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Modulation of nuclear cyclic AMP-dependent protein kinase in dibutyryl cyclic AMP-treated rat H4IIE hepatoma cells

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Biochemical and immunochemical studies were undertaken to quantify the effects of cyclic AMP on cyclic AMP-dependent protein kinase subunit levels in nuclei of H4IIE hepatoma cells. Dibutyryl cyclic AMP (10 μM) caused a significant biphasic (10 and 120 min after stimulation) increase in total nuclear protein kinase activity. The increase observed 10 min after dibutyryl cyclic AMP stimulation was primarily due to an approx. 3-fold increase of catalytic (C) subunit activity, whereas the change observed 120 min after stimulation consisted of an increase in both C subunit and cyclic AMP-independent protein kinase activities. Analysis of nuclear protein extracts by photoaffinity labelling with 8-azido cyclic [^{32}P]AMP identified only the type II regulatory subunit (RII), but not the type I regulatory subunit (RI). Analysis of nuclear RII variants by two-dimensional gel electrophoresis demonstrated that dibutyryl cyclic AMP caused the appearance of two RII variant forms which were not present in the nuclei of unstimulated cells. Using affinity-purified polyclonal antibodies and immunoblotting procedures, we identified an approx. 2-fold increase in the RII and C subunits in nuclear extracts of dibutyryl cyclic AMP-treated hepatoma cells. Finally, the RI, RII and C subunits were quantified by an e.l.i.s.a. which indicated that dibutyryl cyclic AMP increased nuclear RII and C subunit levels biphasically, reaching peak values 10 and 120 min after the initial stimulation. Nuclear RI subunit levels were not affected. These results provide qualitative as well as quantitative evidence for a modulation by cyclic AMP of the nuclear RII and C subunit levels in rat H4IIE hepatoma cells, and indicate a relatively rapid but temporarily limited dibutyryl cyclic AMP-induced translocation of the RII and C subunits to nuclear sites.

INTRODUCTION

Phosphorylative modification of key regulatory nuclear proteins by cyclic AMP-dependent protein kinase has been implicated in the regulation of gene activity, primarily on the basis of a temporal correlation between activation of nuclear cyclic AMP-dependent protein kinase and phosphorylation of nuclear substrates on the one hand and induction of the synthesis of several RNA species on the other (for review see Jungmann & Kranias, 1977). Although most studies on the regulation of gene expression have emphasized aspects directly related to mRNA and enzyme synthesis, there is relatively little information about the very early events of cyclic AMP action at the level of cyclic AMP-dependent protein kinase.

In rat hepatoma cells, several studies have provided evidence for a functional role of cyclic AMP-dependent protein kinase in tyrosine aminotransferase induction. It has been reported that tyrosine aminotransferase synthesis is tightly coupled to cyclic AMP-mediated protein kinase activation, suggesting a causal relationship between the two processes (Culpepper & Liu, 1983; Wimalasena & Wicks, 1979). Further support for this relationship has come from studies by Boney *et al.* (1983). Using a micro-injection technique, they demonstrated an inductive effect of the C subunit on tyrosine aminotransferase synthesis in H4IIE hepatoma cells. However, micro-injection of a RII-subunit preparation blocked the cyclic AMP-mediated induction of tyrosine aminotransferase. In other cell systems, it has been demonstrated that an active C subunit (Reisine *et al.*, 1985; Grove *et al.*, 1987) and the type II cyclic AMP-dependent protein kinase (Montminy & Bilezikjian, 1987) may be necessary mediators in the cyclic AMP stimulation of gene transcription.

