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# Dibutyryl cyclic AMP enhances the down-regulation of RB protein during $G_1$ phase in the proliferating primary rat hepatocytes, but inhibits their entries into S phase and RB's phosphorylation

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Summary: To elucidate the cAMP's bi-directional dual effect on hepatic regeneration with a special reference to RB protein, we studied the serial change of RB protein in the proliferating primary rat hepatocytes and the effect of dibutyryl cAMP (DBcAMP) on it. RB protein in the hepatocytes was down-regulated during the $G_1$ phase, and the highly phosphorylated / the underphosphorylated ratio of RB protein increased at the S phase. DBcAMP (1mM) addition, inhibitory on the DNA synthesis, enhanced this down-regulation, but did not increase the phosphorylated form of RB protein at the S phase. High dose of cAMP at the late $G_1$ - $G_1$/S phase could block some essential events for $G_1$/S transition including phosphorylation of RB protein. 

Cyclic AMP (cAMP) is a bi-directional growth regulator in hepatocellular proliferation (1,2). Transient increase of intracellular cAMP level prior to the S phase is observed in rat hepatic regeneration after partial hepatectomy (3,4). Since the delay of this cAMP elevation by adrenergic antagonists has resulted in the delay of DNA synthesis (5), and the addition of cAMP-elevating agents such as norepinephrine, glucagon and dibutyryl cAMP (DBcAMP) at low concentrations has resulted in enhancement of the subsequent DNA synthesis (1,6), cAMP is believed as an essential and stimulative factor for the replicative DNA synthesis in hepatic regeneration (7). However, the sustained high concentration of cAMP or the cAMP elevation at the late $G_1$ - $G_1$/S phase has been reported to result in the inhibition of DNA synthesis (8,9). Although the transient increase and the bi-directional effect of cAMP are explained by changes in the dif-

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ferential expression of guanine nucleotide-binding proteins (G-proteins) (8) and cAMP-dependent protein kinase subunits (10), respectively, the whole process of cAMP-mediated growth control in hepatic regeneration is unknown. We attempted to elucidate the cAMP's bi-directional effect on hepatic regeneration with a special reference to retinoblastoma gene (RB) protein, which has been recently recognized as a putative cell-cycle regulator (11-14).

## Materials and Methods

### Materials.

DBcAMP was provided by Daiichi Pharmaceutical Co., Ltd., Tokyo. Collagenase Type I, insulin, dexamethasone and 8-bromo-cAMP were purchased from Sigma Chemical Company, St. Louis, MO. Williams' E (WE) medium and fetal bovine serum (FBS) were obtained from Flow Laboratories Inc., Irvine. [6-$^3$H]Thymidine (>15Ci/mmol, TdR) was obtained from New England Nuclear Corp., Boston, MA. All other chemicals were obtained from Nacalai Tesque Inc., Kyoto.

### Primary culture of rat hepatocytes.

Hepatocytes were isolated from male Sprague-Dawley rats, weighing about 200g (from Japan SLC, Inc., Shizuoka), according to the two-step collagenase perfusion method described by Seglen (15). The hepatocytes were resuspended at a concentration of $2.5\times10^5$cells/ml in WE medium containing insulin (1μM), dexamethasone (1μM) and 10% FBS, plated on plastic dishes at a density of $5\times10^4$cells/cm$^2$ and maintained at 37°C under 5% $CO_2$ in air. Ninety-six-well plates and 35-mm dishes were used for TdR incorporation and DNA determination, respectively. After 4h culture, the medium was renewed. Following further 20h culture period, the medium was replaced with fresh medium containing no serum or hormones.

### Estimation of DNA synthesis in primary rat hepatocytes.

DNA synthesis was estimated by the incorporation of TdR into the DNA of hepatocytes. TdR (0.1μCi/well) was exposed to cultured hepatocytes for 8h prior to harvest. Acid-insoluble fraction of cells were extracted with ice-cold 10% trichloroacetic acid to measure radioactivity. DNA was determined by the fluorometric method. After the culture with DBcAMP or 8-bromo-cAMP (10, 100μM and 1mM) during 4-24hr, TdR (0.1μCi/well) was exposed to cultured hepatocytes for the following 24hs prior to harvest. The cultured media were collected to measure the released LDH, which revealed that DBcAMP had not a cytotoxic effect on cells.

