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The Regulatory and Catalytic Subunits of cAMP-dependent Protein Kinases Are Associated with Transcriptionally Active Chromatin during Changes in Gene Expression*

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Changes in the association of the catalytic subunit and the regulatory subunits of isozymes I and II of cAMP-dependent protein kinases (R_I and R_{II}, respectively) with the transcriptionally active chromatin fraction from rat liver were examined after a glucagon/theophylline injection and also after partial hepatectomy. Chromatin was partitioned into transcriptionally active and bulk, transcriptionally inactive fractions by digestion with micrococcal nuclease under appropriate conditions. In both experimental models, an increased content of catalytic and both R_I and R_{II} subunits was observed in chromatin fractions that were enriched in transcriptionally active DNA, particularly in the fraction associated with the residual nuclear matrix-lamina. The changes in the association of the subunits with these fractions paralleled the increases in intracellular cAMP levels and occurred in a time frame compatible with the changes in gene expression. The catalytic subunits could be removed from the nuclear matrix-lamina fraction by salt, whereas the two regulatory subunits remained tightly bound. The data support the concept of a direct role of the regulatory subunits of cAMP-dependent protein kinases in the induction of gene expression. However, we were unable to confirm that R_{II} possessed an intrinsic topoisomerase activity.

The second messenger cAMP plays a critical role in the 16-h-long prereplicative period that precedes the initiation of DNA synthesis in regenerating liver cells (reviewed in Ref. 1). Inhibitor studies have shown that the first burst of cAMP synthesis occurring between 1 and 4 h after surgery is essential for the cells to successfully complete mitosis, but not essential for the initiation of DNA synthesis (2); whereas the second burst occurring between 12 and 16 h after surgery is essential for cells to initiate DNA synthesis (3). These bursts of cAMP synthesis are followed closely by an accumulation of catalytic and regulatory subunits in the nucleus (4, 5), suggesting that the cyclic nucleotide is effecting changes in gene expression during prereplicative development, although the nature of these gene products is largely unknown.

Cyclic AMP and the two cAMP-dependent protein kinases are also known to mediate changes in gene expression in many diverse tissues, principally in response to stimulation by polypeptide hormones (6, 7). For example, in liver, glucagon induces the synthesis of tyrosine aminotransferase and phosphoenolpyruvate carboxykinase, together with a small number of other enzymes (6). In the cases of tyrosine aminotransferase and phosphoenolpyruvate carboxykinase, actual increases in the rates of transcription of the structural genes in response to stimulation of the cells with cAMP have been demonstrated (8, 9). Although the catalytic subunit has now been shown to be essential for the induction of the phosphoenolpyruvate carboxykinase gene (10), attention has focused recently on a more direct role for the regulatory subunits because of an amino acid sequence homology between these proteins and the catabolite activator protein in bacterial cells (even though this homology extends only to the cAMP-binding sites) and based upon the existence of a putative protein-binding sequence found upstream from the transcription start site of genes that are inducible by cAMP (11–13). This concept has gained more credence with the demonstration that R_{II}[1] possesses DNA-binding and topoisomerase-like activity (14, 15).

There is now evidence that two of the protooncogenes, c-*myc* and c-*fos*, that are expressed transiently during prereplicative development are induced by cAMP (16). Moreover, the above-mentioned upstream consensus sequence has been identified approximately 60 nucleotides upstream from the transcription start site of the c-*fos* gene (17). This suggests that, during prereplicative development, these are two of the critically important genes induced by the surges of cAMP. Furthermore, since neoplastic transformation appears to be accompanied by a disruption of the cAMP (and calcium) signal transduction mechanisms, an understanding of the way in which these second messengers regulate the expression of the protooncogenes is of vital importance (1, 18–20).

Recently, there have been several significant advances in chromatin fractionation techniques with the use of nucleases to partition chromatin into its transcriptionally active and inactive components (21, 22). Using this approach, it has also been shown that active genes are associated with the nuclear matrix (22–25). In this study, we have investigated the changes in the association of the catalytic subunit and both of the regulatory subunits of the cAMP-dependent protein kinases with liver chromatin and the nuclear matrix during prereplicative development and after a single injection of glucagon. The data show that the elevation of intracellular cAMP levels results in an increase in the association of all three subunits with the transcriptionally active fraction of chromatin in a time frame that is compatible with their role

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[1] The abbreviations used are: R_{II} and R_I, the regulatory subunits of isozymes II and I of cAMP-dependent protein kinases, respectively; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

as activators of gene expression. Although we have been unable to confirm the topoisomerase activity of R_{II}, the data indicate that the regulatory subunits are somehow involved in the mechanism by which cAMP effects transient changes in gene expression.

EXPERIMENTAL PROCEDURES

Materials—Micrococcal nuclease, glucagon, Kemptide fragment, ATP, cAMP, bovine serum albumin, pyridoxal phosphate, α-ketoglutarate, tyrosine, theophylline, benzamidine hydrochloride, aprotinin, phenylmethylsulfonyl fluoride, and dithiothreitol were obtained from Sigma. 8-Azido-[^{32}P]cAMP (specific activity ≥ 50 Ci/mmol), [^{3}H]cAMP (specific activity = 15–30 Ci/mmol), and [γ-^{32}P]ATP (specific activity = 10–25 Ci/mmol) were obtained from ICN Radiochemicals. Agarose-hexane-cAMP (type 3) and φX174 replicative form I DNA were from Pharmacia LKB Biotechnology Inc. Calf thymus topoisomerase I was from Bethesda Research Laboratories. Polygram CEL 300 polyethyleneimine/UV_{254}-cellulose plates were obtained from Brinkmann Instruments.

