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cAMP-Dependent Positive Control of Cyclin A2 Expression during G1/S Transition in Primary Hepatocytes

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cAMP positively and negatively regulates hepatocyte proliferation but its molecular targets are still unknown. Cyclin A2 is a major regulator of the cell cycle progression and its synthesis is required for progression to S phase. We have investigated whether cyclin A2 and cyclin A2-associated kinase might be one of the targets for the cAMP transduction pathway during progression of hepatocytes through G1 and G1/S. We show that stimulation of primary cultured hepatocytes by glucagon differentially modulated the expression of G1/S cyclins. Glucagon indeed upregulated cyclin A2 and cyclin A2-associated kinase while cyclin E-associated kinase was unmodified. In conclusion, our study identifies cyclin A2 as an important effector of the cAMP transduction network during hepatocyte proliferation.

Key Words: **hepatocytes; cell cycle; cyclin A2; cyclic AMP; DNA synthesis.**

In mammals, cell division is monitored by a complex biochemical machinery in which Cyclin Dependent Kinases (CDKs) are sequentially activated by a variety of cyclins. Consecutive activation of cyclin/CDK complexes thus allows progression through critical steps of the cell cycle (checkpoints) by phosphorylation of specific sets of substrates involved in different phases of cell division. Cyclin A2 is accumulated in somatic cells from the end of G1 and persists until metaphase of mitosis. It binds to and thus activates CDK2 in G1 and S phases and CDK1 (CDC2) in G2 and M (for a review, see references (1, 2)). These cyclin A2-dependent kinases are required respectively for the G1/S and the G2/M transitions, as both transitions were inhibited when an antisense cDNA construct or antibodies directed against cyclin A2 were microinjected in G1 or G2 cells (3, 4). Cyclin A2 expression is primarily controlled at the transcriptional level. A region called CDE–CHR (for cell cycle dependent element and cell cycle homology region, respectively) appears to be responsible for the promoter downregulation in quiescent fibroblasts and during the G1 phase (5). A CREB/ATF binding site, located immediately upstream the CDE, has also been implicated in the promoter downregulation in response to TGF-β1 in contact inhibited endothelial cells (6, 7) and in hamster fibroblasts (8). In the latter case, cAMP was shown to act in synergy with TGF-β1 to down-regulate the cyclin A2 promoter through the CREB/ATF site (8). We have previously shown that the CREB/ATF site is also responsible for the cAMP inducibility of the cyclin A2 gene in human fibroblasts (9). Since this inducibility is cell cycle dependent and occurs specifically from mid-G1 to early S phase, we suggested that cAMP could positively control cyclin A2 expression at the end of G1 in these cells.

Recently, other connections between the cAMP transduction network and the cell cycle machinery have been uncovered. Upon treatment with cAMP, a G1 arrest is observed in macrophages, fibroblasts and lymphocytes stimulated with mitogens. The molecular mechanism underlying this arrest is the inhibition of CDKs operating in G1, by down-regulation of cyclin D1 in fibroblasts and lymphocytes (10) or accumulation of the CDK inhibitor p27 in macrophages (11). However, cAMP is known to affect in both positive and negative ways proliferation in a variety of cell types (12–14), among which one of the best example is the rat hepatocyte. In cultured primary hepatocytes, cAMP exerts a bidirectional effect, depending on the concentration of the cAMP-elevating agent and the time of addition (15, 16). Thus, dissecting the molecular events triggered by cAMP should allow a more comprehensive comparison between the mechanisms controlling normal cell proliferation and those operating in neoplastic growth.

The experiments presented here were designed to better understand the growth-regulatory effects of cAMP in rat hepatocyte proliferation. Our results dem-

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onstrate that, in cultured primary rat hepatocytes, the cAMP-elevating hormone glucagon upregulates cyclin A2 in the prereplicative period. Increasing the cAMP level differentially modulated cell cycle regulatory components accumulation since cyclins D1 and E levels were not modified, while cyclin A2 and cyclin D3 were upregulated under those conditions.

