THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 264, No. 8, Issue of March 15, pp. 4374–4382, 1989
Printed in U.S.A.

The Expression of cAMP-dependent Protein Kinase Subunits Is Differentially Regulated during Liver Regeneration*

(Received for publication, October 24, 1988)

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The levels of the regulatory (RI and RII) and catalytic (C) components of cAMP-dependent protein kinase and of their messages were studied during the first 36 h of liver regeneration after 70% hepatectomy. Both RIα mRNA and RIIα mRNA started to increase 4 h after the resection, reaching peak levels after 9 h. RI mRNA decreased abruptly 9–12 h after resection, whereas RII mRNA stayed elevated. Cα mRNA was rather constant during the period of study. In accordance with the mRNA data the level of C was constant while RI and RII increased during the prereplicative phase of liver regeneration. RI increased rapidly when its message became elevated. RII, however, increased noticeably only 6–8 h after its mRNA had become elevated. The increased expression of R led to a disproportion between R and C that was most pronounced 14 h after resection, *i.e.* coinciding with the prereplicative cAMP burst. The increased R/C ratio at that time of regeneration diminished the concentration of active C subunit during the cAMP burst. In that way the otherwise inhibitory effect of high concentrations of active C on the DNA replication may have been decreased. The fractional saturation of RI and RII by endogenous cAMP fluctuated in parallel as a function of liver cAMP levels, although there was a tendency that RI was more highly saturated than RII at high concentrations of cAMP.

The swift restoration of the liver mass (compensatory hyperplasia or "liver regeneration") following partial hepatectomy is a striking and much studied example of non-malignant proliferation in the adult mammal reviewed by Bucher and Malt (1971), Alison (1986), and Sobczak and Duguet (1986). cAMP has been implicated in the control of liver regeneration (see the above mentioned reviews and Boynton and Whitfield, 1983; Whitfield *et al.*, 1987). cAMP also affects the proliferation of hepatocytes in primary culture (Friedman *et al.*, 1981; Brønstad *et al.*, 1983).

cAMP carries out its function as a second messenger by binding to cAMP-dependent protein kinases (for reviews, see Beebe and Corbin, 1986; Edelman *et al.*, 1987). These enzymes are tetramers of two regulatory (R)[1] subunits and two catalytic (C) subunits. They exist in two major types, cAKI and cAKII, which differ in the receptor moieties, RI and RII. Subforms (α, β)[2] of RI, RII (Erlichman *et al.*, 1980; Weldon *et al.*, 1985; Jahnsen *et al.*, 1986a, 1986b; Clegg *et al.*, 1988), and C (Uhler *et al.*, 1986; Showers and Maurer, 1986) have been reported, the α subforms being predominant in normal liver (Jahnsen *et al.*, 1986a, Scott *et al.*, 1987, Uhler *et al.*, 1986; Clegg *et al.*, 1988).

The present study focuses on the cAK isozymes in the early stages (mainly the prereplicative period) of liver regeneration. We will show that the levels of RI, RII, and C and of their mRNAs are differentially regulated during that period, and discuss the possible significance of the increased R/C ratio for the effect of the prereplicative cAMP surge (MacManus *et al.*, 1973) on the hepatocyte DNA synthesis. The fractional saturation of RI and RII by endogenous cAMP was determined for several reasons. 1) There might be preferential binding of endogenous cAMP to one isotype of R. 2) Since the direct determination of the intracellular fractional kinase activity proved unfeasible with the presently available methods, this parameter had to be calculated from the degree of saturation of RI and RII. 3) The degree of saturation of R might give clues as to how large proportion of the hepatocytes was involved by the prereplicative cAMP surge, *i.e.* if the cAMP was moderately increased in most cells or strongly increased in a few cells only.

EXPERIMENTAL PROCEDURES

Materials—[5′,8-^{3}H]cAMP (45 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), [L-^{35}S]methionine (>800 Ci/mmol), DNA multiprime labeling kit, rabbit reticulocyte lysate system (code N 90), and Hybond-N nylon membranes were from The Radiochemical Centre, Amersham, United Kingdom. Phosphate acceptor heptapeptide ("kemptide," Leu-Arg-Arg-Ala-Ser-Leu-Gly) was from Sigma. Protein A-Sepharose (Cl4B) was from Pharmacia LKB Biotechnology Inc., Uppsala, Sweden. Triton X-100 and the γ-globulin protein standard were from Bio-Rad. CsCl (ultrapure) was from

* This work was supported by grants from The Norwegian Cancer Society and the Nordic Insulin Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: R, regulatory subunit; cAK, cAMP-dependent protein kinase; cAKI, cAMP-dependent protein kinase type I; cAKII, cAMP-dependent protein kinase type II; C, catalytic subunit; RI, regulatory subunit of cAKI; RII, regulatory subunit of cAKII; DTE, 1,4-dithioerythritol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; kb, kilobase pair.

[2] Publications dealing with the two subforms of C and RI described so far have used the terminologies Cα, Cβ, and RIα, RIβ, respectively. The two known forms of mammalian RII have been termed variously "α and β," "non-neural and neural," and (for rat tissues) "M_r 54 and M_r 51/52." For the sake of consistency we will use α for the non-neural and β for the neural form of RII in the present article.

Bethesda Research Laboratories, Bethesda, MD. Ammonium sulfate (analytical grade) and most other chemicals were from Merck, Darmstadt, Federal Republic of Germany. RIα cDNA and Cα cDNA were gifts from S. McKnight (University of Washington, Seattle) (Lee *et al.*, 1983; Uhler *et al.*, 1986), and RIIβ cDNA from T. Jahnsen (University of Oslo) (Jahnsen *et al.*, 1986b).

RIIα cDNA was prepared as described (Scott *et al.*, 1987). A synthetic peptide corresponding to residues 5–24 of the specific inhibitor of C was prepared as described (Scott *et al.*, 1986). C subunit from bovine heart was prepared by a modification of the procedure of Sugden *et al.* (1976).

Treatment of Animals, Operative Procedures—Male Wistar rats (110–140 g) were given a low protein diet (c 1004, containing less than 2% protein; from Altromin, Lage, West Germany) for 10 days prior to operation. They were subjected either to partial (70%) hepatectomy according to Higgins and Anderson (1931) or laparotomized and the liver elevated and manipulated (sham-operation). The animals were given light ether narcosis during all operative procedures. They were not given access to food (low protein diet) until 24 h after surgery, but had always free access to water.

The biopsies were either the 70% of the liver resected (see above), the liver remnant in various phases of regeneration, or the intact liver of sham-operated animals. They were taken from animals in narcosis by elevating the liver through a midline incision and freeze-clamping with a Wollenberger tong precooled in liquid nitrogen. In all cases the frozen tissue block was ground in a mortar immersed in liquid nitrogen, and the powder kept in liquid nitrogen until homogenization. Some animals had been injected intraperitoneally with [^{3}H]thymidine (0.1 mCi) 0.5 h before killing.