Isolation and Digestion of Nuclei—Nuclei were isolated from livers of male (190–210 g) specific pathogen-free Sprague-Dawley rats following either glucagon/theophylline injections (0.5 mg of glucagon + 1 mg of theophylline/100 g of body weight) or partial hepatectomy as previously described (26). The buffer used throughout the nuclear isolation procedure consisted of 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, and 0.25 M sucrose. This buffer and all the digestion and assay buffers described below contained 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM benzamidine, and 40 kallikrein units/ml aprotinin.

The nuclei were resuspended at a concentration 0.8–1.0 mg/ml DNA in 20 mM Tris-HCl, pH 8.5, and digested with micrococcal nuclease (50 units/ml) for 10 min at 30 °C as described previously (21). The reaction was terminated by adding EDTA to a final concentration of 10 mM, and the suspension was centrifuged at 25,000 × *g* for 15 min at 5 °C in the Ti-50 rotor of a Beckman L8-70 ultracentrifuge to produce a supernatant (S1) containing released, digested chromatin and a pellet (P1). Approximately 85% of the total nuclear DNA was solubilized under these conditions. The pellet (P1) was washed once with 20 mM Tris-HCl, pH 8.5, and centrifuged as described above to produce a second supernatant (S2) and a second pellet (P2). The pellet was resuspended in buffer containing 1.0 M NaCl, incubated for 30 min on ice, and centrifuged as described above to give a third supernatant (S3) containing salt-releasable chromatin fragments (approximately 12% of total nuclear DNA) and a third pellet (P3), which was washed once with 1.0 M NaCl buffer, generating a final pellet (P4) of insoluble nuclear matrix which still contained 2–3% of the total nuclear DNA.

Purification and Quantitation of Regulatory Subunits—R_{II} was purified from rat liver by affinity chromatography using the agarose-hexane-cAMP column essentially as described by Dills *et al.* (27). The intranuclear concentrations of R_I and R_{II} were measured on the various nuclear fractions either by the [^{3}H]cAMP-binding assay of Gilman (Ref. 28; see Ref. 5) or by photoaffinity labeling with 8-azido-[^{32}P]cAMP according to Friedman and Strittholt (29).

Topoisomerase Assay—R_{II} was tested for topoisomerase I activity as described by Constantinou *et al.* (14). The assay mixture for the relaxation of negatively supercoiled DNA contained 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM Na_2EDTA, 30 μg/ml bovine serum albumin, 1 μg of φX174 replicative form I DNA, and either R_{II} or calf thymus topoisomerase I as a standard in a total volume of 20 μl. Assays were carried out in the presence and absence of 10 μM cAMP. After incubation for 30 min at 25 °C, the reaction was terminated by the addition of 4 μl of stop solution (0.1 M Na_2EDTA, 0.5% sodium dodecyl sulfate, 60% sucrose, and 0.05% bromphenol blue). Aliquots containing 0.5 μg of DNA were then analyzed on 0.8% agarose gels run at 40 V (15 mA) for approximately 17 h in a buffer containing 50 mM Tris borate, pH 8.3, and 1 mM Na_2EDTA. After electrophoresis, the gels were stained for 30 min in 1 μg/ml ethidium bromide, destained for 30 min in 1 mM $MgSO_4$, and photographed using a CAMAG transilluminator.

Assay of Catalytic Subunit—The activity of the catalytic subunit of the cAMP-dependent protein kinases was assayed using the Kemptide fragment as substrate (30, 31). After incubation for 15 min at 30 °C, the reaction mixture (which consisted of 20 mM phosphate buffer, pH 7.1, 10 μM cAMP, 100 μM Kemptide fragment, 2.5 mM $MgCl_2$, 100 μM ATP, 5–8 × 10^5 cpm of [γ-^{32}P]ATP, and an aliquot of nuclear extract containing 20–30 μg of protein in a total volume of 20 μl) was analyzed by chromatography on polyethyleneimine-cellulose plates developed in distilled water to separate the phosphorylated substrate from other components of the reaction mixture. After autoradiography on Kodak SB-5 film, the radioactive spots were cut out and counted in a Beckman LS3801 liquid scintillation counter.

Hybridization—Chromatin fractions were deproteinized, and the DNA was isolated and purified as described previously (32). Samples of the DNA, sonicated to an average size of 400 base pairs, were immobilized on Hybond-N membranes (Amersham Corp.) using a Bio-Rad dot-blot apparatus. After irradiation, the membranes were prehybridized for 4 h at 65 °C in 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 20 μg/ml denatured salmon testis DNA and then hybridized for 18 h at 65 °C in fresh prehybridization buffer containing 50 ng/ml ^{32}P-labeled cDNA to either mouse globin or rat liver polysomal poly(A)$^+$ RNA. Following hybridization, the membranes were washed twice with 2 × SSC at 65 °C for 15 min, once with 2 × SSC containing 0.1% SDS at 65 °C for 30 min, and finally with 0.1 × SSC at 65 °C for 10 min. The dried filters were exposed using intensifying screens to Kodak SB-5 film at −70 °C for 48–120 h.