MATERIALS AND METHODS

Isolation and culture of hepatocytes. Male Wistar rats (150–220 g) (IFFACREDO, France) were used. Animals were treated according to the European Community laws for animals care. Rats were maintained on standard light/dark cycles and fed *ad libitum*. Hepatocytes were isolated by *in vitro* collagenase perfusion and low speed centrifugations (17), with modifications as previously described. Cell viability measured as the ability to exclude trypan blue, was 90–97%. The cells were suspended in culture medium and plated (20,000/cm^2) essentially as described (18). The culture medium used was a serum free 1:1 combination of Dulbecco's modified Eagle's medium and Williams medium E with a final glucose concentration 8.4 mM. The medium was bicarbonate-buffered and supplemented with penicillin (67 μg/mL) and streptomycin (100 μg/mL). Insulin (100 nM), dexamethasone (25 nM) and collagen (3 μg/mL) were added at the time of plating (time 0 h), EGF (5 nM) was added at 24 h and glucagon (1 or 10 nM) was added at 3 h. The cultures were kept in 95% air/5% CO_2 at 37°C.

Measurement of DNA synthesis. [^{3}H]Thymidine was added to the cultures (1 μCi/mL, 0.125 Ci/mmol), at different times after plating. The DNA synthesis was measured as the cumulative amount of radioactivity incorporated into DNA (12 h after adding). The cellular material was dissolved with 1 mL 0.5 N NaOH for 3 h at 37°C, collected, mixed with 1.5 mL H_2O, and precipitated with 0.5 mL 50% trichloroacetic (TCA). The acid-precipitable material was transferred to glass fiber filters (GF/C Whatman) and washed twice with 5 mL 5% TCA, followed by liquid scintillation counting of the filters. Results were expressed as cpm incorporated per milligram of total protein.

To determine the percentage of cells in S phase, hepatocytes were incubated with BrdU labeling medium for 12 h. The medium was then removed and the cells washed three times with PBS and fixed with 30:70 acetic/ethanol solution at −20°C for 30 min. The dished were washed three times with 1× PBS and store at +4°C until revelation. The Detection Kit II procedure was used for revelation (Amersham). The percentage of BrdU incorporation was obtained by counting the cells with positive staining in the nucleus compared to the negative cells.

Western blot analyses. Protein extracts were prepared in lysis buffer containing 50 mM Tris pH 7.5, 250 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 2 μg/mL leupeptin, pepstatin and aprotinin. After 15 min on ice, the extracts were centrifuged and supernatants were stored at −70°C. Protein concentration was determined by the Bradford method (Bio-Rad); 20 μg of protein were fractioned on SDS–PAGE and then transferred to PVDF membranes (Millipore). Individual proteins were detected using specific primary antisera (incubation overnight at 4°C): cyclin A2 (C19), cyclin E (M20) and cyclin D3 (C16) were from Santa Cruz Biotechnology (used at 0.25 μg/mL); antisera against cyclin D1 (06–137) was from Upstate Biotechnology (used at 0.25 μg/mL). Horseradish peroxidase-conjugated antibodies (Amersham) were used as second antibodies (dilution 1:2000, incubation for 1 h at room temperature). Immunoreactive bands were visualized with enhanced chemiluminescence (ECL, Amersham) according to the manufacturer's instructions.

