Growth Factor Dependence of Progression through G₁ and S Phases of Adult Rat Hepatocytes in Vitro

EVIDENCE OF A MITOGEN RESTRICTION POINT IN MID-LATE G₁*

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Several hepatocyte mitogens have been identified, but the signals triggering the G₀/G₁ transition and cell cycle progression of hepatocytes remain unknown. Using hepatocyte primary cultures, we investigated the role of epidermal growth factor/pyruvate during the entry into and progression through the G₁ phase and analyzed the expression of cell cycle markers. We show that the G₀/G₁ transition occurs during hepatocyte isolation as evidenced by the expression of early genes such as c-fos, c-jun, and c-myc. In culture, hepatocytes progress through G₁ regardless of growth factor stimulation until a restriction point (R point) in mid-late G₁ beyond which they cannot complete the cell cycle without mitogenic stimulation. Changes in cell cycle gene expression were associated with progression in G₁; the cyclin E mRNA level is low early in G₁, but increases at the G₁/S boundary, while the protein is constantly detected during cell cycle but undergoes a change of electrophoretic mobility in mid-late G₁ after the R point. In addition, a drastic induction of cyclin D1 mRNA and protein, and to a lesser extent of cyclin D2 mRNA, takes place in mitogen-stimulated cells after the R point. In contrast, cyclin D3 mRNA appears early in G₁, remains constant in stimulated cells, but accumulates in unstimulated arrested cells, paralleling the cyclin-dependent kinase 4 mRNA expression. These results characterize the different steps of G₁ phase in hepatocytes.

In the normal liver, hepatocytes can remain for very long periods in a quiescent G₀ state. However, they have the capacity to proliferate after chemical injury or partial surgical resection of the liver (Higgins and Anderson, 1931). Following a two-thirds hepatectomy (PHT), hepatocytes rapidly enter the cell cycle and begin their first round of DNA replication 18–20 h later (Fabrikant, 1968). An active field of research during the last 15 years dealt with the identification of factors able to promote hepatocyte DNA synthesis and to understand this compensatory growth. It has been known for some years that hepatocyte growth factor (HGF) and transforming growth factor-α (TGF-α) are primary mitogens during liver regeneration after partial hepatectomy or administration of CCl₄ (Mead and Fausto, 1989; Michalopoulos, 1990). Mullhaupt et al. (1994) recently reported a rapid increase of EGF levels in the immediate early phase of liver regeneration. Furthermore, HGF, TGF-α, and EGF are well characterized mitogens for hepatocytes in primary culture. However, how the hepatocyte cell cycle is controlled by these external factors remains to be clarified.

It is well established that, in vivo, normal hepatocytes are largely unresponsive to growth factors and become competent only after “priming” induced by specific treatments such as partial hepatectomy, necrosis following injury, metabolic stress, or any phenomenon leading to disruption of cell-cell contacts (Etienne et al., 1988; Sawada, 1989; Ikeda et al., 1989) or digestion of the extracellular matrix (Liu et al., 1994). These metabolic events would trigger the G₀/G₁ transition of hepatocytes in vivo and increase the expression of growth factors, which then induce DNA synthesis. This hypothesis is based on the fact that induction of immediate-early oncogenes such as c-fos or c-jun (Corral et al., 1985; Thompson et al., 1986; Sobczak et al., 1989; Morello et al., 1990), takes place 20–30 min after PHT, while HGF level rises around 2 h post PHT (Lindroos et al., 1991). Previous in vitro studies have also shown that hepatocytes express immediate-early oncogenes during cell isolation and in primary culture, in the absence of mitogens. On the other hand, it was clearly established that these “primed” hepatocytes would undergo DNA synthesis only when they are stimulated by growth factors (McGowan, 1986; Sawada 1989). We therefore decided to determine whether unstimulated hepatocytes are blocked at a given point in G₁, and how stimulated hepatocytes modulate their response to different mitogenic signals in terms of DNA synthesis.