Other Assays—Tyrosine aminotransferase activity was assayed on liver homogenates of glucagon-injected rats by the method of Diamondstone (33). The cAMP content in livers of partially hepatectomized rats was measured by protein-binding assay (28). DNA synthesis in the liver remnant was determined by the incorporation of [^{3}H]thymidine (1.0 μCi/g of body weight) administered 1 h prior to death as described previously (4). Protein concentrations were measured by the method of Lowry *et al.* (34).

RESULTS

Characterization of Nuclear Subfractions—Micrococcal nuclease under appropriate conditions can be used to digest and release bulk chromatin into S1, leaving most of the transcriptionally active chromatin in P1 (21, 22). Fig. 1 shows dot blots of S1 and P1 DNA probed with cDNA to poly(A)$^+$ RNA to indicate the localization of transcriptionally active genes and cDNA to a globin gene which is not expressed in liver cells. The globin sequences were released along with bulk chromatin into S1, whereas the transcriptionally active genes were enriched in the fraction remaining in P1. The majority of the transcriptionally active chromatin can then be released from P1 with salt, leaving an insoluble pellet which, although containing only 2–3% of nuclear DNA, is highly enriched in transcribed sequences (22–25). Typical protein profiles of these fractions are shown in Fig. 2. Histone H1 and the four core histones were the most predominant proteins in S1, together with many non-histone proteins of higher molecular weight. When S1 samples were subjected to sucrose density gradient centrifugation (see Fig. 10 of Walker *et al.* (32)), these non-histone proteins remained at the top of the gradient, indicating that they were non-DNA-binding nucleoplasmic proteins; whereas the histone-containing transcriptionally inactive chromatin fragments moved into the gradient. The chromatin released into S3 by salt also contained both core histones and H1, but was highly enriched in non-histone proteins. The residual P4 protein profile contained all the proteins characteristic of the nuclear matrix and nu-

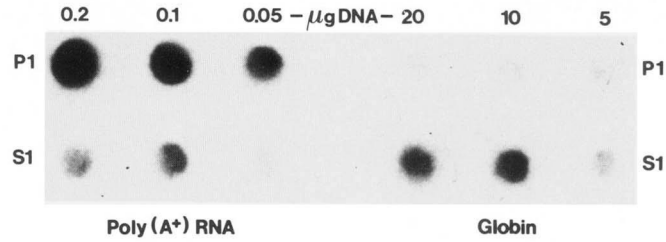

FIG. 1. **Dot-blot hybridization of chromatin fractions.** S1 and P1 DNA samples were blotted in the amounts indicated and hybridized to either globin cDNA or cDNA to polysomal poly(A)$^+$ RNA as described under "Experimental Procedures."

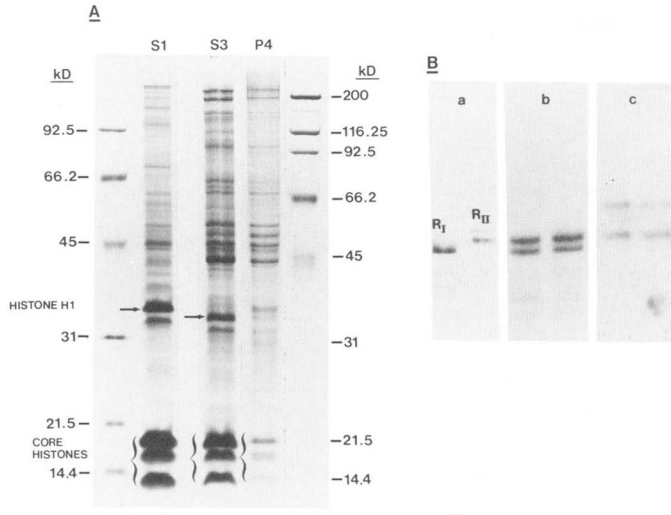

FIG. 2. **Protein analysis of liver chromatin fractions.** *A*, nuclei were fractionated into subfractions S1, S3, and P4 as described under "Experimental Procedures," and their protein content was analyzed by SDS-PAGE (10% (w/v) gel). The electrophoresis was run at a constant current of 30 mA/gel, and the gels were stained with Coomassie Blue after alcohol fixation. The molecular size markers used were: lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31.0 kDa; ovalbumin, 45.0 kDa; bovine serum albumin, 66.2 kDa; phosphorylase *b*, 92.5 kDa; β-galactosidase, 116.25 kDa; and myosin, 200.0 kDa. Low range molecular size markers refer to S1, and high range ones to S3 and P4. *B*, 10% (w/v) SDS-PAGE of purified R_I and R_{II} stained with Coomassie Blue (*panel a*), 8-azido-[^{32}P]cAMP binding carried out on P4 as described under "Experimental Procedures" (after transfer to nitrocellulose the membrane was autoradiographed on Kodak SB-5 x-ray film for 18 h (*panel b*), and Western blot of the same fraction as in *panel b* using a rat liver R_{II}-specific polyclonal antibody as described in the text (*panel c*).

clear lamina, together with small amounts of core histones.