The primary goals of our laboratory are to understand the role of cyclic AMP-dependent protein kinase subunits in cyclic AMP-mediated enzyme induction. To that effect, we have previously shown that isoprenaline enhances transcription of the lactate dehydrogenase A-subunit gene in rat C6 glioma cells via a cyclic AMP-mediated mechanism (Jungmann *et al.*, 1983). The onset of lactate dehydrogenase mRNA transcription was shown to be preceded by an activation of cytoplasmic and nuclear cyclic AMP-dependent protein kinase (Jungmann *et al.*, 1979) and phosphorylative modification of several histones (Harrison *et al.*, 1982) and RNA polymerase II subunits (Lee *et al.*, 1984). Although the precise functional significance of these nuclear events in the regulation of lactate dehydrogenase mRNA transcription remains unknown, we decided to determine whether cyclic AMP-mediated gene regulation in rat H4IIE hepatoma cells is preceded or accompanied by a similar modulation of

Abbreviations used: C subunit, catalytic subunit; RI and RII subunit, type I and type II regulatory subunit; PAGE, polyacrylamide-gel electrophoresis.

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cyclic AMP-dependent protein kinase at the nuclear level. If so, these findings would provide an experimental basis to investigate a potential functional correlation between modulation of the cyclic AMP-protein kinase system and enzyme induction. In this paper we present a detailed quantitative analysis of the effects of dibutyryl cyclic AMP on nuclear cyclic AMP-dependent protein kinase subunit levels in rat H4IIE hepatoma cells during a time period preceding induction of phosphoenolpyruvate carboxykinase and tyrosine aminotransferase.

EXPERIMENTAL

Chemicals

8-Azido cyclic [^{32}P]AMP (sp. radioactivity 90 Ci/mmol) was from International Chemical and Nuclear, Irvine, CA, U.S.A., and [γ-^{32}P]ATP (sp. radioactivity 3000 Ci/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. Benzamidine was from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; phosphocellulose paper (P81) was from Whatman, Clifton, NJ, U.S.A.; nitrocellulose membranes were from Schleicher and Schuell, Keene, NH, U.S.A. Enzyme-grade sucrose was obtained from Schwarz/Mann Co., Orangeburg, NY, U.S.A. Kemptide (a synthetic heptapeptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly), ATP, cyclic AMP, N^6,$O^{2'}$-dibutyryl cyclic AMP, phenylmethanesulphonyl fluoride, EGTA, EDTA and all other analytical-grade chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. XRP-5 X-ray film was obtained from Eastman Kodak Co., Rochester, NY, U.S.A.

Cell culture

A cloned cell line (H4IIE) derived from Reuber H35 rat hepatoma cells was kindly provided by Dr. Daryl K. Granner (Vanderbilt University, Nashville, TN, U.S.A.). The cells were maintained as monolayer cultures at 37 °C in an atmosphere of 5 % CO_2 in air in Swim's 77 medium supplemented with 2.5% (v/v) fetal-calf serum, 2.5% (v/v) newborn-calf serum, 50 units of penicillin/ml, 50 μg of streptomycin/ml, 1.2 mg of glucose/ml, 2.4 mM-$CaCl_2$, 60 mM-cystine and 60 mM-Tricine. Cells were grown to confluence (about 4 days after seeding) in 850 cm^2 roller bottles containing 100 ml of culture medium; 18 h before addition of dibutyryl cyclic AMP (0.5 mM), the medium was replaced with serum-free Swim's 77 medium and incubation was continued at 37 °C until cell harvest.

Preparation of cytosol

Confluent H4IIE hepatoma cells were scraped into 5 ml of ice-cold serum-free Swim's 77 medium, sedimented by centrifugation at 10000 *g* for 10 min, and washed with serum-free Swim's 77 medium. The cell pellet was resuspended and homogenized in homogenization buffer (HB buffer), consisting of 10 mM-Tris/HCl, pH 7.4, 4 mM-$MgCl_2$, 20 mM-benzamidine, 0.5 mM-EDTA, 0.5 mM-EGTA, 0.5 mM-phenylmethanesulphonyl fluoride, 2 mM-dithiothreitol, 0.25 mM-sucrose and 0.1 % Triton X-100. The homogenate was centrifuged for 10 min at 10000 *g*. The supernatant fraction was centrifuged at 105000 *g* for 60 min, yielding a soluble fraction defined as cytosol.

