained from primary cultures plated under growth cycle stimulating conditions ($1x10^6$ hepatocytes/35 mm dish; standard arginine-free media containing 0.4 mM l-ornithine; supplemented with 10 μg each of insulin, hydrocortisone and inosine per ml and pretested heat-inactivated dialyzed fetal bovine serum [15% v/v]). "**Growth Cycle**" samples were examined between day zero (freshly isolated cell suspensions prior to plating) and 12d post-plating as indicated. On day 12, "**Growth Reinitiation**" assays were performed by shifting cultures into fresh serum- and supplement-free standard media without or with 10 or 50 ng human TGFα. RNA samples from these cultures or from unchanged control dishes were obtained 24h later on d13 as indicated. Normalized relative cyclin D1 mRNA levels (in arbitrary units, where day 1 levels = 1 [refs.22,27]) are indicated numerically ("-fold") and plotted in Fig.2.

Cyclin D1 mRNA expression during hepatocyte growth cycles in primary culture. A typical growth curve for adult hepatocytes *in vitro* is shown in **Fig.2A**. The population doubling time (T_{avg}) was ≈90h. As shown in **Fig.1** ("IN VITRO"), cyclin D1 mRNAs were not detected in isolated hepatocyte suspensions at the time of plating (day 0) or during stationary phase (12-13d); but, they were expressed periodically during the intervening lag and late log phases of the population's asynchronous growth cycle (1-4 and 6-10 days post-plating, respectively). Quantitative measurements of relative cyclin D1 mRNA levels revealed a bimodal curve (**Fig.2A**). It consisted of a sharp "major peak" 4d post-plating, when rates of hepatocyte DNA synthesis, nuclear labelling indices and the proportions of S-phase cells determined by flow microfluorimetry were elevated maximally (7,33-35); and, a broad "minor peak" at the tail-end of log phase between 8-10 days post-plating before quiescence was reached (**Fig.2A**).

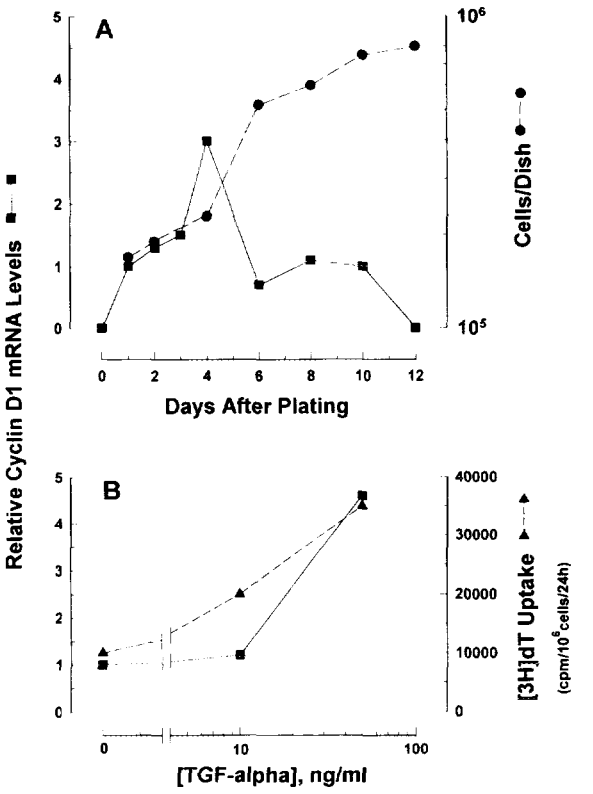

FIG.2. Relative rat cyclin D1 mRNA levels during hepatocellular growth cycles and synchronous proliferative transitions *in vitro*. Quantitative measurements of mRNA levels were made from growth cycle (**panel A**) and growth reinitiation assays (**panel B**) described under Fig.1. The relative cyclin D1 mRNA levels (solid lines) were normalized from internal control measurements of α-tubulin mRNAs. Hepatocyte growth curve determinations were made by standard cell counts (dashed line, **panel A**). DNA synthesis rates in growth reinitiation assays were made on d13 by standard measurements of [^{3}H]dT uptake (7) after a 24h pulse (dashed line, **panel B**). Rates of [^{3}H]dT uptake in unchanged cultures were ≈5000 cpm/10^6 cells/24h).