The G₁ phase has been divided into subphases (Pledger et al., 1977, 1978; Tushinski and Stanley, 1985) during which external signals must impinge on the machinery that regulates the G₁ to S phase transition. In fibroblasts, Pardee (1992) has also described a point in G₁ at which the cells have acquired growth factor independence. He called it the restriction point or R point. It was located near but not concomitant with the initiation of S phase. However, comparison of the data obtained from different in vitro models clearly emphasizes the notion that there are several different regulatory points in G₁ and that each cell type would be defined by a specific behavior in G₁ characterized by its own check point(s) (Pardee, 1992).

Many attempts have been made to identify the proteins which control the progression of the cell cycle through these G₁ check points. Of the proteins characterized to date, the cyclin-dependent kinases (Cdks) and their cyclin partners play a...
crucial role in cell cycle regulation (Sherr, 1993). Cyclins bound to Cdc2 or Cdk2 appear to be involved in regulating DNA initiation and/or synthesis (Pagano et al., 1992; Zindy et al., 1992) and G2/M transition (Pagano et al., 1992; Sherr, 1993), respectively. The cyclin E-Cdk2 complex is activated at the end of G1 and is considered to be a limiting step at the G1/S boundary (Koff et al., 1991; Dulic et al., 1992; Sherr, 1993). The D type cyclins also play a crucial role in G1, via their association with Cdk2, Cdk4, and Cdk6 (Xiong et al., 1992; Baldin et al., 1993; Quelle et al., 1993; Meyerson and Harlow, 1994). The sequential activation of these complexes and their substrate specificities could be the key to their regulatory function throughout the G1 phase (Aïchenbaum et al., 1993; Sherr, 1993).

Analysis of the expression and activation of Cdc2 and Cdk2 in regenerating liver revealed that Cdc2 was expressed and active in S, G2, and M phases but not in G1, whereas Cdk2 was constantly expressed during the cell cycle but inactive in G1 (Loyer et al., 1994). The expression and role of the different cyclins and their corresponding Cdk partners in the hepatocyte cell cycle is poorly documented.

In the present report, we used primary cultures of normal rat hepatocytes that were unstimulated or stimulated by EGF to define the different subphases of G1 and to examine the related expression of various cell cycle markers including proto-oncogenes, cdks, and cyclins.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The anti-p34^cdc2^ is a polyclonal antiserum specifically directed against C-terminal part of human p34^cdc2^ (Pagano et al., 1992). The anti-cyclin E is a rabbit polyclonal antibody directed against the total protein of the Xenopus cyclin E protein (from M. Philippe, Rennes, France). The anti-cyclin D1 is a monoclonal antibody generated against bacteria-cultured recombinant D1 type cyclin (from C. Sherr, Memphis, TN). For all the Western blot analyses, bands of molecular weights, similar to those found in previous reports using the same antiserum, have been obtained.

**cDNA Probes**—The RNA blots were analyzed with the murine cdck2 cDNA, kindly provided by Dr. P. Nurse (London). Murine cDNAs of cyclins, D1, D2, D3, E, and cdks and cdk4 have been provided by Drs. M. Roussell and C. Sherr (Memphis, TN). Human cyclin A cDNA (Wang et al., 1990) was obtained from Dr. C. Bréchot (Paris). Murine cDNAs of c-jun, junB, and junD (Hirai et al., 1989) have been provided by Dr. C. Babinet (Paris). We obtained p53 (Caron de Forestele et al., 1987) from Dr. D. Galibert, cyclin A cDNA of mouse (Batey et al., 1983) and cyclin D1 (Chang et al., 1982) from Dr. J. Kruh (Paris). Rat albumin cDNA (Sargent et al., 1979) and 18 S rRNA (human genomic 5.7-kb EcoRI fragment) were used as controls.