### Immunoblotting of RB protein in primary rat hepatocytes.

The hepatocytes were suspended at a concentration of $2\times10^5$cells/ml in WE medium containing serum and hormones. Ten milliliters of suspensions were plated on Falcon flasks (75cm$^2$) and cultured under the same conditions described above. After two washes of the cells with cold $Ca^{++}$- and $Mg^{++}$-free PBS, 1ml of cold lysis buffer (10mM Tris-HCl, pH 7.4, 150mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 1mM EDTA and 10μg/ml aprotinin) was added to each flask. The extracts were scraped with silicone scrapers and collected in microfuge tubes, which

were placed on ice for 30min with intermittent vigorous mixing. The lysates were centrifuged, and the supernatant was saved and immunoprecipitated with 3μg of monoclonal antibody to RB protein (G3-245, Pharmingen, San Diego, CA) and protein A-Sepharose 4B. Immune complexes were separated on 4-20% linear gradient polyacrylamide gel containing 0.1% SDS and transferred to PVDF membranes for immunoblotting, which was performed with monoclonal antibody to RB protein and peroxidase-conjugated anti-mouse IgG. Also, immunoblotting was enhanced with "BLAST" amplification (New England Nuclear Corp., Boston, MA.) as previously described (16). Another sample of cell lysate was used for the determination of intracellular cAMP by radioimmunoassay.

## Results and Discussion

We used the in vitro hepatic regeneration model, rat hepatocytes in primary culture at a low cell density, which is known to enter the S phase under the growth stimulation with fetal serum or EGF and hormones (17,18). Hepatocytes were isolated from adult normal rat, and cultured for the first 24hrs under growth stimulation with FBS and insulin, and the following 24hrs with serum-free medium. Assay of TdR incorporation into DNA of hepatocytes, determined every 8hrs of culture period, showed that DNA synthesis initiated just before 24hr and peaked during 32-40hr in culture period of this model (Fig.1A). Intracellular cAMP level increased from 4h to 20h in the serum-supplemented culture and decreased thereafter. Addition of high and non-cytotoxic doses of DBcAMP or 8-bromo-cAMP (10μM-1mM) during 4-

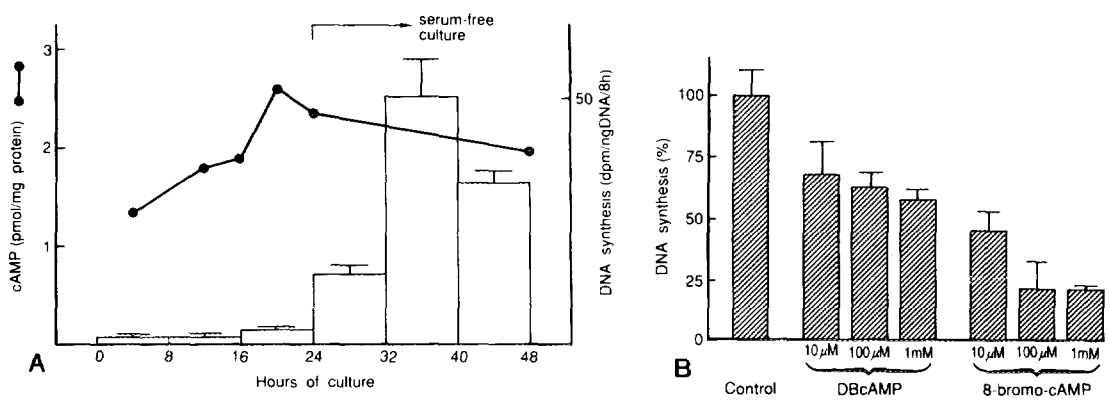


Fig.1. (A) The serial changes of DNA synthesis and intracellular cAMP level in the proliferating primary rat hepatocytes under the growth stimulation with fetal bovine serum and insulin.
(B) Effect of DBcAMP or 8-bromo-cAMP on DNA synthesis in the hepatocytes.
All data are means (+SD) of three assays. DBcAMP or 8-bromo-cAMP (10, 100μM and 1mM) was added during 4-24hr in the culture period. Then, TdR (0.1μCi/well) was exposed to cultured hepatocytes for the following 24hs prior to harvest. Other culture conditions are described in Materials and Methods.