Cyclin D1 mRNA expression is stimulated by TGFα in quiescent primary culture. When quiescent stationary phase cultures were fluid changed into defined media without or with TGFα, rates of DNA synthesis 24h later (d13) were increased ≈2-fold in the absence of mitogen, as expected (4,7,19), and ≈4-8-fold in its presence over a range of 0.2-0.8 nM (**Fig.2B**). Under these conditions[1], which reinitiate proliferative transitions synchronously with kinetics that simulate regenerative transitions *in vivo* (4,7,20-22), graded increases in cyclin D1 mRNA levels were observed at 24h (**Fig.1** ["IN VITRO"]). Quantitative measurements

[1]Full details of kinetics of reinitiation of hepatocyte DNA synthesis and mitosis by EGF, TGFα and other peptides under similar culture conditions are given in refs. 4, 7, 15-17, 19-21 and 35. The [^{3}H]dT labelling conditions used here integrate the collective entry of cells into the S-phase between 12-24h post-fluid change.

revealed small but detectable (≈1.2-fold) and significantly elevated levels (4.6-fold) of these mRNAs in cultures treated with 0.2 and 0.8 nM TGFα, respectively (Fig.2B).

DISCUSSION

Growth control findings made with primary hepatocyte cultures support the hypothesis that cyclin D1 mRNA expression during liver regeneration is regulated periodically and directly by a Type I mitogen like TGFα. Results from *in vivo* studies indicate further that cyclin D1 mRNA levels are elevated earlier during the prereplicative phase (mid-G_1) than previously supposed (27,28).

These and prior observations, causally linking Na^+-dependent TGFα/EGF receptor-activation of c-FOS expression to hepatocyte cell cycle entry (2-4,21), suggest that receptor-mediated signal transduction events associated with "immediate-early" proto-oncogene expression are required for cyclin D1 mRNA elevations. Recent findings of TGFα-like response elements embedded in the promoter region of the human cyclin D1 gene (36) support this prediction. Unexpectedly, however, $p36^{cycD1}$ appears to be present constitutively in quiescent rat liver (29); and, recent reports suggest that regeneration-associated cyclin D1 mRNA elevations are regulated post-transcriptionally (28,30). Moreover, although the known rat cyclins (37) display "cascades" of elevated mRNA expression during the $G_0 \rightarrow G_1$ (E→C→D1), $G_1 \rightarrow S$ (cyclin A) and $G_2 \rightarrow M$ (B1 and B2) phases of both rat and mouse liver regeneration (27,28), the patterns of $G_0 \rightarrow G_1$ cyclin expression differ from other animal cell systems reported thus far (27-30). It would appear, therefore, that the significance and mechanism of elevating cyclin D1 mRNA expression during the prereplicative phase of liver regeneration remain to be determined.

Complex regulatory mechanisms are also anticipated from studies of obligatory growth controlling roles proposed for cyclin D1 by virtue of its temporal patterns of expression in relation to other cyclins (38), and as a consequence of its physical associations with specific CDKs (39), the Rb tumor suppressor (40), the DNA polymerase-δ regulatory subunit PCNA (41) and two cyclin inhibitors in non-hepatic systems (41,42). In addition, there is reason to believe that obligatory cyclin/CDK-regulatory networks in hepatocytes (43) may enlist alternative tissue-specific forms or tissue-specific patterns of the various cell cycle components or component functions. For example, anti-human CDK2 antisera detect "novel" rat liver CDK2-like proteins with mobilities on SDS-PAGE gels that correspond to CDK3 (M_r ≈35-36 kDa) and PLSTIRE-forms (M_r ≈37 kDa [ref.44])[2]. Possible interactions among these potentially "novel" rat liver CDKs and rat cyclin D1 are unknown. Their candidacy should be considered because of

[2]Unpublished results.

the fact that the predicted physiological kinase partners of rat hepatic cyclin D1 ($p34^{CDK4}$, ref.39) and cyclin G (37) are unknown. Further experiments with quiescent primary hepatocytes should help to unravel and identify these complex regulatory circuits.