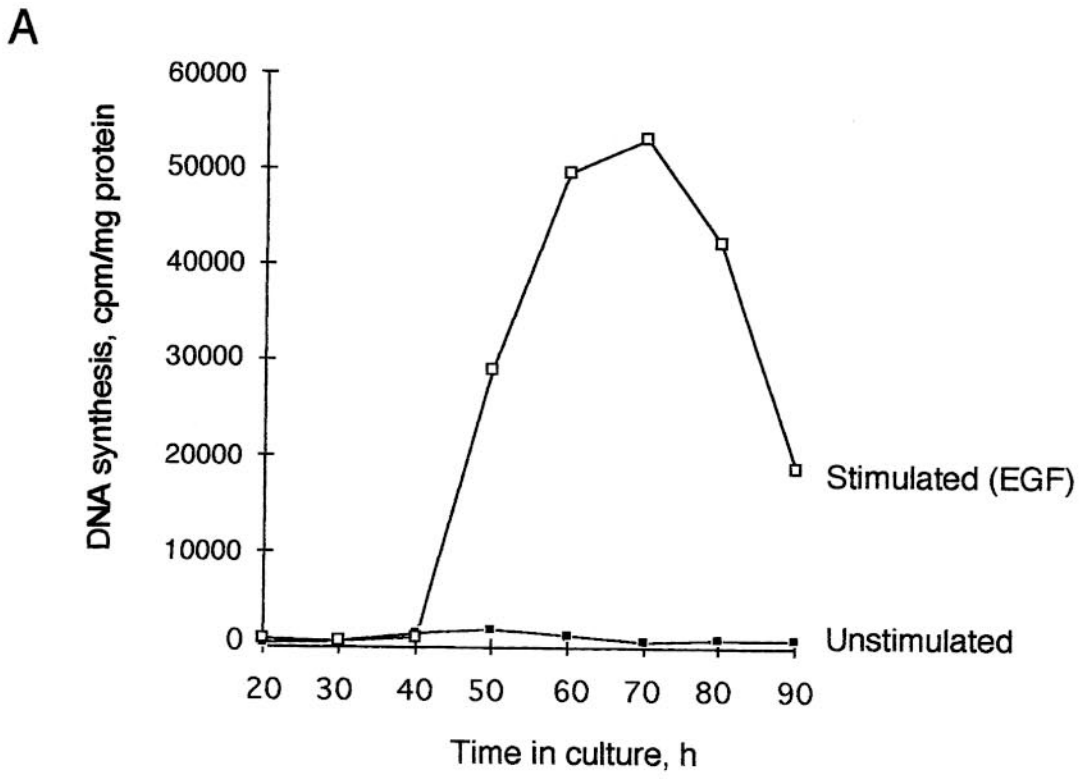
Histone H1 kinase activity assay. These experiments were performed with extracts prepared as described above. Four hundred micrograms of proteins were immunoprecipitated with the same antibodies used for western analyses (1 μg per reaction). Lysates were precleared for 1 h at 4°C with 100 mL of protein A beads that had been equilibrated in lysis buffer (50:50). Lysates were incubated for 1 h at 4°C with the antibodies against cyclin A2 or E, and then 25 mL of protein A–Sepharose beads (50:50) were added and the mixture was incubated for another hour. For determination of histone H1 kinase activity, the protein A beads were washed twice with the lysis buffer and then three times with the H1 buffer (50 mM Hepes–NaOH pH 7.6, 15 mM $MgCl_2$, 1 mM DTT) supplemented with 0.1 mg/mL of BSA. The protein A beads were then incubated for 30 min at 30°C in 50 mL of H1 buffer containing 2 μg of histone H1 (Boehringer-Mannheim), 25 μM ATP, and 10 μCi [γ-^{32}P]ATP, and the reaction was stopped by the addition of 4× Laemmli buffer. The reaction products were separated by SDS–PAGE, gels were fixed, dried and exposed to X-ray film (Kodak).

RESULTS

The aim of our study was to investigate the possible role of cAMP in the control of cyclin A2 expression during rat hepatocyte proliferation. We examined, in primary rat hepatocyte culture, the effect of cAMP on expression levels of cyclin A2 and other G1 and G1/S cyclins. Cultured hepatocytes are characterized by a long G1 and mitogenic stimulation is necessary for the progression of primed hepatocytes towards S phase. In our experiments, hepatocytes were placed in culture and stimulated by the addition of EGF, 24 h after seeding (19). DNA synthesis was assessed by measuring either [^{3}H]thymidine or BrdU incorporation. In stimulated cultures, no [^{3}H]thymidine incorporation was observed during the first 2 days, DNA replication started after 40 h, reached a maximum at 70 h and then rapidly decreased (Fig. 1A). BrdU incorporation analysis revealed that DNA replication occurred in at least 70% of hepatocytes (Fig. 1B).