Preparation of Cytosol and Particulate Fractions of Tissue and Isolated Cells—Tissue powder (100 mg) was homogenized (2 × 10 s) with a Polytron PT 10/35 emulsifier (at a setting of 4) in 2 ml of ice-cold buffer (homogenization buffer): 50 mM Hepes-NaOH, pH 7.2, containing 5 mM EDTA, 3 mM EGTA, 120 mM NaCl, 1 mM DTE, and the protease inhibitors: aprotinin (0.05 mg/ml), soybean trypsin inhibitor (1 mg/ml), leupeptin (100 μM), pepstatin (20 μM), chymostatin (10 μM), antipain (10 μM), and benzamidine (10 mM).

The homogenate was split in three. One portion was kept for measurement of total DNA. The second portion was made 1% in Triton X-100, incubated for 1 h at 0 °C and used for determination of RI, RII, and C in the unfractionated homogenate. The third portion was centrifuged (120,000 × g_{av}) for 10 min at 4 °C in the A-95 rotor of a Beckman Airfuge to separate the cytosol (supernatant) and particulate fraction (sediment). The sediment was washed (resuspended and recentrifuged as above) in 2 ml of ice-cold homogenization buffer and finally incubated for 1 h at 0 °C in 1 ml of homogenization buffer containing 1% of Triton X-100. The fractions (crude and Triton-treated homogenate, cytosol, and Triton-treated particulate) were stored in small aliquots in liquid nitrogen until further processing.

Determination of Protein Kinase Subunits—The determination of RI or RII was based on measurement of the binding capacity for [^{3}H]cAMP. For this, a 0.1-ml sample (Triton-treated homogenate, cytosol or Triton-treated particulate) was mixed with 0.1 ml of homogenization buffer (containing 1% Triton in the case of cytosol) and 0.8 ml of 30 mM Hepes-NaOH, pH 7.2, containing 10 mM EDTA, 1 μM [^{3}H]cAMP (5 Ci/mmol), 0.6 mM of 3-isobutyl-1-methylxanthine, 1 mg/ml of bovine serum albumin, 20 mM 2-mercaptoethanol, 1 mM DTE, and 30 μM leupeptin. After incubation for 30 min at 37 °C (to exchange the endogenously bound cAMP with [^{3}H]cAMP), aliquots were mixed with Protein A-Sepharose (suspended in homogenization buffer) and anti-RI or anti-RII serum and gently rocked for 2 h at 4 °C. R-protein not complexed with antibody-Sepharose was removed by washing the Sepharose in columns (Econo columns from Bio-Rad) equipped with a 10-μm filter. Finally RI- or RII-bound [^{3}H]cAMP was eluted with 0.3 M acetic acid and mixed with scintillation liquid before counting. For details of the procedure and of preparation and determination of the specificity of antisera, see Ekanger *et al.* (1985) and Ekanger and Døskeland (1988).

The amount of C subunit was determined by assaying its phosphotransferase activity. The samples to be tested were diluted 40- and 80-fold in 50 mM potassium phosphate, pH 7.0, containing 1 mM EGTA, 0.3 mM EDTA, 2 mM DTE, 0.15% Triton X-100, 0.9 mg/ml bovine serum albumin, and 0.09 mg/ml of soybean trypsin inhibitor. The assay was started by mixing such diluted sample with 2 volumes of assay mixture, pH 7.0, to give the following final concentrations of assay components: 50 mM potassium phosphate, 15 mM Hepes, 10 mM magnesium acetate, 70 μM kemptide, 0.1 mM [^{32}P]ATP (2 μCi/mmol), 0.5 mM EGTA, 0.1 mM EDTA, 1 mM DTE, 0.2 mM 3-isobutyl-1-methylxanthine, and 10 μM cAMP (when present). The assay was run at 30 °C. Aliquots (25 μl) were removed after 4, 8, 12 min of incubation by spotting on phosphocellulose paper strips, according to Roskoski (1983). In order to achieve uniform and low blanks (0.03–0.05% of added radioactivity), the filters (up to 30/beaker) were immersed into beakers containing 1 liter of wash solution (25 mM phosphoric acid) under continuous shaking, and the wash solution was changed every 10 min for the next 90 min. The kinase activity is expressed as units, *i.e.* nanomole of phosphate incorporated into kemptide/min under the above conditions.

Determination of the Fractional Saturation of RI or RII with Endogenous cAMP—The method is based on the use of ammonium sulfate, glycerol, and low temperature to stabilize the endogenously formed complexes of R and cAMP, and was performed as described (Ekanger and Døskeland, 1988) with the following modifications: 10% glycerol was included in the homogenization buffer, and 0.2 ml of 5% casein was added to the homogenate before it was mixed with 4 volumes of 2.4 M aqueous ammonium sulfate and spun. These modifications made it possible to obtain quantitative recovery of RI and RII even in fat-laden livers (see also the first paragraph of "Results" for a discussion of this point). The fractional saturation of RI or RII is the ratio between the endogenously bound cAMP and the cAMP-binding capacity.

Isolation of Total RNA—RNA was isolated by the method of Chirgwin *et al.* (1979), modified as follows. Liver tissue (0.5–1 g) was homogenized in 10 ml of 25 mM sodium citrate buffer, pH 7, containing 5 M guanidinium thiocyanate and 1.3 M of 2-mercaptoethanol, using a Polytron PT 10/35 homogenizer for 45 s (at a setting of 9). Sodium lauroyl sarcosine was added to 0.5% (w/v). Cellular debris was removed by centrifugation (3000 × *g* for 10 min). 3 ml of the supernatant was overlaid a 2-ml cushion of 5.7 M CsCl in 100 mM EDTA, pH 7. After spinning for 15 h (156,000 × g_{av} at 20 °C in a Beckman SW 50.1 rotor), the pellet was carefully dissolved in 0.3 ml of 10 mM Tris-HCl buffer, pH 7.4, containing 1% sodium dodecyl sulfate and 5 mM EDTA. The solution was extracted once with 2 volumes of chloroform/1-butanol (4:1), precipitated with 2 volumes of 4.5 M sodium acetate, pH 7, at −20 °C overnight, the precipitate washed once with 70% ethanol and dissolved in H_2O. The $A_{260\,nm}/A_{280\,nm}$ ranged from 1.80 to 2.05, being usually close to 2.0. The size distribution of the RNA molecules was checked by ethidium bromide staining after gel electrophoresis.

Hybridization with cDNA Probes for RIα, RIIα, and Cα—Total liver RNA was electrophoresed in 1.5% agarose gel in the presence of 6.6% formaldehyde (Maniatis *et al.*, 1982), transferred to nylon membranes, and hybridized with ^{32}P-labeled dCTP probes made by random primed labeling (Feinberg and Vogelstein, 1982) of RIα-cDNA, RIIα cDNA, RIIβ cDNA, or Cα cDNA fragments. Filters were hybridized for 24–48 h at 42 °C in 5 × SSPE, 5 × Denhardt's solution, 100 μg/ml sheared denaturated salmon sperm DNA, 50% (v/v) deionized formamide, and 0.5% sodium dodecyl sulfate. They were then washed 5 times (22 °C, 5 min) with 2 × SSPE containing 0.1% sodium dodecyl sulfate and 2 times (50 °C, 15 min) with 0.1 × SSPE containing 0.1% sodium dodecyl sulfate (1 × SSPE is 10 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, and 149 mM NaCl; 1 × Denhardt solution contains 10 mg of Ficoll, 10 mg of polyvinylpyrrolidone, and 10 mg of bovine serum albumin/100 ml of solution). Autoradiography was performed with preflashed film at −70 °C, 3–10 days exposure. The hybridization signals were measured using the LKB UltroScan XL Laser Densitometer. The molecular weight of the RNA bands was estimated using a commercially obtained RNA ladder (BRL) and the position of 18 S and 28 S ribosomal RNA.