Isolation and purification of nuclei

H4IIE hepatoma cells were homogenized in HB buffer as described above. The homogenate was centrifuged for 10 min at 10000 *g*. The 10000 *g* pellet was resuspended in HB buffer, layered on to 1.0 M-sucrose, and centrifuged at 200000 *g* for 60 min. The pelleted nuclei were resuspended in 0.15 M-NaCl for 15 min and re-pelleted by centrifugation for 10 min at 10000 *g*. This treatment prevents the nonspecific adherence of dissociated cytoplasmic C subunit to nuclei (Keely *et al.*, 1975). The nuclei were resuspended in 50 mM-Tris (pH 8.3)/40% (v/v) glycerol/5 mM-$MgCl_2$/0.1 mM-EDTA/0.5 mM-EGTA/2 mM-dithiothreitol. The concentration of nuclei was determined with a Coulter Counter. DNA concentration was determined as described by Burton (1956).

Extraction of nuclear non-histone proteins

Nuclear non-histone proteins were extracted from purified nuclei with 0.35 M-NaCl/0.32 M-sucrose/10 mM-Tris/HCl (pH 7.4)/4 mM-$MgCl_2$/0.5 mM-phenylmethanesulphonyl fluoride/0.5 mM-EGTA/0.5 mM-EDTA/5 mM-2-mercaptoethanol for 60 min as described by Harrison *et al.* (1982). Protein in the nuclear non-histone fraction was measured by the method of Bradford (1976). Under these experimental conditions, extraction of protein kinase subunits from nuclei was quantitative.

Processing of H4IIE hepatoma cells and nuclei for electron microscopy

H4IIE hepatoma cells or isolated nuclei were pelleted at 4500 rev./min for 10 min. Each pellet was resuspended in 2.5% (v/v) glutaraldehyde in 0.1 M-sodium phosphate, pH 7.4, and gently mixed for 1 h. After this treatment, cells and nuclei were re-pelleted at 200 *g* for 15 min. Cells and nuclei were washed for 10 min in 0.1 M-sodium phosphate containing 7% (w/v) sucrose before being re-pelleted as above. The cell and nuclear pellets were processed and morphologically evaluated by electron microscopy as described previously by us (Kuettel *et al.*, 1985; Jungmann *et al.*, 1988).

Assay of protein kinase activity

Cyclic AMP-dependent protein kinase activity was assayed as described previously by us (Harrison *et al.*, 1982), with the synthetic phosphate-acceptor heptapeptide Kemptide as substrate. The reaction was started by the addition of approx. 50 μg of nuclear non-histone protein. Incubations were carried out for 10 min at 30 °C and terminated by the addition of 1.0 ml of 75 mM-H_3PO_4. Reaction rates were linear in the absence or the presence of cyclic AMP (10 μM) for at least 15 min.

Marker-enzyme assay

Lactate dehydrogenase activity was measured in 100000 *g*-supernatant fractions as well as in nuclear non-histone protein fractions. Lactate dehydrogenase activity was assayed spectrophotometrically by converting pyruvate into lactate and measuring the decrease in absorbance of NADH at 340 nm (Derda *et al.*, 1980).

Photoaffinity labelling of the regulatory RI and RII subunits, gel electrophoresis and autoradiography

Photoaffinity labelling of nuclear non-histone protein or cytosolic protein with 8-azido cyclic [^{32}P]AMP,

SDS/PAGE and autoradiography were carried out as described by us previously (Laks *et al.*, 1981). Autoradiographs obtained after exposure of Kodak X-Omat XRP-5 film to dried gels for 3–7 days at −70 °C were scanned with a Zeineh soft-laser densitometer integrated with a Hewlett–Packard computer to calculate the relative peak areas automatically. RI and RII subunits were identified by their migration relative to protein standards (pyruvate dehydrogenase, M_r 58000; glutamate dehydrogenase, M_r 53000; fumarase, M_r 49000). Two-dimensional gels were run as described by O'Farrell (1975), with the following modifications: isoelectrofocusing gels (4% acrylamide) were prepared by mixing Ampholines, pH 4.0–6.5, with Ampholines, pH 3.0–10.0, in the ratio 4:1 (v/v). Second-dimension electrophoresis was carried out in 10%-polyacrylamide gels.