To elevate the intracellular levels of cAMP, glucagon was added to the culture medium, 3 h after seeding. The time course of the effect of this hormone on DNA synthesis was examined. The S phase entry started at about 40 h in the absence or presence of glucagon; then the rate of DNA synthesis was enhanced in the hepatocytes treated with glucagon (either with 1 or 10 nM) compared to control hepatocytes that had only received EGF, insulin, and dexamethasone (Figs. 2A and 2B). This results confirms, as previously described (16), that glucagon, when employed early after plating, accelerates the transit through the prereplicative period, thereby increasing the pool of hepatocytes that are prepared for G1 exit.

We next monitored by western blot the appearance of cyclins D1, D3, E and A2 in cycling hepatocytes and examined the changes in the levels of these proteins after glucagon treatment. We observed, in accordance with the observations of other groups on the expression of cyclin proteins in cycling hepatocytes (4, 19–21), that cyclin E levels increased from 32 h and remained stable throughout G1 and G1/S phases (Fig. 3). Cyclins



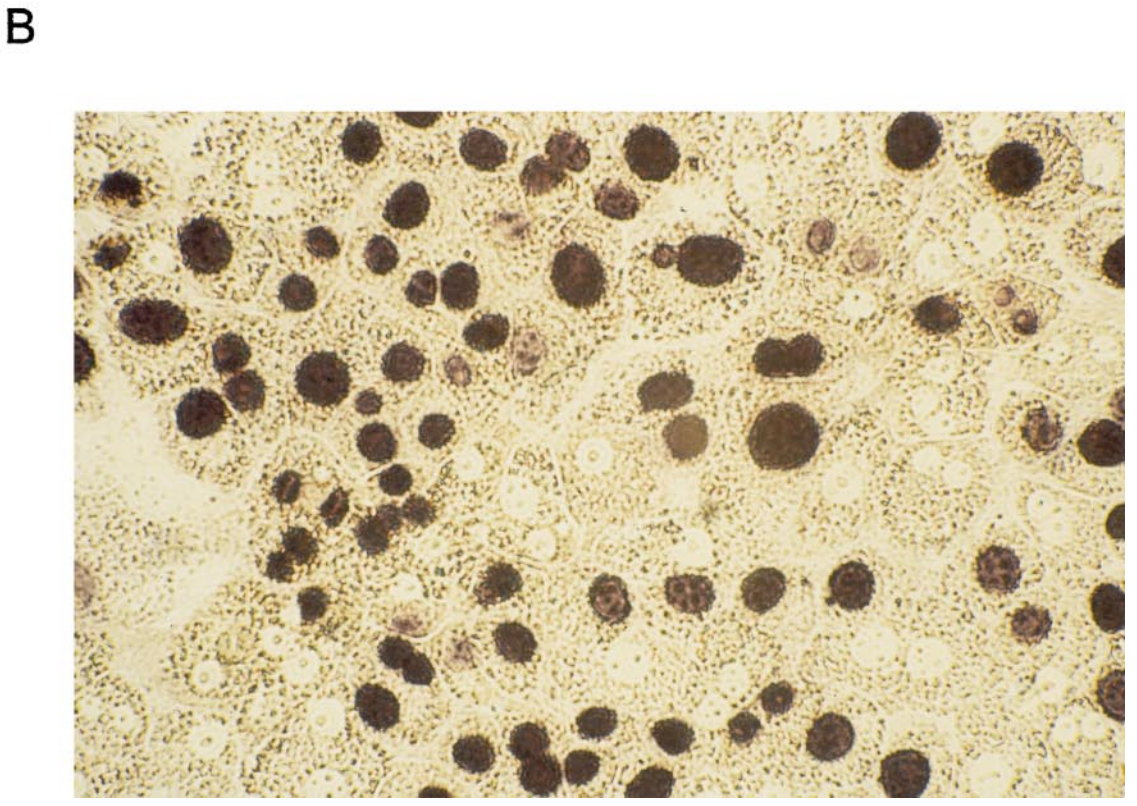


FIG. 1. Cell cycle progression of primary rat hepatocytes. Hepatocytes were cultured in serum-free medium with EGF (5 nM), insulin (100 nM), and dexamethasone (25 nM). EGF was added 24 h after plating. DNA synthesis was evaluated by [^{3}H]thymidine incorporation (A) and BrdU incorporation (B) (see Materials and Methods). Cultures were made in duplicate.