In Vitro Protein Synthesis—Translation of heated RNA was carried out with a rabbit reticulocyte lysate system in the presence of labeled methionine for 90 min at 30 °C, and the samples digested with deoxyribonuclease and ribonuclease (Fukami *et al.*, 1986). Aliquots of 5 μl were mixed with 20 μl of water and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (1970), except that two sets of running gels (7% and 12% acrylamide, respectively) were used to achieve resolution of both large and small peptides. For autoradiography of the dried gels β-max film (Amersham) was used, and the films scanned as described above.

Determination of cAMP, Protein, DNA, and [^{3}H]Thymidine Incorporated into DNA—Pulverized liver tissue (100 mg) was precipitated in 2 ml of ice-cold 5% (w/v) aqueous trichloroacetic acid in 0.1 M HCl and centrifuged (20,000 × g_{av}) at 4 °C for 10 min. The trichloroacetic acid was removed from the supernatant by repeated ether extractions and the cAMP measured by a competitive binding assay

(Døskeland and Kvinnsland, 1980).

Protein was determined by the Bio-Rad version of the assay of Bradford (1976), using bovine γ-globulin as the standard. It may be noted that the protein values obtained using this protein standard were about 2.5 times higher than with bovine serum albumin as the standard.

The determination of DNA was by a modification (Vintermyr and Døskeland, 1987) of the procedure of Patterson (1979). Before spectrophotometric determination of DNA, an aliquot of the sample was removed to determine the amount of [^{3}H]thymidine incorporated into DNA by liquid scintillation counting.

Calculation of the Fractional Activity of cAMP Kinase from Data on Fractional Saturation of R—The fractional activity of C is defined as the ratio between free and total C. The concentration of free C can be expressed as a function of total C, total R, and the fractional saturation of R (Equation 1): Free C = Total C − Total R·(1 − fractional saturation of R), where the fractional saturation of R is (fractional saturation of RI·total RI + fractional saturation RII·total RII)/(total RI + RII). The values for total RI, RII, and C were taken from Fig. 1, and the values for fractional saturation of RI and RII from Fig. 8, A and B.

The assumptions implicit in Equation 1 are that the affinity of R is so high for cAMP and C that R exists complexed with either cAMP or C intracellularly, and that the binding of cAMP and C to R can be considered mutually exclusive for operational purposes. The first assumption is supported by the finding that the apparent equilibrium binding dissociation constant for R and C is in the subnanomolar range (Builder *et al.*, 1981) and for RI and cAMP in the nanomolar range (Døskeland and Øgreid, 1984), *i.e.* 2 orders of magnitude below the normal intracellular concentrations of cAMP and C. The second assumption is supported by the tight coupling between cAMP occupancy and fractional kinase activity when the isozymes are assayed *in vitro* (Ueland and Døskeland, 1976; Øgreid and Døskeland, 1982), and by the requirement of supraphysiological concentrations of cAMP-liganded R to form appreciable amounts of ternary complex[3] (Builder *et al.*, 1981). On the other hand the slightly lower cooperativity of cAMP binding observed *in vivo* (Ekanger *et al.*, 1985) than under standard kinase assay conditions (Øgreid and Døskeland, 1982) is compatible with a slight amount of ternary complex formation *in vivo* according to the theoretical treatise of Swillens (1984). If appreciable amounts of inactive ternary complex should exist in the liver, the calculated fractional activity of C will be an overestimate. The values to be presented for the fractional activity of cAK are therefore to be considered as maximum values.

RESULTS

Methodological Considerations—Losses of activity and other pitfalls are associated with separation of RI and RII by, *e.g.* ion exchange chromatography (Malkinson *et al.*, 1983). Autoradiography of 8-azido-[^{32}P]cAMP-labeled RI and RII after electrophoretic separation (Walter and Greengard, 1983) is also associated with problems. The incorporation into the binding sites is most often nonquantitative (Øgreid and Døskeland, 1982; Bubis and Taylor, 1987), and the labeled photoaffinity analog is chemically unstable (Cartwright *et al.*, 1976; Øgreid and Døskeland, 1982) in the buffers usually employed during extraction of R, and during the exchange between affinity analog and endogenous cAMP.

We therefore measured RI, RII, and C directly in unfractionated liver extracts, relying on immobilized, isozyme-specific antibodies to discriminate RI and RII (Weber *et al.*, 1981; Ekanger *et al.*, 1988). Since the binding capacity for [^{3}H]cAMP was measured, complete exchange between endogenous cAMP and added [^{3}H]cAMP was essential. This was not achieved even after overnight incubation at 4 °C, but at 37 °C complete exchange of RI- or RII-associated cAMP was obtained after 30 and 5 min, respectively. Since a large fraction of RI was liganded with endogenous cAMP in the prereplicative phase of liver regeneration (see Fig. 8), omitting the exchange step would have lead to significant underestimation of R, especially RI. The considerable dilution of the liver extract and the presence of protease inhibitors during the 37 °C step ensured that immunoreactive R was stable for at least 1 h. In the absence of protease inhibitors, immunoassayable RII was rapidly lost.

The immunoestimation of RI and RII appeared reliable by several criteria. The amount of cytosolic RI and RII was determined using unlabeled cAMP instead of [^{3}H]cAMP, avoiding the exchange step at 37 °C, and found to be similar to the values determined by the assay using labeled cAMP. The amount of RI + RII determined in extract corresponded to the [^{3}H]cAMP binding capacity determined with the ammonium sulfate precipitation method (Døskeland and Øgreid, 1988). Partially purified rat liver cAKI and cAKII or pure rat muscle RI or RII, added to homogenates of regenerating liver, were quantitatively recovered. The fraction of liver extract not immobilized with antibody against RI was found to contain more than 95% of the total assayable RII and less than 5% of RI. This was true whether crude Triton-solubilized homogenate, cytosol, or the particulate fraction was tested, and indicates that the antibodies do not immobilize both RI and RII, as might be expected if the two isoreceptors coexisted in Triton micelles recognized by either antiserum. A final point of concern was that cAMP carried over from the homogenate to the incubations might invalidate the determination of R by diluting the [^{3}H]cAMP. For tissue samples containing high levels of endogenous cAMP, the determination of R was therefore performed with two different dilutions of sample and with the normal as well as twice the normal concentration of [^{3}H]cAMP. In all cases the R level determined was independent of dilution.