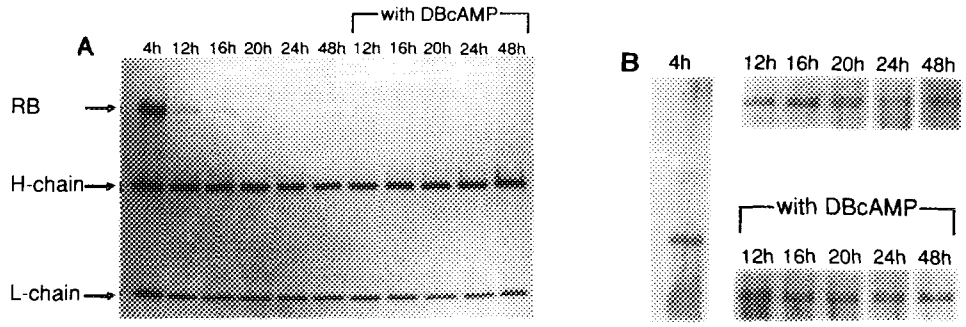


Fig.2. (A) Immunoblotting of RB protein in the proliferating primary rat hepatocytes. (B) Immunoblotting with "BLAST" amplification of RB protein in the hepatocytes. Each time point is indicated in figures. H-chain and L-chain indicate heavy and light chains of immunoprecipitated monoclonal antibody to RB protein, respectively. DBcAMP (1mM) was added during 4-24h in the culture period.

24hr in culture period reduced the DNA synthesis during 24-48hr in a dose-dependent manner (Fig.1B). Changes of RB protein determined by immunoblotting showed a gradual decrease in control culture, and an early drastic decrease in DBcAMP-added culture (Fig.2A). Moreover, the immunoblotting with the "BLAST" amplification based on the catalyzed reporter deposition technology (16) revealed the splitted band of RB protein, suggested to be consisting of the highly-phosphorylated and the underphosphorylated form (Fig.2B). The highly-phosphorylated form of RB protein is known to be usually elevated from $G_1$/S boundary to M phase (19). In the control culture, the highly-phosphorylated / the underphosphorylated form ratio of RB protein increased at 24hr and kept the elevation of this ratio at 48hr. On the contrary, in DBcAMP-added culture, this ratio did not increase at 24hr and apparently decreased at 48hr. In summary, DBcAMP enhanced the down-regulation of RB protein during the prereplicative phase of the hepatocytes proliferation, but inhibited the increase of the highly-phosphorylated / the underphosphorylated form ratio of RB protein at the S phase.

The down-regulation of RB protein in the proliferating hepatocytes seems to be an essential process in cell-cycle progression, and DBcAMP-induced enhancement of this down-regulation might be stimulative for the progression, since RB protein itself is considered to be a negative regulator of the progression. Therefore, the increase of intracellular cAMP prior to the S phase in the proliferating hepatocytes could be associated with the down-regulation of RB protein as a process in

cell-cycle progression. On the other hand, the subsequent decrease of cAMP at the S phase could be associated with the phosphorylation of RB protein as another important process in the progression which is inhibited by the sustained high concentration of cAMP. These possible associations may be responsible for the cAMP's bi-directional dual effect on hepatic regeneration in part. However, there is a report that the down-regulation of RB protein is involved in the experimental differentiation-induction of the human leukemic cells HL-60 (20). Thus, although the down-regulation of RB protein is suggested to be stimulative for the progression in the normal hepatocytes proliferation, significance of the down-regulation may be varied in other cell lines.

Although the detailed relation between cAMP and RB protein is not yet clear, transforming growth factor (TGF)-β is a candidate factor associated with that relation at the late $G_1$. TGF-β is a potent growth-inhibitory factor in hepatic regeneration and to the proliferating hepatocytes (21-23). It is reported that TGF-β inhibits the phosphorylation of RB protein in the rat liver epithelial cell line WB-F334 (24). Also, it is reported that cAMP enhances the expression of TGF-$\beta_2$ in human prostate carcinoma cells (25). These reports suggest that cAMP-induced inhibitions of growth and RB phosphorylation are mediated by TGF-β. However, the expression of TGF-β is usually found in non-parenchymal liver cells such as endothelial cells, but not in hepatocytes (26). Thus, the cAMP-induced TGF-β expression is not likely in our in vitro hepatocytes culture system. The cAMP-mediated and the TGF-β-mediated processes may be cross-linked in their signal transductions (9).

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