Identification of Regulatory Subunits—Changes in the amounts of regulatory subunits associated with the various subnuclear fractions were estimated by the [^{3}H]cAMP-binding assay. This assay reproducibly measures the sum total of R_I and R_{II}, but does not easily discriminate between the two. The identity of the subunits was confirmed by photoaffinity labeling with 8-azido-[^{32}P]cAMP as shown in Fig. 2*B*. *Panel a* shows the migration of purified R_I and R_{II} in gels stained with Coomassie Blue. *Panel b* shows an autoradiograph of photoaffinity-labeled (with azido-cAMP) proteins from P4 which were subjected to electrophoresis under the same conditions as in *panel a*. Two bands, corresponding to R_I and R_{II}, were clearly visible. Because of variability in labeling, autoradiographic exposure times, etc., photoaffinity labeling was not used quantitatively; however, in general, trends similar to those observed by ^{3}H binding were apparent. *Panel c* confirms the identity of the R_{II} band with a Western blot of the same fraction probed with a rat liver R_{II}-specific antibody affinity-purified from rabbit antisera (kindly supplied by Dr. J. Kwast-Welfeld, Northwestern University, Chicago). This antibody detected a protein corresponding to the upper of the two bands detected by azido labeling, although a second protein of M_r = 68,000 was also recognized. Taken together, the data in Fig. 2*B* show that azido labeling effectively detects both R_I and R_{II}.

Changes in the Concentration of Regulatory Subunits in Nuclear Subfractions following Glucagon Injection—Tyrosine aminotransferase was used as an indicator of a change in gene expression following a single intraperitoneal injection of a glucagon/theophylline mixture. The enzyme's activity remained unchanged for 15 min and then rose during the next 3 h after injection, giving a 2–3-fold increase in activity, followed by the onset of a decline at 4 h (Fig. 3*A*). This change in enzyme activity parallels exactly the change in tyrosine aminotransferase mRNA levels following a single injection of cAMP (8). Since there is also an increase in the actual rate of transcription of the gene (8), it is clear that glucagon effects a change in gene expression mediated by cAMP.

The combined content of R_I and R_{II} was estimated with the [^{3}H]cAMP-binding assay in three of the nuclear subfractions (S1, S3, and P4) following a single injection of glucagon/theophylline (Fig. 3). There were 3-fold increases in the levels of the regulatory subunits in S3 and P4 which commenced about 15 min after hormone injection and paralleled the changes in tyrosine aminotransferase activity. The correlation between these changes is shown more clearly in Fig. 3*B*, in which change in enzyme activity is plotted against change in R subunit concentration in both S3 and P4. Linear regression analysis of the data showed that a similar line can be drawn through both sets of data with correlation coefficients of 0.97 and 0.96 for S3 and P4, respectively. The concentration of R subunits also increased in S1 during the first 2 h after hormone injection, but declined to uninduced levels by 4 h. Sucrose density gradient analysis of S1 fractions showed that the [^{3}H]cAMP activity remained on top of the gradient, which indicated that the subunits were free rather than associated with the chromatin fragments (data not shown).

Photoaffinity labeling experiments with 8-azido-[^{32}P]cAMP revealed the presence of both R_I and R_{II} in all three of the nuclear subfractions (Fig. 4). Analyses were carried out on control, uninjected animals and animals at 1, 2, and 3 h after hormone injection. Although both R_I and R_{II} bands were detectable in S3 fractions, most of the labeling was associated with the characteristic degradation products of these proteins (27). We were unable to prevent this degradation despite using all of the conventional protease inhibitors in our extraction and assay buffers. Furthermore, since approximately equal amounts of non-histone proteins were subjected to

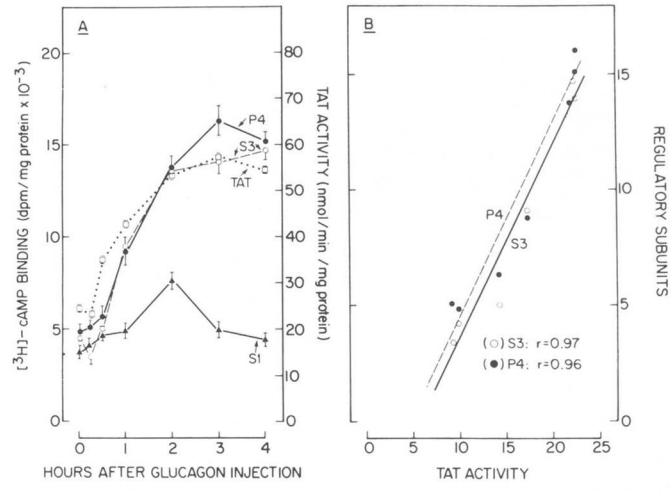

FIG. 3. **Changes in the concentration of the regulatory subunits from the cAMP-dependent protein kinases in nuclear subfractions and the induction of tyrosine aminotransferase activity in liver following an injection of glucagon/theophylline.** *A*, the concentration of regulatory subunits was measured using the [^{3}H]cAMP-binding technique as described under "Experimental Procedures." Tyrosine aminotransferase (*TAT*) activity was measured in total liver homogenates, and each point is the mean ± S.E. from four to eight determinations. *B*, linear regression analysis of the relationship between changes in tyrosine aminotransferase activity and the concentrations of regulatory subunits in S3 and P4 chromatin fractions. *r*, correlation coefficient.