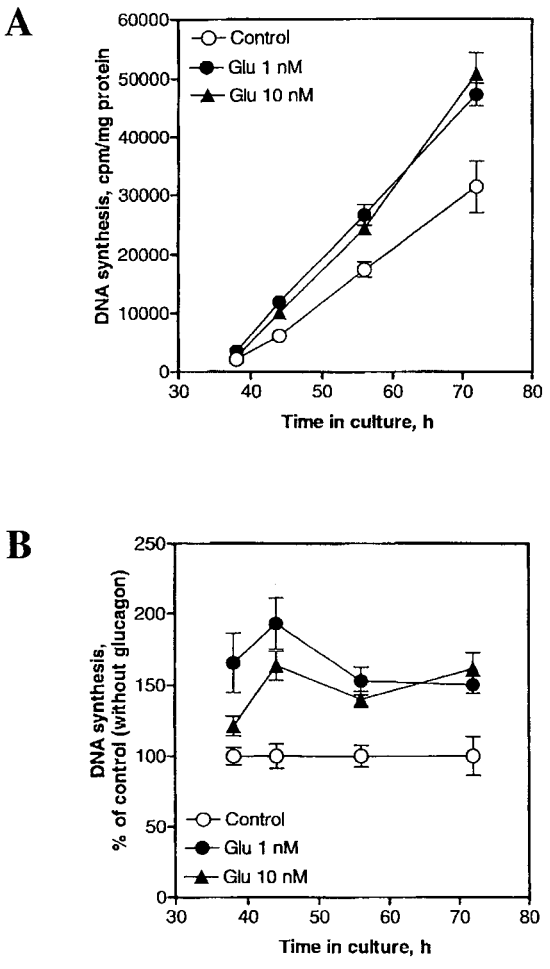


FIG. 2. Time course of the stimulatory effect of glucagon on hepatocyte DNA synthesis. (A and B) Hepatocytes were cultured in serum-free medium with EGF (5 nM), insulin (100 nM), and dexamethasone (25 nM), with or without glucagon (1 or 10 nM). All additions were at time of plating except for glucagon (time 3 h) and EGF (time 24 h). The DNA synthesis was measured as [^{3}H]thymidine cumulatively incorporated in DNA. Data represent means ± SEM of three cultures from one representative of three experiments.

A2 and D3 accumulated from 44 h when the majority of cells were entering the S phase, followed by the accumulation of cyclin D1 from 56 h (Fig. 3). Increasing the intracellular concentration of cAMP by adding glucagon in the culture medium led to a markedly advanced expression of cyclins A2 and D3. In both cases, proteins were detected 32 h after seeding (Fig. 3). By contrast, we did not observe any significant change in the expression of cyclins D1 and E after glucagon treatment. We then examined whether accumulation of cyclin A2, triggered by glucagon, led to an activated cyclin A2-CDK2 kinase. Figure 4 shows that the cyclin A2 associated kinase was indeed activated at 44 h after stimulation. In contrast, there was no modification in the cyclin E-associated kinase compared to control hepatocytes under these conditions. Our data suggest a differential effect of the cAMP signal transduction path-