Purification of cyclic AMP-dependent protein kinase subunits

The RI and RII subunits were purified to homogeneity from rat liver as described by Dills *et al.* (1979). Contaminating RI subunit was removed from the RII subunit with an anti-RI–Protein A–Sepharose 4B affinity column. C subunit was purified from bovine heart as described previously (Schwoch *et al.*, 1980).

Preparation and characterization of antisera

Preparation of the antisera against bovine heart C subunit and rat liver RI and RII subunits in rabbits and their characterization have been described in detail (Kuettel *et al.*, 1985).

Immunoblotting

Immunoblot analysis was carried out by a modification of the method of Towbin *et al.* (1979). The modification was described by us in detail previously (Kuettel *et al.*, 1985).

E.l.i.s.a.

Competitive e.l.i.s.a. was carried out by the method of Schwoch & Hamann (1982), with slight modifications as described by us (Kuettel *et al.*, 1985).

RESULTS

Assessment of nuclear morphology and purity

The morphology of isolated nuclei was assessed by electron microscopy and compared with the morphology of nuclei in intact H4IIE hepatoma cells (Jungmann *et al.*, 1988). Isolated nuclei possessed a morphologically intact nuclear envelope. Nuclear substructures (i.e. heterochromatin, euchromatin, nucleolus) were morphologically comparable in isolated nuclei and in intact cells (micrographs not shown).

Marker-enzyme assay, such as lactate dehydrogenase, showed an activity of 1.39 units in the cytosol from 10^8 cells and consistently less than 0.01 unit in the nuclear pellet, indicating a minimal contamination of nuclei with a cytoplasmic component. The protein/DNA ratio of isolated nuclei was consistently between 3.5 and 4. Furthermore, comparison of the DNA content of the homogenate with that in isolated nuclei showed a routine recovery of DNA of about 77%.

The potential of artifactual contamination of nuclei with dissociated cytoplasmic RI, RII or C subunits was assessed by determination of the binding and retention by nuclei of exogenously added RI, RII and C to the homogenate before isolation of nuclei. In this experiment, purified rat liver RI and RII subunits, photoaffinity-labelled with 8-azido cyclic [^{32}P]AMP, or purified bovine C subunit were added to hepatoma-cell homogenates at concentrations of approx. 2.8 pmol (5×10^5 c.p.m. of ^{32}P-labelled RI or RII) per homogenate from one roller bottle. Nuclei were subsequently isolated, and the nuclear