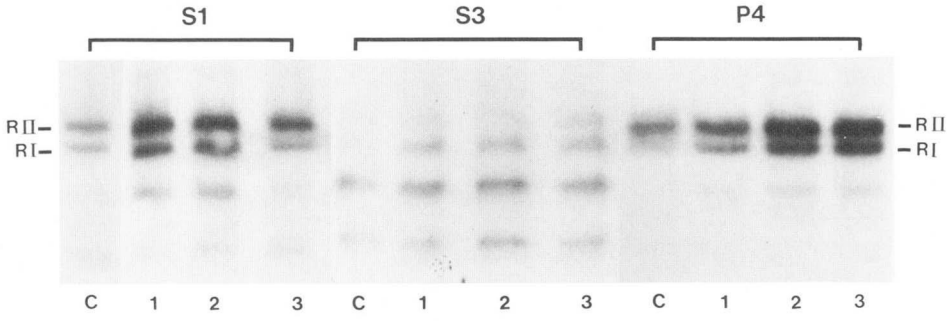

FIG. 4. **Photoaffinity labeling of cAMP-binding proteins in nuclear fractions following an injection of glucagon/theophylline.** 8-Azido-[^{32}P] cAMP binding was carried out for 60 min at 4 °C, followed by UV irradiation and SDS-PAGE (10% (w/v) gel). The proteins were then transferred to nitrocellulose at 10 V (100 mA) for 18 h and autoradiographed on Kodak SB-5 x-ray film. *Lane C*, control uninjected animal; *lanes 1–3*, 1, 2, and 3 h after hormone injection, respectively.

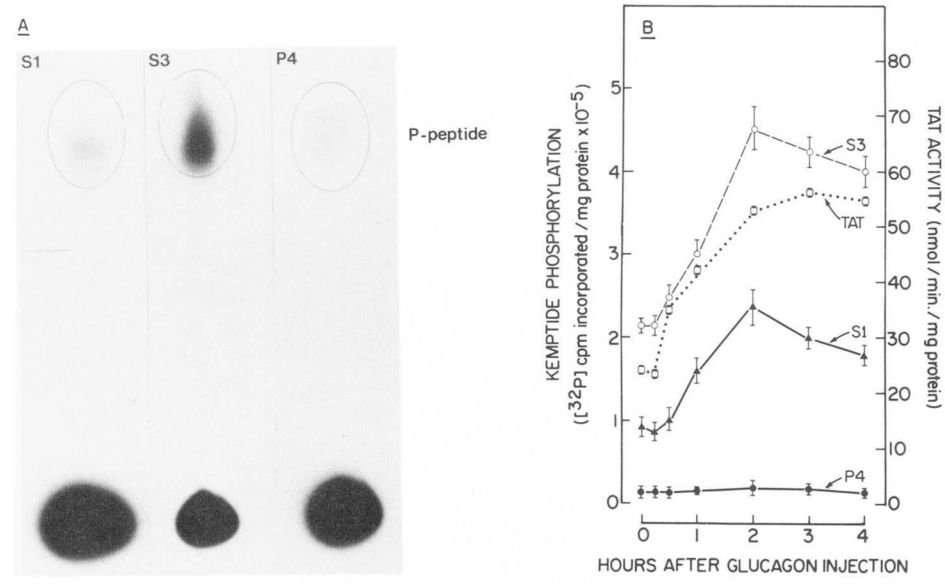

FIG. 5. **Changes in the activity of catalytic subunits in the nuclear fractions following a single injection of glucagon/theophylline.** Subunit activity was quantitated by phosphorylation of the Kemptide fragment. After a 15-min incubation at 30 °C with [γ-^{32}P]ATP, the reaction mixture was analyzed by chromatography on polyethyleneimine-cellulose plates developed in distilled water to separate the phosphopeptide (*P-peptide*) from the components of the reaction mixture (*A*, Kemptide phosphorylation by chromatin fractions at 2 h after hormone injection). After autoradiography, the radioactive phosphopeptide spots were cut out, and the radioactivity was determined by liquid scintillation counting. The data points in *B* represent mean ± S.E. from four to eight experiments. *TAT*, tyrosine aminotransferase.

photoaffinity labeling (*cf.* Fig. 2*A*), in each fraction, it appeared that either photoaffinity coupling was partially blocked in fraction S3 or the subunits were degraded to the small fragments which, although still capable of binding cAMP (35), could not be photoaffinity-labeled. There were also minor levels of degradation in S1 and P4 fractions, but the majority of the labeling was associated with the regulatory subunits in these fractions (*cf.* Fig. 2). Moreover, there was no evidence for the presence of any other cAMP-binding proteins in any of the nuclear fractions. The data show that glucagon/theophylline caused increases in both R_I and R_{II} in all these fractions, particularly in P4.

Changes in the Activity of Catalytic Subunits in Nuclear Fractions following Glucagon Injection—The activity of catalytic subunits released from the cAMP-dependent protein kinase holoenzymes was measured by phosphorylation of the synthetic Kemptide peptide fragment. This allows the specific detection of catalytic subunit activity in the presence of a number of other nuclear protein kinases. Assays were performed on S1, S3, and P4 following a single injection of glucagon/theophylline as shown in Fig. 5. As with the regulatory subunits, there was a 2.5–3-fold increase in the concentration of catalytic subunits in S3 which paralleled exactly the time course of induction of tyrosine aminotransferase. A similar increase was seen in unbound catalytic subunits released into S1. In contrast, only a low basal level of catalytic subunit activity was observed in P4 which remained unchanged following hormone injection.