The method used for determination of the degree of saturation of RI and RII with endogenous cAMP has recently been described, and validated for normal liver (Ekanger and Døskeland, 1988). We found that it had to be slightly modified to ensure complete recovery of R in fat-rich organs, like the regenerating liver. At a high ratio of tissue to homogenization buffer, and concentrations of ammonium sulfate above about 2.8 M in the medium used during the first centrifugation step, part of the R proteins tended to flotate rather than sediment. This was probably due to formation of low density complexes between R and lipoproteins in the antichaotropic ammonium sulfate solution. The problem was overcome by diluting the homogenization buffer with glycerol (to 10%), reducing the final concentration of ammonium sulfate in the centrifugation medium to 2.5 M, and adding casein as a co-precipitant to increase the density of the precipitates formed. With these modifications all soluble R was recovered. It was verified that the modified procedure was reliable by the criteria previously used for the original procedure (Ekanger and Døskeland, 1988).

We noted that the animals began eating 19–23 h after the liver resection. In order to standardize the period of postoperative fasting the animals were not given access to food until 24 h after the operations. Whereas deprivation from normal diet led to a significant decrease of cytosolic cAKI in both normal and sham-operated animals (data not shown; see also Chan *et al.*, 1979; Ekanger *et al.*, 1988) deprivation from the protein-poor diet used in the present study had little effect on the kinase subunits (data not shown). Furthermore, such diet decreases the diurnal variations of the levels of insulin and glucagon (Tiedgen and Seitz, 1980), hormones implicated

[3] A "ternary complex" between R, C, and cAMP in this context means all complexes where one or both of the subunits of the dimeric R_2 simultaneously binds cAMP and C. This means that all complexes containing R_2, C, and cAMP are included except $R_2C(cAMP)_2$, where presumably one R subunit binds two cAMP molecules and the other one binds C (Connelly *et al.*, 1986).

in the control of hepatocyte growth (Bucher *et al.*, 1977; Alison, 1986).

To ensure that this feeding regimen did not perturb the proliferative capacity of the liver, total liver DNA and the incorporation of labeled thymidine into DNA were determined after resection. The incorporation of [^{3}H]thymidine increased rapidly from 17 h after liver resection, and after 24 h it was 30-fold higher than in the sham-operated animals. As judged by the estimations of the total liver weight, protein, and DNA, the liver mass was 75% restored 120 h after operation. The regenerative capacity of the liver in the rats used in the present experiments thus did not grossly differ from that reported in previous studies (Bucher and Malt, 1971; MacManus *et al.*, 1973).

Evidence for Disproportionate Expression of cAMP-dependent Protein Kinase Subunits during Liver Regeneration—The hepatocyte content of RI started to increase about 4 h after the two-thirds hepatectomy, being 60% above the control level between 8 and 12 h postresection. A rather abrupt decline of RI to a level 20% above the starting level was noted between 12 and 18 h after the operation (Fig. 1*A*). RII started to increase 10 h postresection, stayed 50% increased 14–18 h after the operation, and thereafter declined slowly to a level 30% above the starting point (Fig. 1*A*). A comparison with the hepatocyte protein content (Fig. 1*B*) shows that the concentrations of R proteins fluctuated independently of the general protein level. In contrast, the hepatocyte content of C subunit grossly followed the protein level, showing an early decline followed by a slow increase to 15% above the starting level (Fig. 1*A*). Assuming that cytosol protein reflects the cytosol volume it can therefore be concluded that the hepatocyte concentration of C stays unaltered during liver regeneration.

The fluctuations of R protein levels observed were specific for the regenerating liver, since they did not occur in sham-operated animals (Fig. 2). The data shown in Figs. 1 and 2 allowed the calculation of the RI:RII ratio, which was higher in the regenerating liver remnant than in the liver of sham-operated animals at all time points studied.

The selective increase of R (RI + RII) relative to C in the regenerating liver led to a significant disproportion between R and C, which was maximal 12–15 h postresection (Fig. 3). The increase of R/C ratio in the period 6–22 h postresection was highly significant ($p < 0.0005$) when tested statistically against the preresection R/C ratio (legend to Fig. 3). In a thorough study Hofmann *et al.* (1977) found that the proportion between R and C subunits was 1:1 in all normal rabbit tissues tested. To ensure that this was also the case in the liver of the unoperated rats we compared the ratio of the kinase activity and cAMP binding activity in the liver extracts, in a preparation of homogeneous cAK from rat muscle, and in two preparations of partially purified cAK from rat liver. Taking the R/C ratio of the holoenzymes as 1:1, the R/C ratio of normal liver was 1.1:1 (range 1.04–1.18).

The percentage of particulate-associated RII stayed constant (at 42%) and of RI nearly constant (at about 27%) during the regenerative period studied (Fig. 4). This suggests that the newly formed R must be relatively rapidly distributed between the cytosolic and particulate fractions. In contrast, when amino acids are fed to starved rats, the increase in hepatocyte RI occurs nearly exclusively in the cytosol fraction (Ekanger *et al.*, 1988). Similar values of particulate-associated R were found whether determined directly in the washed particulates or by subtracting the cytosol value from the homogenate value. That the overall increase of RI and RII in the whole particulate fraction was proportional to that in the cytosol does not rule out higher increase of R in some subcompartments of the particulate fraction. A considerable increase of R has been described in the nuclear fraction (see Sikorska *et al.*, 1988, and the references therein) and on the external face of the plasma membrane (Whitfield *et al.*, 1987).

In view of reports of induction of an altered form of RI that does not easily complex with C (Prashad *et al.*, 1979; Prashad, 1981) we wanted to know if the RI accumulating in the

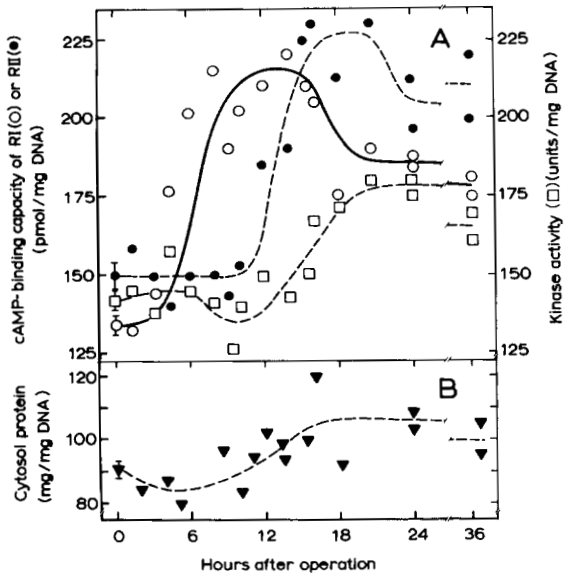

FIG. 1. **The hepatocyte content of RI, RII, C, and cytosolic protein as a function of time after partial hepatectomy.** *Panel A*, samples of liver tissue were removed at various times after 70% liver resection, and the amount of cAMP-dependent protein kinase subunits determined in the homogenate. RI (○) and RII (●) are expressed as cAMP-binding capacity/mg of homogenate DNA (*left ordinate*). C (□) is expressed as units/mg homogenate DNA (*right ordinate*). For definition of units of C and for further experimental details, see "Experimental Procedures." The points with *error bars* represent the values obtained for biopsies removed at the time of resection, and are the means ± S.E. of data from 24 animals. The other points represent mean values of biopsies from 2–3 animals. *Panel B*, for comparison, the amount of cytosol protein (▼), expressed as milligrams/mg of homogenate DNA, is shown. The protein was determined in the cytosol fraction of the same homogenates as were used for determination of protein kinase subunits.