**Cell Isolation and Culture**—Hepatocytes from adult male Sprague-Dawley rats, weighing 180–200 g, were isolated by a two-step collagenase perfusion procedure as described previously (Guguen et al., 1975). Hepatocytes were seeded at 7.5 × 10^4 cells/cm^2 on plastic dishes or 75-cm^2 flasks for total RNA preparation, in a mixture of 75% minimum essential medium and 25% medium 199, supplemented with 10% fetal calf serum, and per ml: 100 IU of penicillin, 100 μg of streptomycin sulfate, 1 mg of bovine serum albumin, and 5 μg of bovine insulin. After cell attachment (4 h later), the medium was renewed with the same medium deprived of fetal calf serum and supplemented with 7 × 10^−7 M hydrocortisone hemisuccinate. It was renewed every day thereafter.

**Cyclin D1**—The concentration of coupled p9 was 3 mg/ml of gel. The p9 beads used in this study are the same as those described by Azzi et al. (1992). These p9 beads are largely used to affinity purify both Cdc2 and Cdk2 from cell extracts. They have been used here for Western blots or measurement of kinase activity of the two Cdns using histone H1 as substrate.

**Gels** were dried prior to exposure to hyperfilm MP.

**RESULTS**

Permanent EGF / Pyruvate Stimulation induces DNA Synthesis in Most Rat Hepatocytes in Primary Culture—Normal rat hepatocytes were exposed to EGF/pyr just after seeding and all throughout culture. DNA synthesis was assessed by measuring either [^3H]methylthymidine or BrdU incorporation over a 4-day period. In stimulated cultures, no [^3H]methylthymidine incorporation was observed during the first 2 days; DNA replication started after 48 h, reached a maximum at 78 h, and then rapidly decreased (Fig. 1). In control cultures, incorporation was very low. BrdU incorporation analysis revealed that DNA replication occurred in 70–80% of the hepatocytes in EGF/pyr culture conditions between 48 and 120 h (data not shown). These data demonstrated that DNA synthesis was induced in most of the hepatocytes in the presence of EGF/pyr.
Hepatocytes Spontaneously Enter the G1 Phase during Enzymatic Liver Disruption by Collagenase Perfusion—Using Northern blot analysis, we examined the expression of c-fos, c-jun, and c-myc mRNA levels in hepatocytes during isolation and establishment of primary culture (Fig. 2). mRNA levels of these proto-oncogenes were basically undetectable in normal liver and during Hepes washing. However, levels of both c-fos and c-jun mRNAs rapidly increased during collagenase perfusion, reached a maximum in freshly isolated hepatocytes, and drastically decreased thereafter, while c-myc mRNAs appeared as a faint band in freshly isolated hepatocytes and increased in amount in cultured cells only 4 h after plating.

Completion of G1 Phase and Transition to S Phase Require a Mitogenic Signal—In order to investigate the ability of hepatocytes to progress through G1, we examined by Northern blot analysis the mRNA levels of the proto-oncogenes, junB, junD, c-myc, and c-Ki-ras, and the p53 anti-oncogene. Their expression was found to be induced in cultured hepatocytes (Fig. 3). A few hours after c-fos, c-jun, junB, junD, and c-myc mRNA levels increased in isolated cells and reached a maximum 6 h after plating, p53 mRNAs were detected 24 h after plating and finally, c-Ki-ras mRNAs at 48 h. This orderly sequence of events allowed us to distinguish the different steps in G1 phase as immediate-early, early, and mid G1. These results were compared to the albumin mRNA level which was very high in normal liver and in freshly isolated hepatocytes and greatly decreased in primary culture as previously reported (Guguen-Guillouzo et al., 1983).

We next addressed the question whether the unstimulated as well as the EGF-stimulated cells were able to progress through G1, by analyzing the mRNA levels of c-myc and p53 which were used as markers of early and mid G1 respectively. The two cultures expressed c-myc mRNA at similar levels at the same time. However, at day 3, both mRNAs gradually disappeared in EGF-treated cells (Fig. 4A).