Changes in Regulatory and Catalytic Subunits of cAMP-dependent Protein Kinases in Nuclear Subfractions following Partial Hepatectomy—Changes in the association of the catalytic subunits and R_I and R_{II} with nuclear subfractions S3 and P4 were measured during the prereplicative period of liver regeneration at the times (1–4 and 12–16 h) when cAMP levels are elevated (3). We have previously demonstrated (4, 5) that there are increases in total nuclear levels of catalytic subunits and both type I and II regulatory subunits during these time periods. As shown in Fig. 6*A*, there were increases in the amount of catalytic subunit activity associated with the salt-extracted, transcriptionally active chromatin in S3 which paralleled the increases in intracellular cAMP concentration at both time intervals (1–4 and 12–16 h) after partial hepatectomy. However, as with the glucagon-injected animals, there was no significant change in the low basal level of catalytic subunits associated with P4. There were similar increases in [^{3}H]cAMP binding in S3 and P4 in response to partial hepatectomy at 1–4 and 12–16 h after operation (Fig. 6*B*).

Photoaffinity labeling experiments confirmed the increased content of both R_I and R_{II} in S3 and P4 in hepatectomized animals at these time periods (data not shown).

Assay of Purified R_{II} for Topoisomerase Activity—R_{II} was purified as described under "Experimental Procedures," phosphorylated with purified catalytic subunit as described in the legend to Fig. 7, and assayed for its ability to relax supercoiled ϕX174 DNA in an ATP-independent manner. Fig. 7*A* shows a silver-stained gel of purified liver R_{II} before phosphorylation. Apart from the presence of a low level of the characteristic degradation fragments and a minor contamination with R_I, the protein was essentially pure. Fig. 7*B* shows the effect of increasing amounts of phosphorylated R_{II} on the degree of supercoiling of ϕX174 DNA in the presence and absence of cAMP. Although even the lowest concentration of R_{II} tested was able to convert the supercoiled DNA to its fully relaxed

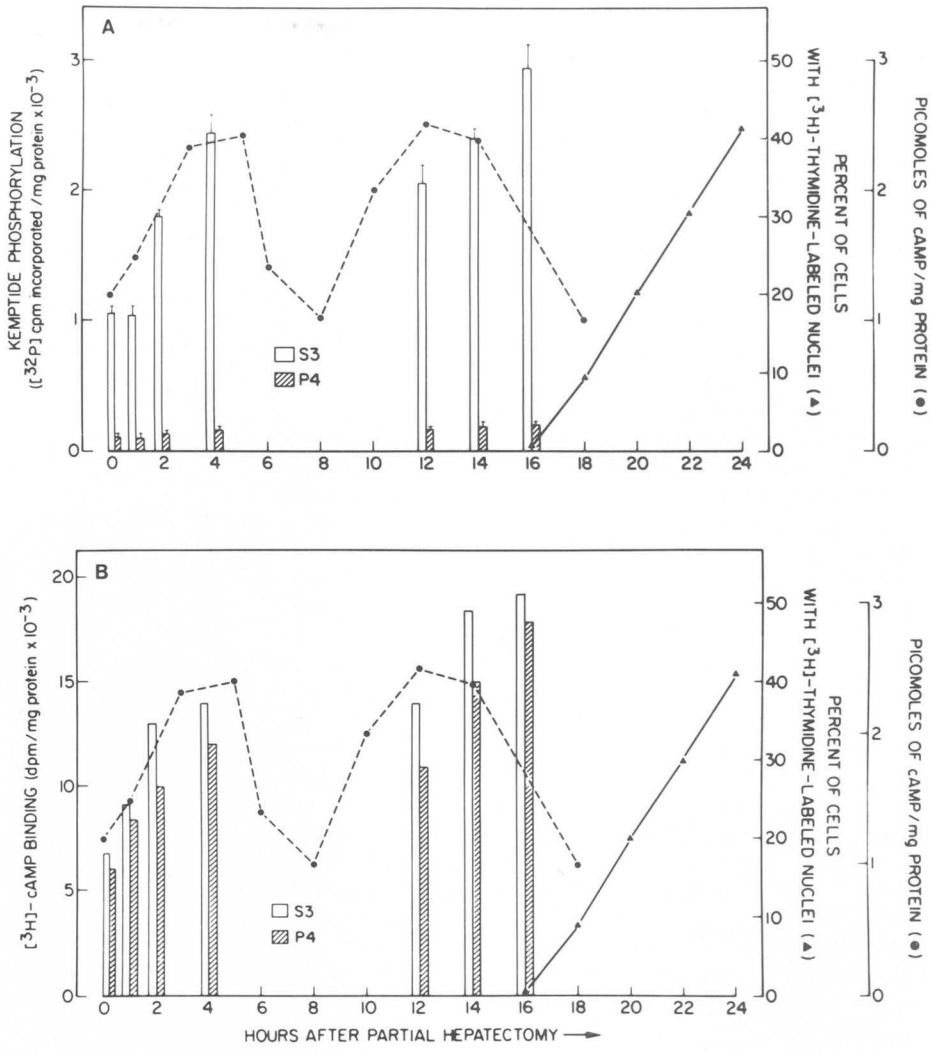

FIG. 6. **Changes in the concentration of regulatory subunits and the activity of catalytic subunits after partial hepatectomy.** *A*, the activity of catalytic subunits was estimated in subnuclear fractions S3 and P4 using the Kemptide phosphorylation assay as described in the legend to Fig. 5. Each point is the mean ± S.E. from five to seven determinations. *B*, the concentration of the regulatory subunits in S3 and P4 was estimated with the [^{3}H]cAMP-binding assay and the cAMP content in liver by the cAMP protein-binding assay. DNA synthetic activity is expressed as the percentage of parenchymal cell nuclei which incorporated [^{3}H]thymidine during a 1-h period before death. Each point is the mean ± S.E. from five to seven determinations.