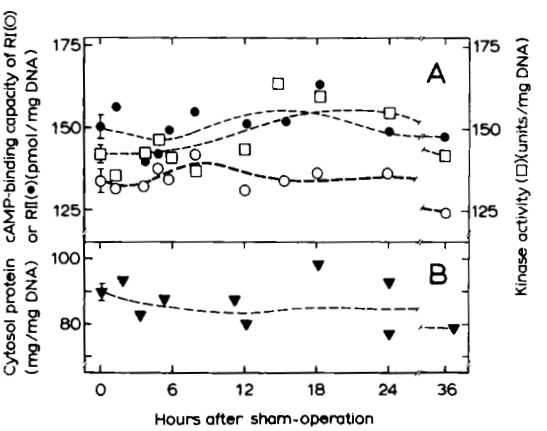

FIG. 2. **The hepatocyte content of RI, RII, C, and cytosolic protein after sham-operation.** Rats were subjected to sham-operation, *i.e.* laparotomy was performed, the liver elevated and manipulated but not resected. *Panel A* shows the levels of homogenate RI (○), RII (●), and C (□). *Panel B* shows the level of cytosolic protein (▼). Experimental details and presentation were as described in the legend to Fig. 1.

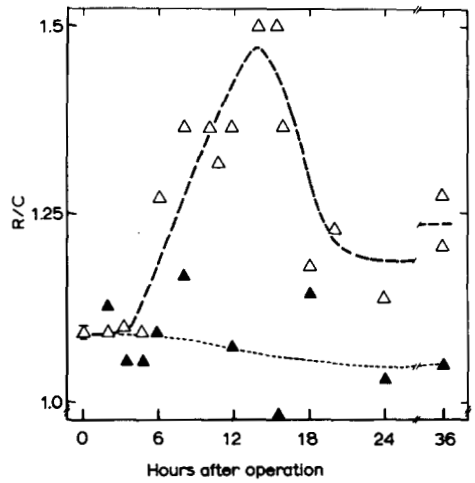

FIG. 3. **The proportion between homogenate R and C after liver resection or sham-operation.** Homogenate R (RI plus RII) and C were determined (see legend to Fig. 1 and the "Experimental Procedures") in the liver remnant at various times after liver resection (△) or sham-operation (▲), and the R/C ratio calculated as described under "Experimental Procedures" and "Results." The point with an *error bar*, representing the livers removed at the time of resection, is the mean ± S.E. ($n = 24$). The other points represent the mean values of biopsies from 2–3 animals. The values of the R/C ratio in the period 6–22 h after resection ($n = 14$) were compared to the values of R/C in the livers at the time of resection ($n = 24$) using the Wilcoxon non-paired comparison test, and the difference in R/C ratio was found to be highly statistically significant ($p < 0.0005$). When the R/C ratio was compared between the liver remnant 6–22 h after resection and the biopsy from the same liver at the time of resection, it was higher in 12 out of 14 cases. In the remaining two cases (one 6-h and one 22-h animal) there was no difference. Using the Wilcoxon paired comparison test this translates to a highly significant ($p < 0.00025$) difference in R/C ratio between regenerating (6–22 h post resection) and normal rat liver.

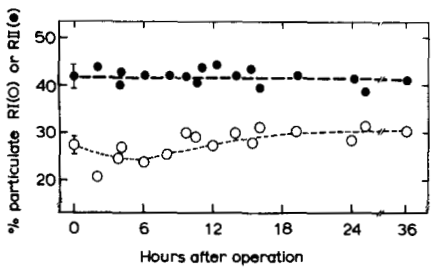

FIG. 4. **The proportion of particulate-associated RI and RII during liver regeneration.** To see if the increases of homogenate RI and RII, occurring in the liver after partial hepatectomy (see Fig. 1*A*), were based on selective increase in either the cytosolic or the particulate fraction, the amounts of particulate associated RI (○) and RII (●) were determined. The amount of particulate R is given as a fraction of the total (homogenate) R. The points with an *error bar*, representing the livers at the time of resection, are means ± S.E. ($n = 24$). The other points are means of biopsies from 2–3 animals.

regenerating liver (Fig. 1) had normal kinetics with respect to interaction with cAMP (Fig. 5*A*) and C subunit (Fig 5*B*). We noted no difference in the rate of spontaneous dissociation (Fig. 5*A*) or C subunit-induced dissociation (Fig. 5*B*) of the complex of RI and [^{3}H]cAMP. Incidentally, the very slow rate of cAMP dissociation from site B of RI in freshly prepared cytosol is an additional argument that the more rapid kinetics of exchange often observed in purified preparations of RI is due to alterations of R during purification, *i.e.* "aging" (Døskeland and Øgreid, 1984). That the increased rate of cAMP dissociation from RI in the presence of C subunit was specifically due to the C subunit (and not, *e.g.* proteolysis) was