We used Cdc2 and the histone H1 kinase activity associated with p9-beads, as markers to see whether unstimulated and EGF/pyr-stimulated hepatocytes in culture underwent the G1/S transition (Fig. 4, A and B). We also analyzed the cdc2 expression at the mRNA level. In the absence of EGF/pyr, cdc2 transcripts were detected as a faint band, if any (Fig. 4A). In contrast, in the continuous presence of EGF/pyr, cdc2 mRNA was detected between 48 and 54 h post plating and was highly expressed at 72 h, while the protein was first detected at 60 h and maximally expressed at 84 h (Fig. 4B). Cdc2 binds to p9 regardless its phosphorylation status or association with cyclins. Therefore, the Cdc2 Western blot after p9 purification represented the real Cdc2 expression in total cell lysates. The kinase activity, measured by the ability of the Cdc2-related proteins (Cdc2 and Cdk2) to phosphorylate histone H1, was detected from 60 h and reached a maximum between 72 and 84 h, correlating mainly with S and M phases as we previously observed in regenerating liver (Loyer et al., 1994).

These results led us to conclude that, under conditions of continuous EGF/pyr stimulation, most hepatocytes reached the G1/S boundary approximately 58–60 h after seeding. In contrast, in the absence of mitogenic factor, hepatocytes entered the G1 phase, progressed up to mid-G1 but failed to complete the G1/S transition.

A Mitogen-associated Restriction Point Is Located in Mid-late G1—To determine the point in G1, beyond which hepatocytes could not progress through without a mitogenic signal, we evaluated the ability of these cells to respond to a 24-h exposure to EGF/pyr, depending on whether this stimulation started immediately, 6, 24, 48, or 72 h after cell plating. For each treatment, DNA synthesis was monitored over 5 days and compared to that in unstimulated cultures (Fig. 5). Three main observations could be made: 1) the total amounts of [3H]meth-

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**Fig. 1.** Time course of [3H]methylthymidine incorporation into DNA in unstimulated and EGF/pyr-stimulated primary hepatocyte cultures. Hepatocytes were maintained under unstimulated conditions ( ) or in the presence of EGF/pyr ( ). Cultures were incubated with 2 μCi/ml [3H]methylthymidine (2-h period) either at seeding or every 8 h for 48 h; and thereafter every 2 h for 94 h. EGF/pyr was added just after cell seeding and every day thereafter with renewal of medium. Cultures were made in duplicate.

**Fig. 2.** Levels of c-jun, c-fos, and c-myc mRNAs in hepatocytes during liver dissociation and in primary culture. Total RNAs (20 μg) extracted from normal liver (1), liver after Hepes perfusion (2), or after collagenase perfusion (3); hepatocytes after the first (4) and the second wash in Hepes buffer (5); freshly isolated hepatocytes (6) and hepatocytes 4 h after seeding (7) were analyzed by Northern blot hybridization with cDNA probes for c-jun, c-fos, c-myc, albumin (Alb), and 18 S as a control of the total amounts of RNAs in each lane.
ylthymidine incorporation varied according to the culture conditions. In cultures stimulated during the first 24 h, DNA synthesis was low; it greatly increased in cells stimulated at 24 h, reached a maximal level in cells stimulated at 48 h, but significantly decreased in cells stimulated at 72 h; 2) the peak of thymidine incorporation was either poorly defined in cells stimulated early after seeding (6 h), or maximally abrupt in cells submitted to late stimulation in culture; and 3) cell entry in S phase occurred at approximately 55 to 60 h in hepatocytes stimulated at 6 h (cell spreading) or at 24 h. In contrast, DNA synthesis began only at 68–70 h and 95–98 h in cells exposed to EGF/pyr at 48 and 72 h, respectively.

These data clearly show that hepatocyte entry in S phase could be delayed by late mitogenic stimulation. The results also suggest that the cells were blocked and arrested at an R point presumably located between 24 and 48 h. In addition, they indicate that hepatocytes remained responsive to EGF/pyr treatment for at least three days of culture.