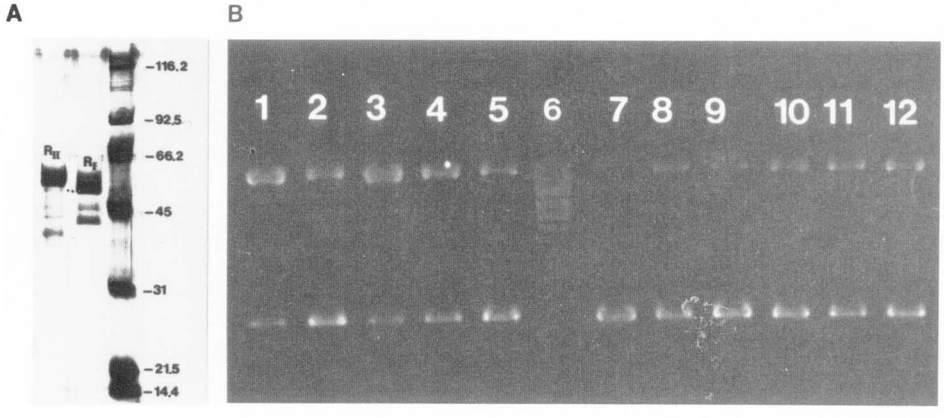

FIG. 7. **Effect of purified rat liver R_{II} on the relaxation ϕX174 supercoiled DNA.** *A*, silver-stained 10% SDS-polyacrylamide gel showing the R_{II} preparation used in the relaxation assay and an R_I preparation for comparison, together with molecular size markers (listed in the legend to Fig. 2). Purified R_{II} was phosphorylated with the catalytic subunit in the presence of $MgCl_2$ and ATP. The components of the reaction mixture were removed by ion-exchange chromatography on a Mono Q column in the presence of 10 μM cAMP, and phosphorylated R_{II} was eluted with a salt gradient (0–0.6 M). Phosphorylated R_{II} was used in the relaxation assay. *B*, ethidium bromide-stained 0.8% agarose gel of ϕX174 DNA after it was incubated for 30 min at 25 °C with various amounts of phospho-R_{II} cAMP (*lanes 1*, *3*, *5*, *9*, and *11*) or phospho-R_{II} (*lanes 2*, *4*, *8*, *10*, and *12*). The detailed protocol for the relaxation assay is given under "Experimental Procedures." The amounts of R_{II} tested ranged from 10 pg to 5 μg/1 μg of ϕX174 DNA/assay. *Lanes 1* and *2*, 5 μg; *lanes 3* and *4*, 2 μg; *lanes 5* and *8*, 1 μg; *lanes 9* and *10*, 500 ng; *lanes 11* and *12*, 100 ng; *lane 6*, 20 units of calf thymus topoisomerase I; *lane 7*, no enzyme.

form, there was no indication of the typical ladder of intermediate topoisomers that was generated by the purified calf thymus topoisomerase used as a standard (*lane 6*). Therefore, purified R_{II} did not have topoisomerase-like activity, but either it had an intrinsic endonuclease activity or the preparation was contaminated by an endonuclease. It was noteworthy that cAMP stimulated this endonuclease activity, leading to the accumulation of more fully relaxed and linearized DNA molecules.

DISCUSSION

The catalytic and R_I and R_{II} subunits of the cAMP-dependent protein kinases are associated with the transcriptionally active fraction of chromatin. The two regulatory subunits are also bound to the nuclear matrix, but not to bulk, transcriptionally inactive chromatin. Moreover, since no other cAMP-binding proteins are detectable in the nucleus, the effects of cAMP on gene expression must be mediated via one or all of these three proteins. Within minutes after glucagon binds to the membrane of hepatocytes and at two critical stages of prereplicative development when cAMP levels increase, there is an accumulation of all three subunits in the transcriptionally active fraction of liver chromatin. These observations indicate a direct kinetic link between the binding of these components of the cAMP signal transduction system to active chromatin and an increase in the rate of gene transcription.

Although we have demonstrated a rapid accumulation of all three subunits in the nucleus, the chain of events between the elevation of cytoplasmic and, presumably, nuclear (7) levels of cAMP and the increased detection of these proteins in association with transcriptionally active chromatin and the matrix is still not clear. There is general agreement that catalytic subunits translocate into the nucleus in response to stimulation of cells or tissues with cAMP or agents which elevate cAMP levels (reviewed in Ref. 7), but there is still no consensus on the movement of the regulatory subunits or, indeed, whether they are present in nuclei under basal conditions. For example, R_{II} was not found in the nucleus in brain cells (36), rapidly growing MDA-MB-231 breast cancer cells, retinoblastoma cells, or *Dictyostelium* cells (37–39). However, when the retinoblastoma cells were induced to differentiate (38) and during developmental growth of *Dictyostelium* (39), there was an accumulation of R_{II} in the nucleus. Several other workers (15, 19, 40) have shown that regulatory subunits, principally R_{II}, are translocated to the nucleus in cAMP-stimulated Chinese hamster ovary, 3T3, H4IIE, and H35 cells. In contrast, Nigg *et al.* (41) reported that catalytic subunits, but not regulatory subunits, translocate to the nucleus in the cAMP-stimulated Madin-Darby canine kidney cells. Whereas there is general agreement that the catalytic subunit and both regulatory subunits are present in liver nuclei under basal conditions (42, 43), there is considerable confusion over their changes in response to agents that affect gene expression. Thus, Koide *et al.* (42) observed that all three subunits increase in liver nuclei after glucagon treatment, whereas Kuettel *et al.* (43) reported that only catalytic subunits are translocated in response to the same hormone. Furthermore, R_I along with the catalytic subunits, but not R_{II}, were shown to accumulate in the nucleus at 16 h after partial hepatectomy (40).