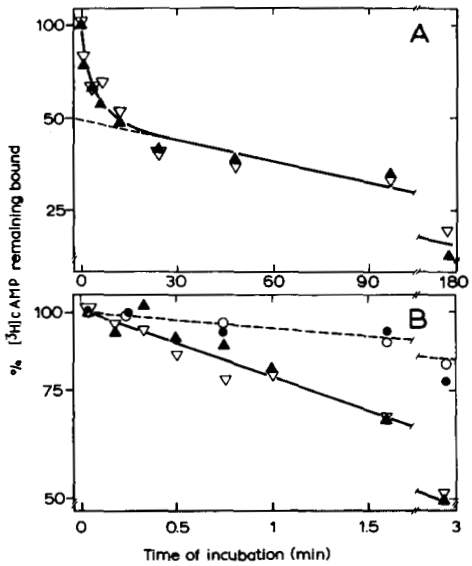

FIG. 5. **The spontaneous and C-induced dissociation of [^{3}H] cAMP bound to RI in control and regenerating liver.** Liver cytosol was obtained from liver tissue removed at the time of resection (*open symbols*) and from the liver remnant of the same animal 10 h after resection (*solid symbols*). *Panel A* shows the (spontaneous) dissociation of [^{3}H]cAMP from cytosolic RI in 3.2 M NaCl at 30 °C. *Panel B* shows the rate of dissociation of [^{3}H]cAMP from RI in the presence of added C subunit (0.15 M NaCl, 25 °C). The *upper curve* of *panel B* (○, ●) represents the dissociation rate in the presence of excess C inhibitor peptide. Details of the experiments were as follows: each cytosol sample was mixed with 1 volume of the homogenization buffer described under "Experimental Procedures" supplied with 1.2 μM [^{3}H]cAMP, 3 mM 3-isobutyl-1-methylxanthine, and 10 times the usual level of all protease inhibitors. The mixtures were left for 30 min at 37 °C to achieve exchange between added [^{3}H]cAMP and any endogenously bound cAMP, and divided into three portions. One portion (*panel A*) was made 3.2 M in NaCl, equilibrated for 5 min at 30 °C, and the amount of [^{3}H]cAMP remaining bound determined at various times (*abscissa* of *panel A*) after the addition of unlabeled cAMP (to a final concentration of 0.2 mM). The other portions (*panel B*) were equilibrated at 25 °C in 0.15 M NaCl and the rate of exchange measured after the simultaneous addition of catalytic subunit (final concentration 40 nM), Mg (acetate)$_2$ (15 mM), ATP (1 mM), and unlabeled cAMP (0.2 mM) without (▲, ▽) or with (○, ●) 100 nM of a synthetic peptide inhibitor of C. For the experiments in either panel aliquots of 40 μl were removed and rapidly mixed with 1.5 ml of 15 mM Tris-HCl, pH 8.5, at −5 °C containing 0.4 M ammonium sulfate, 2.4 M glycerol, 5 mM EDTA, and one-fifth the concentration of protease inhibitors normally present in the homogenization buffer. The amount of [^{3}H]cAMP bound specifically to RI was determined by incubation with antibody against RI as described (Ekanger and Døskeland, 1988). Data similar to those shown were obtained with other cytosols (from animals 12 and 14 h after liver resection). In separate experiments it was found that the rate of exchange in the presence of inhibitor peptide under the conditions of *panel B* was similar to that when Mg/ATP was absent.

shown by the ability of an inhibitor peptide directed towards the active site of C to abolish the C effect (Fig. 5*B*).

As an alternative to measuring the amount of C by its phosphotransferase activity (Figs. 1 and 2) we determined C activity by the ability of selected cytosols to enhance the dissociation of [^{3}H]cAMP from rabbit muscle RI. This way of measuring C is not susceptible to errors resulting from, *e.g.* phosphoprotein phosphatase or substrate depletion. We found a complete correlation between the two methods to determine C (data not shown).

The C-specific inhibitor peptide (Scott *et al.*, 1986) abolished more than 90% of the kinase activity under the conditions used. In order to rule out that endogenous heat stable inhibitor (Walsh and Ashby, 1973) might influence the assay,

we showed that addition of up to 10 nM of this inhibitor to liver homogenates did not affect the kinase determinations and that interference by higher concentrations added were detected by lack of proportionality between homogenate concentration and kinase activity (data not shown; see also Døskeland and Ueland, 1975). For all the liver extracts whose C content is reported (Figs. 1 and 2) the kinase activity was inversely proportional to dilution, and the homogenate value equal to the sum of the cytosol and particulate values, which would ensure that the assay was not affected by the heat-stable inhibitor protein.

The mRNA Signals for cAMP-dependent Protein Kinase Subunits—Fig. 6 shows typical mRNA signals after Northern blotting. Three mRNA size variants hybridized specifically with the probe for RIα. All three variants increased during the early stage of liver regeneration, there being a tendency for the 3.0- and 3.2-kb signals to rise slightly earlier and decline slightly earlier than the main 1.7-kb signal. Signals of 5 and 2.4 kb were detected by the RIIα and Cα probes, respectively (Fig. 6). No signal for the RIIβ probe was detected under conditions when a clear signal was detected in Northern blot of RNA isolated from adult rat testis. The Northern blots of the testis RNA also differed in that a 2.2-kb signal for RIIα, not detected in liver, dominated over a very faint 5–6-kb species, and that the 3–3.2-kb messages for RIα dominated over the 1.7-kb RIα signal.

The levels of the various messages were quantified by densitometric scanning of autoradiograms like the one shown in Fig. 6, and the level of each message expressed relative to the levels in the liver biopsies removed at the time of partial hepatectomy (Fig. 7). The mRNA signals for both RI and RII increased 3–4-fold between 3 and 9 h after resection. Whereas the increase in mRNA for RI was transient, the RII message stayed elevated during the whole period (36 h) studied. The level of mRNA for C did not change significantly (Fig. 7). The levels of mRNA for RI and RII were only slightly increased about 6 h after sham-operation (data not shown).

Several of the RNA samples were used in a cell-free protein synthesis system, and the resulting [^{35}S]methionine-labeled proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In all cases high molecular size (apparent $M_r > 100{,}000$) labeled proteins were detected, indicating that mRNA breakdown was minimal. In agreement with previous

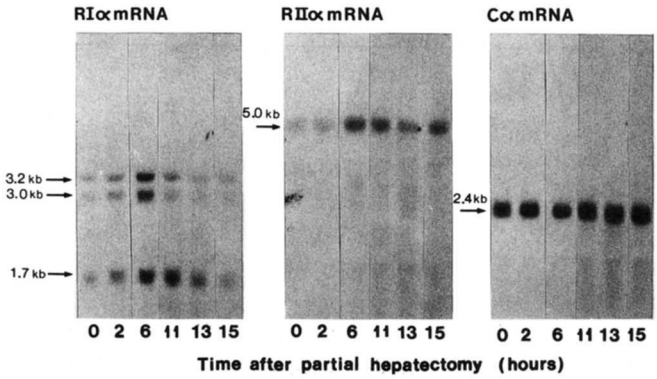

FIG. 6. **Hybridization of RIα, RIIα, and Cα cDNA probes to Northern blots of RNA from liver at various stages of the prereplicative period of regeneration.** Northern blots were made of total RNA isolated from rat liver at various times after liver resection. 25 μg of total RNA was loaded onto each lane. The figure shows a typical autoradiograph of a Northern-blotting membrane after hybridization with cDNA probes for RIα, RIIα, and Cα. The three messages for RIα (3.2, 3.0, 1.7 kb), the message for RIIα (5 kb) and for Cα (2.4 kb) are all indicated by *arrows*. For further details, see "Experimental Procedures."

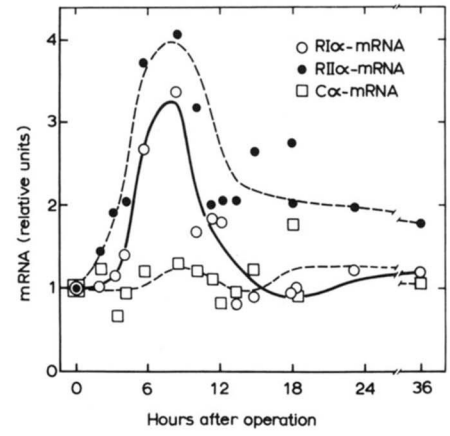

FIG. 7. **The levels of mRNA for cAMP-dependent protein kinase subunits during liver regeneration.** The levels of mRNA for RIα (○), RIIα (●), and Cα (□) were quantified by densitometric scanning of autoradiographs of hybridized Northern blots like the ones shown in Fig. 6. An equal amount of (total) liver RNA was loaded into each lane before the electrophoretic separation. Data are given as ratios of the signal strength at various times after partial hepatectomy (*abscissa*) and at the time of resection. It was verified that the signal measured was proportional to the amount of RNA loaded. Each point represents the mean value of three analyses. Since the strength of the mRNA signals is related to total liver RNA rather than DNA, they can be directly compared with the protein data of Fig. 1 if the ratio RNA/DNA is known. This ratio was not determined in the present study because it has been thoroughly studied by Lieberman and Kane (1965), who found that RNA/DNA was stable during the first 9 h postresection, and then increased linearly to reach a level 30% above initial 17 h after the operation (see also: Fausto, 1984).

studies of general gene expression during liver regeneration (Friedman *et al.*, 1984; Petropoulos *et al.*, 1985), little difference was noted between the pattern of labeled proteins using RNA from normal and regenerating liver (data not shown). The amount of protein synthesized per unit RNA was 20–50% increased in samples taken from the liver remnant 6–18 h after resection, as expected from earlier studies (Fausto, 1984) showing an increase of polyadenylated RNA during liver regeneration.