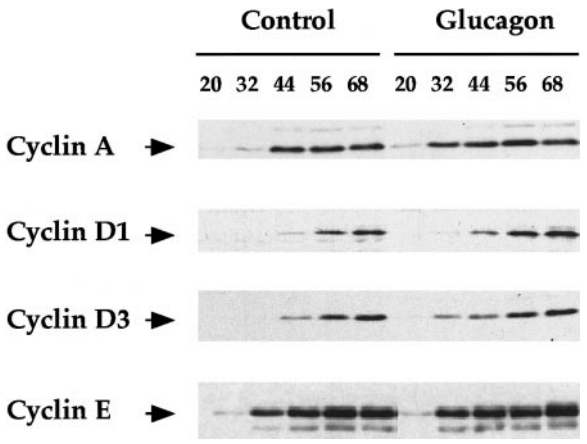


FIG. 3. Expression of cyclins A2, cyclin D1, cyclin D3 and cyclin E in primary culture of hepatocytes treated with or without glucagon. After isolation and plating, hepatocytes were cultured in the presence of insulin (100 nM), EGF (5 nM) and dexamethasone (25 nM). In half of the cultures, glucagon was added at the concentration of 1 nM. Cell lysates were prepared at different times (20, 32, 44, 56, 68 h) for both of the cultures (control and glucagon 1 nM). The expression of different cyclins was analyzed by immunoblotting as described under Materials and Methods.

way on the cyclin-CDK machinery with an upregulation of cyclin A2-, and not cyclin E-associated, kinases during the G1/S transition.

DISCUSSION

Our results suggest that cyclin A2 is implicated in the cAMP mediated regulation of *in vitro* hepatocyte proliferation. The kinase response to elevated cAMP is well characterized in the liver. The pre-replicative burst of cAMP during liver regeneration apparently is a positive signal for the onset of DNA synthesis, while the eventual decrease of cAMP levels is necessary before the cells can enter S phase. A selective increase in protein kinase A regulatory subunits occurs before cAMP levels drop, and may be interpreted as a means

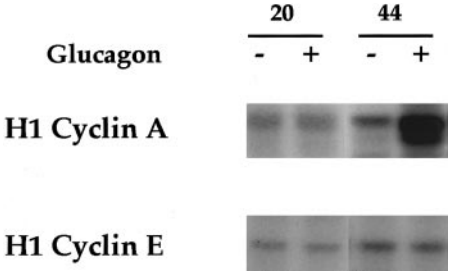


FIG. 4. Cyclin A2- and E-associated kinase activity in primary culture of hepatocytes treated with or without glucagon. Extracts from cells harvested at the indicate time after culture were immunoprecipitated with antibodies against cyclin A2 or cyclin E and were assayed for the ability to phosphorylate histone H1 as described under Materials and Methods.

of down-regulating the kinase response to elevated cAMP preceding DNA replication (22). Our results suggest that accumulation of cyclin A2 may be one of the molecular events triggered by cAMP that lead to hepatocyte DNA replication. We have also shown that selective accumulation of cyclin A2 is followed by activation of cyclin A2-associated kinase upon stimulation by glucagon. We have previously shown that cyclin A2 gene transcription is stimulated upon elevation of intracellular cAMP levels, providing a link between the cAMP dependent effector system and cyclin A2 expression (9). Several lines of evidence indicate that cAMP is a major signal transducer in hepatocyte physiology (23–25). This view has been recently substantiated by the demonstration of CREB phosphorylation and inducible cAMP Early Repressor (ICER) expression in regenerating rat liver coincident with the first peak of cAMP, showing that the cAMP signaling pathway is fully functional during hepatocyte proliferation (26). In addition, CREM−/− mice show a major impairing DNA synthesis after partial hepatectomy (27). In contrast, the targets mediating the effect of cAMP in liver cell cycle are not defined. In other cell type, like parotid gland and thyroid cells, accumulation of cyclin A2 upon activation of the cAMP-transduction pathway has been also reported (28, 29). Our investigation, therefore, expands these results by identifying cyclin A2 as a target of the cAMP effector system during *in vitro* hepatocyte G1/S progression. Furthermore, we show that the cAMP transduction pathway exerts a differential effect on cyclins A2 and D3, compared to cyclins D1 and E. Such differential effect of cAMP on cyclins expression was previously also shown in Swiss 3T3 cells and our results indicate its relevance in highly differentiated epithelial cells (30).

Collectively, our study leads us to propose cyclin A2 and cyclin A2-associated kinase as important elements to link the prereplicative cAMP burst and DNA synthesis in proliferation hepatocytes.

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