In order to address the question whether different growth factors, known to be overproduced during liver regeneration process, were like EGF, capable of overriding the hepatocyte block in mid-late G1, we compared the DNA synthesis of primary cultures stimulated by EGF/pyr, TGF-α/pyr, and FCS/pyr. Four h after seeding, cells were maintained in a mitogenic factor-free medium for two days and then, stimulated by EGF/pyr, TGF-α/pyr, or FCS/pyr for a 24 h period. An active DNA replication simultaneously started in the EGF- and TGF-α-stimulated cultures approximately 64 h after seeding and displayed similar kinetics, with maximum DNA synthesis occur-
ring 80–88 h post seeding. In contrast, FCS did not significantly induce DNA synthesis (Fig. 6, inset).

Cell DNA Replication in Response to EGF/Pyr Depends on the Length of Stimulation and on the Location of the Cells in the G1 Phase—To further analyze growth factor dependence of adult hepatocytes in their progression through S phase following EGF/pyr stimulation, we determined the different levels of DNA synthesis obtained in cells exposed to EGF/pyr at 48 h of culture and for increasing time periods from 1 to 42 h. Fig. 7 shows that \[^3H\]methylthymidine incorporation occurred with only 1-h exposure, but the level was very low; then it increased with longer exposures, reached a maximum for the 24-h time period, and remained at the same level upon longer stimulation, at least up to 42 h. This indicated that a 18–24-h period represents the time of EGF/pyr stimulation needed to induce maximal DNA replication.

Differences in the amounts of \[^3H\]methylthymidine incorporated into the cells were also correlated with the time of culture at which the mitogenic factor was added (see Figs. 5 and 6), suggesting that the hepatocyte response to EGF/pyr could vary during the G1 phase. Indeed, when EGF/pyr was added for a 24-h period at different times after seeding, the DNA synthesis was low in cultures stimulated just after plating and increased for later stimulations, and the maximal incorporation was observed when stimulation took place at 36 or 42 h, near the R point (data not shown).

These data indicate that the ability of hepatocytes to enter S phase, in response to mitogen, varied according to the length of stimulation and the location of the cells in G1. This led us to consider the possibility that these variations could be associated with changes in the major cell cycle control proteins which regulate the G1 progression.
A Drastic Increase of Cyclin D1 Accompanies the R Point Overcrossing in Mid-late G1—We examined the expression of different Cdk's and cyclins associated with the G1 phase, in both hepatocytes stimulated by EGF/pyr at 24 h of culture and unstimulated cells (Fig. 8A). Of the Cdk proteins analyzed, the following observations were made: 1) cdc2 mRNAs became detected only at around 54 h (the beginning of S phase) in stimulated cells; 2) cdk2 mRNAs were constantly expressed throughout G1 independently of the mitogen addition, but there was a gradual increase in mRNA expression from 30 h in mid-G1, to 60 h in S phase; and 3) cdk4 transcripts were expressed early (18 h) and did not vary significantly in amounts through the G1 and S phases. Interestingly, only the cdk4 transcripts were much more abundant in unstimulated cells than in their EGF-stimulated counterparts. Of the cyclins analyzed, three different kinetics of expression were found: 1) cyclin A transcripts were barely detectable in both unstimulated and mitogen-treated cells in G1, but drastically increased between 54 and 60 h in stimulated cells only, a time corresponding to the beginning of S phase; 2) cyclin D3 mRNAs were detected throughout G1 with an increase in the S and G2 phases, and unexpectedly, accumulated in the absence of mitogen; and 3) cyclin D1 mRNAs appeared as a faint band from early to mid-G1 but drastically increased in amounts at 42 h in mitogen-stimulated hepatocytes, a time corresponding to the R point, and then remained at high levels for the remaining time course studied. The kinetic of cyclin D2 expression in G1 resembled that of cyclin D1 with a significant increase at 42 h.

Cyclin E mRNAs were detected at a low level during G1 but their level increased near the G1/S boundary, in the presence of mitogen. The transient exposure to FCS during cell attachment had no incidence on the expression pattern of the different proteins studied in both unstimulated and stimulated cultures.