The Fractional Saturation of RI and RII by Endogenous cAMP after Liver Resection—The cAMP level was elevated throughout the period (36 h) of regeneration studied, and during the first 8 h after sham-operation (Fig. 8*C*), which is in general agreement with most previous studies (MacManus *et al.*, 1973; Thrower and Ord, 1974; Whitfield *et al.*, 1987). The cAMP elevation was accompanied by increased degrees of saturation of the binding sites of RI (Fig. 8*A*) and RII (Fig. 8*B*) in partially hepatectomized as well as sham-operated animals. The fractional saturation of RI was slightly higher than that of RII in the liver remnants with high cAMP levels. A similar tendency has been noted in intact livers from fed and 3-day fasted rats (Ekanger *et al.*, 1988), in the liver of overnight fasted rats, and isolated hepatocytes from fasted rats (data not shown), and probably reflects a slightly higher apparent cooperativity for endogenous cAMP binding to RI than to RII in liver (Ekanger *et al.*, 1988).

Since the cAMP surge occurring about 14 h postresection has been causally related to the ensuing DNA replication (Whitfield *et al.*, 1987) it was of interest to know if it involved the hepatocytes generally or was limited to only a fraction of the hepatocytes, *e.g.* the cells at a restriction point in late G1. In the latter case one would expect that only a fraction of the total liver RI and RII should be exposed to high levels of cAMP, and therefore that the degree of saturation of R should

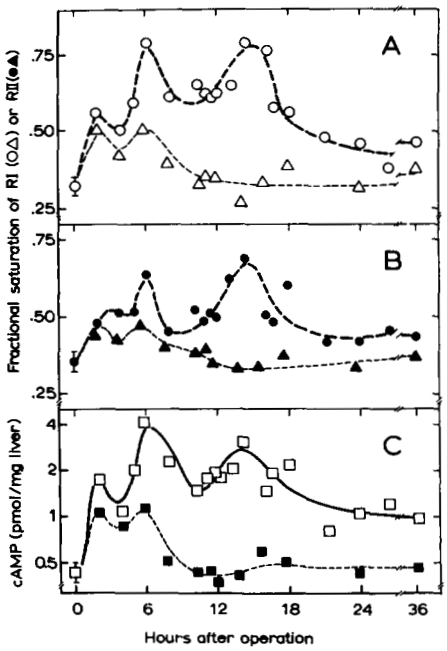

FIG. 8. **The degree of endogenous saturation of RI and RII during the fluctuations of liver cAMP occurring in the early stages of liver regeneration.** The fractional endogenous saturation of RI (*panel A*) and RII (*panel B*), as well as the cAMP concentration (*panel C*) was determined in the liver remnants of rats subjected to partial hepatectomy (○, ●, □). The corresponding values (△, ▲, ■) for liver biopsies from sham-operated animals are shown for comparison. The points with *error bars*, representing the results in the livers removed at the time of resection, are means ± S.E. (n = 24). The other points represent the mean values of determinations in biopsies from 2–3 animals. For further details, see "Experimental Procedures."

be disproportionally low. The fact that more than 75% of RI (Fig. 8*A*) and almost 70% of RII (Fig. 8*B*) was saturated with endogenous cAMP clearly showed that the prereplicative surge of cAMP involved the major part of the liver cell mass.

Byus *et al.* (1977) reported that the fractional kinase activity was 0.4 at the time of operation and increased to about 0.75 14 h postresection. We determined the fractional kinase activity, "activity ratio" (Corbin, 1983), in liver extracts trying several published methods (Cherrington *et al.*, 1976; Byus *et al.*, 1977; Schwoch, 1978; Erlichman *et al.*, 1983) for extraction. Unfortunately, using any of those procedures, when sample from normal liver and liver from an animal given glucagon intraportally (see Ekanger and Døskeland, 1988, for the procedure) was co-homogenized, the apparent degree of activation was higher than could be accounted for by the combined amounts of active kinase in each tissue powder measured separately. Furthermore, the degree of endogenous saturation of RI in selected samples of regenerating liver were significantly higher when the tissue was extracted according to the above mentioned procedures, than according to the presently used method (data not shown). For these reasons we restrained from direct measurement of the kinase activity ratio, and calculated the fractional activity of C as explained in the last paragraph under "Experimental Procedures" (Equation 1). The assumption was made that the degree of endogenous saturation was similar for soluble and particulate R, as is the case in isolated hepatocytes (Ekanger *et al.*, 1985).

The calculated fractional activity of C was 0.23 at the time of partial hepatectomy and was increased in all stages of regeneration, being 0.63, 0.33, 0.56, and 0.31, respectively, 6, 10, 14 and 22 h postresection. In the liver 6 h after sham-operation the fractional activity of C was 0.43. For comparison, the fractional saturation of R (RI + RII) was 0.70, 0.53, 0.72, and 0.45 at the same time points (6, 10, 14, and 22 h) after resection, and 0.46 6 h after sham-operation. It appeared thus that less kinase was active in the liver remnant 10 h after resection than in the liver 6 h after sham-operation even if the cAMP level and fractional saturation of R both were higher 10 h postresection (Fig. 8). This is explained by the excess of R relative to C 10 h postresection (Fig. 3).

DISCUSSION

A vigorous DNA synthesis commences in the liver remnant about 16 h after two-thirds hepatectomy in the rat and peaks 22–24 h postresection (Bucher and Malt, 1971). The present study includes the first 36 h after resection, the main emphasis being on the prereplicative period of regeneration. In the middle of the prereplicative period (8–10 h postresection) there was a severalfold increase of the mRNAs for RIα and RIIα, but not for Cα (Figs. 6 and 7). Comparative analysis of the peptides translated *in vitro* from mRNA isolated from regenerating and normal liver have disclosed very few differences (Friedman *et al.*, 1984; Petropoulos *et al.*, 1985; the present study). This means that the mRNA for the R subunits belongs to the smaller group (Friedman *et al.*, 1984; Petropoulos *et al.*, 1985) of messages that increase significantly during regeneration, whereas the mRNA content for C conforms to the rule. The increase of mRNA for RI and RII occurs at about the same time as the peak of mRNA for the p53 oncogene (Thompson *et al.*, 1986).