Most of these studies were based upon immunocytochemical techniques which have some limitations (7), and it is conceivable that the antibody may not detect subunits that enter the nucleus and become tightly bound to other nuclear components in such a way that their antigenic determinants are shielded. Based upon the nuclear accumulations of subunits observed in this study, cAMP would appear to mediate a translocation of the catalytic subunit and the two regulatory subunits from the cytoplasm to the nucleus. Whether this requires prior dissociation of the holoenzymes or whether an intact ternary cAMP-holoenzyme complex (18) is translocated remains to be established. On the other hand, holoenzyme proteins may be bound to chromatin or some nuclear substructure under basal conditions and only become detectable under conditions that cause their dissociation (5). It is not inconceivable therefore that cAMP may cause either an intranuclear redistribution of the protein kinase subunits to the active chromatin fraction or a change in the structural microenvironment surrounding holoenzyme molecules already bound near their sites of action.

Whatever the mechanism may be, it rapidly leads to the accumulation of these proteins in the active fraction of chromatin within a time frame that is compatible with induction of the tyrosine aminotransferase gene in glucagon-treated animals and at times after partial hepatectomy when there are changes in gene expression (44, 45). The close correlation between the accumulation of the subunits in active chromatin and the appearance of tyrosine aminotransferase protein suggests that these are linked phenomena. Moreover, the time course of induction indicates that the proteins must be bound to active chromatin for the duration of the response. Indeed, the half-life of association, which must be a function of the level of cAMP, may determine the duration of the increase in gene transcription. Multiple injections of cAMP have been shown to be required to maintain high rates of transcription of the tyrosine aminotransferase gene (8), presumably by driving the association of the kinase subunits with active chromatin. When cAMP levels fall, free regulatory subunits accumulate and recombine with catalytic subunits to form inactive holoenzyme molecules which leave the nucleus. This provides a mechanism by which the cAMP-inducible gene is shut off once the stimulus has decayed.

Since both regulatory subunits remain tightly bound to the residual nuclear matrix fraction even after the catalytic subunits have been dissociated by high salt (Figs. 3 and 6), they appear to have specific, high affinity binding sites in this fraction. This observation raises the possibility that more than one of the subunits is necessary for cAMP-mediated changes in gene expression, particularly since the same changes are observed in response to two different stimuli, glucagon injection and prereplicative development.

There is now good evidence that the catalytic subunit is essential for increasing the rate of transcription of the tyrosine aminotransferase gene (10). Furthermore, the specific phosphorylation of a 23-kDa basic nuclear protein accompanies the induction of prolactin by forskolin in a cAMP-mediated reaction (46), and histone H1 is phosphorylated in regenerating liver (47), implying that phosphorylation events do indeed accompany or precede changes in gene expression. If this is the case, then there must be a kinase-phosphatase cycle to regulate the level of the phosphoprotein intermediate(s); and since R_I and R_{II} have been shown to be inhibitors of the phosphatase reaction (48, 49), their presence in the nucleus may be required to control this cycle. The regulatory subunits are therefore important components of the gene activation since these are the molecules that actually monitor the level of cAMP and eventually stop the induction process as described above.

More recently, attention has been focused on possible additional roles for the regulatory subunits (12). Most, but not all, cAMP-inducible genes have a common nucleotide sequence upstream from the transcription start site (11–13, 17),

and R_{II} appears to have a high affinity for this sequence (15). However, although recent work (13) has shown that this site alone is insufficient to confer inducibility, it may still bind one of the components either directly or via a binding protein which also has an affinity for one of the subunits. The observation that plasmids carrying this sequence are not expressed in cells that have only the type I cAMP-dependent kinase but are induced by the introduction of the type II holoenzyme suggests that R_{II} is particularly important in the gene activation process (13). The demonstration that R_{II} may possess an intrinsic topoisomerase activity (14) provided a possible role for this protein. Topoisomerases are enzymes that manipulate the torsional stress on DNA and as such appear to be necessary during replication and transcription of the genetic material (50). However, we have been unable to confirm the presence of a topoisomerase activity (Fig. 7), although the R_{II} preparation appears to be contaminated with an endonuclease activity which can cleave supercoiled DNA.

Finally, this work helps to define better the role of the cAMP signal transduction system in the regulation of prereplicative development in regenerating liver. The catalytic and regulatory subunits accumulate in transcriptionally active chromatin at the two stages when there are critical changes in gene expression (1, 44) including the expression of the c-*fos* and c-*myc* protooncogenes which may play key roles in the regulation of the expression of other genes including those for the enzymes of DNA replication. Clearly, cAMP and its dependent protein kinases are critical regulators of prereplicative development, and a knowledge of how they act will contribute substantially to our understanding of neoplastic transformation, a principal component of which is the disruption or by-passing of both the calcium- and cAMP-dependent signal transduction mechanisms (1, 18–20).

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