To study the correlation between mRNAs and corresponding proteins we further analyzed the expression of cyclins D1 and E by Western blotting (Fig. 8B). These two cyclins were chosen since their mRNA levels were increased at two different times of G1 phase, respectively the R point (mid-late G1) and the G1/S boundary. Cultures were performed following the same protocol as in Fig. 8A. Cyclin D1 was very low in unstimulated cultures. In stimulated cells, it strongly increased at around 60 h and until 96 h, appearing shortly after the induction of the mRNAs. Cyclin E was expressed in both unstimulated and stimulated cultures with very low quantitative variation but displayed a shift in electrophoretic mobility in mitogen-stimulated cells only.

To determine whether induction of cyclin D1 was associated with the ability of the cells to progress in late G1 after the R point, hepatocytes were exposed to EGF/pyr for a 24-h period as early as 6 h or 24 h after plating, or as late as 48 or 60 h (Fig. 9). These late stimulations resulted in a delayed transition to S phase, as expected by data shown in Fig. 5. The amounts of cyclin D1 mRNAs drastically increased in all conditions, and their location in G1 and the levels of the peaks of expression varied according to the stimulation: when cells were stimulated at 6 h, the peak was clearly seen at 24–30 h, but at a low rate because of the poor synchrony and the lower number of cells, which progressed up to S phase in these conditions. The maximal expression was observed for exposition to the mitogen at 48 h, a condition corresponding to maximal DNA synthesis. The occurrence of induction was always located after mitogenic stimulation and, therefore, was delayed in cultures late exc-
It is generally assumed that division of mammalian cells is mainly controlled during the G<sub>1</sub> phase by signals from the external environment (Pardee, 1992) varying from one cell type to another, defining for each cell type, different characteristic checkpoints. In normal liver, in vivo, hepatocytes are arrested in G<sub>0</sub>. One hallmark of the G<sub>0</sub>/G<sub>1</sub> transition is the sequential overexpression of immediate early and early proto-oncogenes such as c-fos, c-jun, c-myc, and p53 (Corral et al., 1985; Thompson et al., 1986; Sobczack et al., 1989; Morello et al., 1990). However, the factors which control this transition remain poorly understood. One hypothesis is that hepatocyte re-entry into G<sub>1</sub> after PHT is a consequence of metabolic changes regardless of growth factors (Corral et al., 1987; Fausto, 1992). These metabolic changes could be associated with alterations in cell-cell interactions (Etienne et al., 1988). Also the possibility that early activation of a growth factor occurs via a cascade of events related to alteration of the extracellular matrix, cannot be ruled out. Indeed, Liu et al. (1994) have shown that partial degradation of the extracellular matrix of the liver in vivo could also trigger the G<sub>0</sub>/G<sub>1</sub> transition.

It may be argued that normal rat hepatocytes, when seeded in culture, have already entered the early G<sub>1</sub>. In this study, we show that they also progress up to mid-late G<sub>1</sub>, in the absence of growth factor and serum in the medium. This is consistent with the sequential overexpression of the early G<sub>1</sub> oncogenes c-fos and c-jun during collagenase perfusion, followed by c-myc and junB expression, 4–6 h after seeding, and finally, of c-Ki-ras and p53 expression, after 24 h of culture. In addition, in the absence of mitogen signal, these cells were found to be arrested in mid G<sub>1</sub> and further progression to the G<sub>1</sub>/S boundary was strikingly dependent on growth factor addition. This was supported by the following observations: 1) in the absence of mitogen, cyclins D1 and D2 were low, cyclin A and Cdc2 were not expressed, and DNA synthesis was not observed; and 2) the onset of DNA synthesis was delayed by late addition of EGF on days 2 and 3, and a sharp peak of labeling was observed in these conditions, reflecting the high synchrony of the hepatocyte population arrested at the R point.

From our data, we estimate that the mitogen-dependent R point in rat hepatocytes occurs at the end of the first two-thirds of the G<sub>1</sub> phase, approximately 42–48 h after seeding under our conditions. Considering effects of early and late exposure of hepatocytes to EGF/pyr, the lag time between the R point and the onset of DNA synthesis appears to be approximately 18–20 h. This R point has some similarities with the start point of the yeast G<sub>0</sub>/S transition; in both systems, the R point occurs in mid G<sub>1</sub> close to but distinct from the G<sub>1</sub>/S boundary, and both are dependent on external signals (Reed, 1992).