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REFERENCES

1. Leffert, H.L., and Koch, K.S. (1977) In Growth, Nutrition and Metabolism of Cells in Culture (G.H. Rothblat and V.J. Cristofalo, Eds.). Vol.III, pp. 225-294. Academic Press, New York.
2. Leffert, H.L., Koch, K.S., Lad, P.J., Shapiro, P., Skelly, H., and de Hemptinne, B. (1988) In The Liver: Biology and Pathobiology (I. Arias, H. Popper, D. Schacter, and D. Shafritz, Eds.) 2nd Edit., pp. 833-850. Raven Press, New York.
3. Koch, K.S., and Leffert, H.L. (1994) J. Tumor Mark. Oncol. **9**, 35-56.
4. Brenner, D.A., Koch, K.S., and Leffert, H.L. (1989) DNA Cell. Molec. Biol. **8**, 279-285.
5. Meade, J.E., and Fausto, N. (1989) Proc. Natl. Acad. Sci. USA **86**, 1558-1562.
6. Richman, R.A., Claus, T.H., Pilkis, S.J., and Friedman, D.L. (1976) Proc. Natl. Acad. Sci. USA **73**, 3589-3593.
7. Koch, K.S., and Leffert, H.L. (1979) Cell **18**, 153-163.
8. Kan, M., Huang, J., Mansson, P.E., Yasumitsu, H., Carr, B., and McKeehan, W.L. (1989) Proc. Natl. Acad. Sci. USA **86**, 7432-7436.
9. Koch, K.S., Lu, X.P., Brenner, D.A., Fey, G.H., Martinez-Conde, A., and Leffert, H.L. (1990) In Vitro Cell. Devel. Biol. **26**, 1011-1023.
10. Miyazawa, K., Tsubouchi, H., Naka, D., Takahashi, K., Okigaki, M., Arakaki, N., Nakayama, H., Hirono, S., and Sakiyama, O. (1989) Biochem. Biophys. Res. Communic. **163**, 967-973.
11. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. (1989) Nature **342**, 440-443.
12. Gherardi, E., Sharpe, M., Lane, K., Sirulnik, A., and Stoker, M. (1993) Symp. Soc. Expt. Biol. **47**, 163-181.
13. Leffert, H.L., Alexander, N.M., Faloona, G., Rubalcava, B., and Unger, R. (1975) Proc. Natl. Acad. Sci. USA **72**, 4033-4036 (1975).
14. Bucher, N., Patel, U., and Cohen, S. (1978) CIBA Found. Symp. **55**, 95-107.
15. Leffert, H.L. and Koch, K.S. (1978) CIBA Found.Symp. **55**, 61-83.
16. Leffert, H.L., Koch, K.S., Moran T., and Rubalcava, B. (1979) Gastroenterology **76**, 1470-1482.
17. Leffert, H.L., Koch, K.S., Lad, P.J., and de Hemptinne, B. (1983) In Handbook Exper. Pharm. (P. Lefebvre, Ed.) Vol.**66**, pp. 453-484. Springer-Verlag, Berlin.
18. Noguchi, S., Ohba, Y., and Oka, T. (1991) J. Endocrinol. **128**, 425-431.
19. Koch, K.S., and Leffert, H.L. (1980) Ann. N.Y. Acad. Sci. **349**, 111-127.
20. Leffert, H.L. and Koch, K.S. (1985) In Growth and Differentiation of Cells in Defined Environment (H. Murakami, I. Yamane, D.W. Barnes, J.P. Mather, I. Hayashi and G.H. Sato, Eds.). pp.9-18. Springer-Verlag, Berlin.
21. Kruijer, W., Skelly, H., Botteri, F., van der Putten, H., Barber, J., Verma, I., and Leffert, H.L. (1986) J. Biol. Chem. **261**, 7929-7933 (1986).