Little is known about the regulation of the genes for the protein kinase subunits and the turnover of their messages. During the first 12 h after resection the messages for RI and RII were similarly regulated (Fig. 7). Thereafter the RII message selectively stayed elevated. The 50% reduction in mRNA for RI and RII from 9 to 12 h after resection indicated that the half-life of their mRNAs was 3 h or shorter. The about 300% increase in mRNA for RI and RII between 5 and 8 h postresection (Fig. 7) could be due either to increased synthesis or stabilization of the messages. If stabilization were the only mechanism involved, the half-life of the unstabilized messages must be 1 h or less in normal liver to explain the rapid increase 5–8 h after partial hepatectomy.

There was a close temporal relationship between increases and decreases in mRNA for RI (Fig. 7) and the level of RI protein (Fig. 1). This suggests that the rate of turnover of RI protein must be relatively high, and gives no indication that the rate of translation of RI mRNA was altered during regeneration. On the other hand the accumulation of RII protein (Fig. 1) lagged considerably behind the appearance of the RII message (Fig. 7). This could be explained by assuming that newly formed RII mRNA was in a translationally less active form, and became translationally active only after a lag of several hours. A simpler explanation is based on the observation of Weber and Hilz (1986) that the half-life of RII protein is very long (125 h) in normal liver. From this it can be calculated that under equilibrium conditions (rate of synthesis equals rate of breakdown), the hourly synthesis of RII in normal liver is equivalent to 0.4% of the RII pool. If it is presumed that there is proportionality between the concentration of RII mRNA and the rate of RII synthesis, and that the rate of mRNA translation and of RII breakdown are as in normal liver, even a 4-fold increase of mRNA for RII should result in only 2–3% increase of RII/hour.

The selective increase in mRNA for the regulatory subunits (RI, RII) of the cAK isozymes (Fig. 7) led to overexpression of R relative to C in the prereplicative period of liver regeneration (Figs. 1 and 3). A normal proportion between the R

and C levels has been found in rat liver during development (Wittmaack *et al.*, 1983) and during the regeneration of normal liver (Sikorska *et al.*, 1983), but not after liver resection of vitamin D-deficient animals (Sikorska and Whitfield, 1985). Overexpression of R relative to C was first reported by Prashad *et al.* (1979) in neuroblastoma cells stimulated to differentiate by prolonged treatment with dibutyryl cAMP. These cells produced a form of RI that apparently had a decreased affinity for C (Prashad *et al.*, 1981). Also neuroblastoma-glioma hybrids overexpress RI when treated with dibutyryl cAMP (Walter *et al.*, 1979). RIIβ is selectively increased in Friend erythroleukemia cells or estradiol-primed granulosa cells exposed to prolonged treatment with cAMP-elevating agents (Schwartz and Rubin, 1983; Jahnsen *et al.*, 1986b). Liver regeneration differed from the abovementioned systems in that RI and RIIα were both increased (Fig. 1). Unlike the situation in neuroblastoma cells (Prashad *et al.*, 1981) the RI induced during liver regeneration behaved normally with respect to interaction with cAMP and C subunit (Fig. 5). Furthermore, the same three size-variants of the RI mRNA signal were detected in normal and regenerating liver (Fig. 6). The difference between liver regeneration and the other systems may be related to the fact that in neuroblastoma, granulosa, and erythroleukemia cells the increased expression of R is accompanied by differentiation, whereas the regenerating liver cells remain at their differentiation level (Friedman *et al.*, 1984; Petropoulos *et al.*, 1985).

The cAMP level was elevated throughout the period (36 h) of liver regeneration studied (Fig. 8). The selective increase of R may be regarded as a means of "down regulating" the kinase response to this prolonged cAMP elevation. Whitfield *et al.* (1987) consider that the prereplicative burst of cAMP (MacManus *et al.*, 1973) is a positive signal for the onset of DNA replication, but also that a decrease of cAMP is necessary before the cells can enter DNA synthesis. For hepatocyte proliferation in culture we have found that cAMP has a dual effect, being weakly stimulatory when moderately elevated (Brønstad *et al.*, 1983) and inhibitory when strongly elevated (Brønstad *et al.*, 1983; Vintermyr and Døskeland).[4] We consider it most likely that the increased R/C in the period before the hepatocytes start synthesizing DNA (Fig. 3) decreases the concentration of active C and thereby facilitates the entry into S-phase. It is of interest that the isoprenaline-induced DNA replication in rat parotid is preceded by a specific decrease of C (Schwoch, 1987), representing another mechanism of down regulation of the cAMP effector system preceding DNA replication in epitheloid cells.

The fact that the RI/RII ratio was higher in the liver remnant after resection (Fig. 1) than in the sham-operated controls (Fig. 2) adds the regenerating liver to the list (for reviews, see Russell, 1978; Døskeland and Øgreid, 1981) of biological systems where an increased RI/RII ratio is associated with cell anabolism and/or proliferation. It should be mentioned that the fluctuations in the RI/RII ratio found in the present study are nearly opposite of those noted by Sikorska *et al.* (1983), casting some doubt on the notion that a selective increase of cAKII triggers hepatocyte DNA synthesis (Boynton and Whitfield, 1980; Sikorska *et al.*, 1983; Whitfield *et al.*, 1987). The discrepancies between the two studies are not easily explained, the most obvious differences being that Sikorska *et al.* (1983) relied on ion-exchange chromatography to differentiate between RI and RII and that the rat strain was Sprague-Dawley rather than Wistar.

Liver regeneration gives the opportunity to study the endogenous binding of cAMP to RI and RII under conditions when the intracellular level of cAMP is elevated for a very protracted period of time independently of exogenously added agents. This was of special interest since Schwoch (1978) reported a selective activation of liver cAKI in response to long term (1 h) stimulation with glucagon, whereas we have been unable to find any considerable difference between the degrees of saturation of RI and RII in response to short term (90 s) glucagon stimulation of isolated hepatocytes (Ekanger *et al.*, 1985), or of intact normal liver from fed or fasted animals (Ekanger and Døskeland, 1988; Ekanger *et al.*, 1988). The finding of similar degrees of saturation of RI and RII during various phases of liver regeneration (Fig. 8) indicated that the intracellular interaction between R and cAMP had not been altered by the prolonged increase in cAMP or other postresection events in the hepatocytes. This does not rule out that RI and RII have separate roles in controlling hepatocyte DNA replication. We are currently testing this possibility by comparing the effects on the DNA synthesis of hepatocytes in culture of cAMP analog combinations with specificity for one of the R isotypes (Beebe *et al.*, 1988; Øgreid *et al.*, 1985).

[4] Our studies on the relation between cAK subunits and the inhibitory effect of cAMP on DNA replication in primary hepatocyte cultures (to be submitted for publication elsewhere) have shown a clear correlation between the amount of free (active) C subunit of the kinase and the degree of inhibition of DNA synthesis. The RI/RII ratio was consistently increased after treatment with the mitogen epidermal growth factor and decreased by treatment with dexamethasone, which inhibited hepatocyte DNA replication.

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