In fibroblasts, G<sub>1</sub> progression requires growth factors (Campisi et al., 1982; Croy and Pardee, 1983), but after the R point, they become growth factor-independent (Yen and Pardee, 1978; Zettenberg and Larsson, 1985). In addition, fibroblasts require at least two factors, platelet-derived growth factor and EGF or insulin-like growth factor, to stimulate the transition of quiescent cells from G<sub>0</sub> to G<sub>1</sub> and from G<sub>1</sub> to S phases, respectively. In contrast, in murine macrophages, one growth factor, the colony-stimulating factor 1, is sufficient to induce transition from quiescence to S phase (Tushinski and Stanley, 1985). Moreover, in this model, colony-stimulating factor 1 must be present throughout G<sub>1</sub> in order to maintain cell cycle progression until S phase. Here we demonstrate that, for cell cycle progression of hepatocytes, growth factor is required in G<sub>1</sub>, for a while, to override the R point. The fact that G<sub>0</sub>/G<sub>1</sub> transition of hepatocytes occurs spontaneously during collagenase perfusion and that G<sub>1</sub>/S transition takes place only...
after stimulation of the cells by growth factors strongly argues for a dual factor requirement to trigger these two transitions. A first unidentified factor, synthesized and/or secreted or released from the extracellular matrix (Liu et al., 1994) during the tissue disruption, would induce G0/G1 transition but not G1/S transition. This last step would require a growth factor acting later in mid-late G1. Taken together, these results define a specific behavior of hepatocytes regarding the cell cycle progression in G1 in relation with growth factor stimulation.

It is generally assumed that G1 cyclins and their corresponding Cdk(s) are integrators of growth factor-mediated signals that drive the cell cycle. However, with the exception of three studies (Lu et al., 1992; Zindy et al., 1992; Albrecht et al., 1995), there is no report describing the kinetic expression of different G1 proteins in hepatocytes and their association with different steps in G1, has never been defined in these cells. We confirm the expression of cyclin A and Cdc2 at the S phase entry and showed that cdk2 mRNA is expressed throughout G1 with an H1 kinase activity was mainly detected in S and M phases. The cyclin E mRNAs were found as a weak band in unstimulated culture and their level greatly increased in late G1 only after mitogenic stimulation. It may reflect an activation of this cyclin only in cells which have overcrossed the R point. In addition, cyclin E Western blot was performed on p9-Cdk-cyclin affinity purified complexes. Since this protein does not bind p9 directly but via its binding to a Cdk, likely Cdk2 as reported by Koff et al. (1991) and Dulic et al. (1992), we may assume that the kinetic corresponded to the levels of cyclin E complexed with Cdk2 along the cell cycle. We did not find significant variation in the level of cyclin E protein, but interestingly, a change in the electrophoretic mobility, probably related to a modification of its phosphorylation status, was evidenced in mid-late G1 after mitogenic stimulation. This likely reflects an activation of the complex in cells which have overcrossed the R point.

In contrast, we show that cyclin D1 mRNA levels and protein expression are both greatly increased after mitogenic stimulation. Accumulation of mRNAs correlated with the R point onset, whereas the cyclin D1 protein was detected 10–15 h later. Accumulation of mRNAs correlated with the R point on- ward progression of cyclin D3, cdk4, and to a lesser extent cdk2 mRNAs, in the absence of mitogen stimulation. The role of these cyclins accumulated in unstimulated cells must be further analyzed.

Altogether, these observations are consistent with the idea that the D-type cyclins may be multifunctional regulators that could target different Cdk partners (Sherr, 1993, 1994). Experiments are now in progress to determine which of these Cdns directly associate with the cyclins to form complexes that serve to link growth factor stimulation with cell cycle progression of hepatocytes and which activators or inhibitors may control this process.

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