22. Lu, X.P., and Leffert, H.L. (1991) J. Biol. Chem. **266**, 9276-9284.
23. Leffert, H.L., and Weinstein, D.B. (1976) J. Cell Biol. **70**, 20-32.
24. Hayashi, I., and Carr, B.I. (1985) J. Cell. Physiol. **125**, 82-90.
25. Nakamura, T., Arakaki, R., and Ichihara, A. (1988) Expt. Cell Res. **179**, 488-497.
26. Yasuda, H., Mine, T., Shibata, H., Eto, Y., Hasegawa, Y., Takeuchi, T., Asano, S., and Kojima, I. (1993) J. Clin. Investig. **92**, 1491-1496.
27. Lu, X.P., Koch, K.S., Lew, D.J., Dulic, V., Pines, J., Reed, S., Hunter, T. and Leffert, H.L. (1992) J. Biol. Chem. **261**, 2841-2844.
28. Albrecht, J.H., Hoffman, J.S., Kren, B.T., and Steer, C.J. (1993) Am. J. Physiol. **265**, G857-G864.
29. Loyer. P., Glaise, D., Cariou, S., Baffet, G., Meijer, L., and Guguen-Guillouzo, C. (1994) J. Biol. Chem. **269**, 2491-2500.
30. Trembley, J.H., Kren, B.T., and Steer, C.J. (1994) Cell Growth Diff. **5**, 99-108.
31. D'Urso, F., Marraccino, R., Marshak, R., and Roberts, J. (1990) Science **250**, 786-791.
32. "The Cell Cycle" (1991) Cold Spring Harb. Symp. Quant. Biol. **55**, 1-782.
33. Leffert, H.L., Moran, T., Boorstein, R., and Koch, K.S. (1977) Nature **267**, 58-61.
34. Brown, J., Lad, P.J., Skelly, H., Koch, K.S., Lin, M., and Leffert, H.L. (1983) Differentiation **25**, 176-184.
35. Koch, K.S. (1987) Doctoral Dissertation, University of California at San Diego. pp. 1-406.
36. Motokura, T., and Arnold, A. (1993) Genes Chrom. Canc. **7**, 89-95.
37. Tamura, K., Kanaoka, Y., Jinno, S., Nagata, A., Ogiso, Y., Shimizu, K., Hayakawa, T., Nojima, H., and Okayama, H. (1993) Oncogene **8**, 2113-2118.
38. Motokura, T., and Arnold, A. (1993) Curr. Opin. Genet. Dev. **3**, 5-10.
39. Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J.Y., Hanks, S.K., Roussel, M.F., and Sherr, C.J. (1992) Cell **71**, 323-334.
40. Dowdy, S.F., Hinds, P.W., Louie, K., Reed, S.I., Arnold, A., and Weinberg, R.A. (1993) Cell **73**, 499-511.
41. Xiong, Y., Zhang, H., and Beach, D. (1992) Cell **71**, 505-514.
42. Toyoshima, H., and Hunter, T. (1994) Cell **78**, 67-74.
43. Zindy, F., Lamas, E., Chenivesse, X., Sobczak, J., Wang, J., Fesquet, D., Henglein, B., and Brechot, C. (1992) Biochem. Biophys. Res. Communic. **182**, 1144-1154.
44. Meyerson, M., Enders, G., Wu, C., Su, L., Gorka, C., Nelson, C., Harlow, E., and Tsai, L. (1992) EMBO J. **11**, 2909-2917.