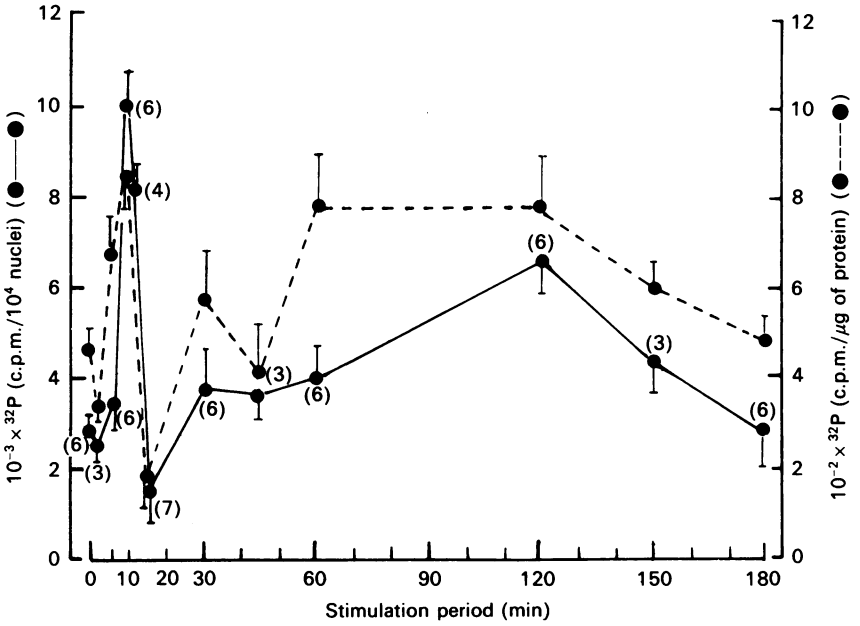

Fig. 1. Nuclear protein kinase activity in H4IIE hepatoma cells

Protein kinase activity was assayed in nuclear non-histone-protein extracts prepared from H4IIE hepatoma cells stimulated for the times indicated with 0.5 mM-dibutyryl cyclic AMP. Kinase activity is expressed as ^{32}P c.p.m. incorporated/μg of protein (●----●) or as ^{32}P c.p.m. incorporated/10^4 nuclei (●——●). Values shown are means ± S.E.M.; the numbers in parentheses indicate the total number of experiments carried out at each stimulation time period.

Table 1. Effect of the heat-stable inhibitor on H4IIE hepatoma-cell nuclear protein kinase activity

H4IIE hepatoma cells were stimulated with 0.5 mM-dibutyryl cyclic AMP for either 10 or 120 min. Nuclear 0.35 M-NaCl extracts were prepared and assayed for protein kinase activity without or with a saturating concentration of heat-stable inhibitor. Details are described in the Experimental section. Results are means ± S.E.M. from three experiments

		10^{-3} × Specific activity			
		(^{32}P c.p.m./min per μg of protein)		(^{32}P c.p.m./min per 10^4 nuclei)	
Treatment	Inhibitor...	−	+	−	+
None		4.4±0.4	0.3±0.2	4.3±0.5	0.3±0.1
Dibutyryl cyclic AMP (10 min)		10.7±1.2	0.4±0.2	14.1±0.9	0.5±0.3
Dibutyryl cyclic AMP (120 min)		7.2±0.8	2.2±0.6	8.8±1.1	2.7±0.7

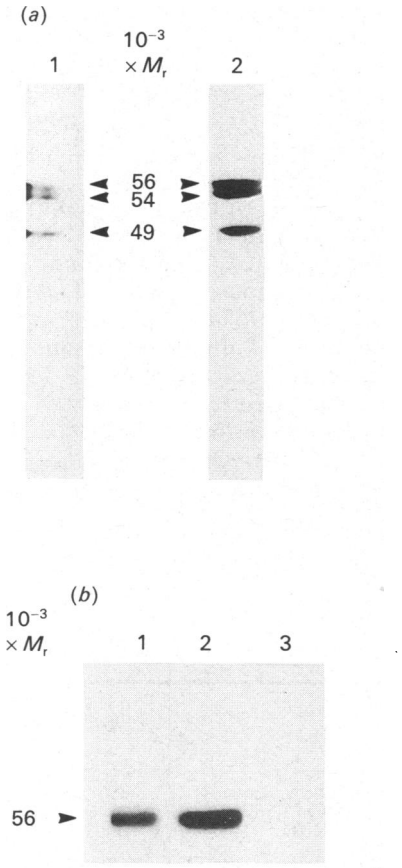

Fig. 2. Electrophoretic resolution of cytosolic and nuclear regulatory subunits from H4IIE hepatoma cells after photoaffinity labelling with 8-azido cyclic [^{32}P]AMP

(*a*) Cytosol protein from unstimulated cells was photoaffinity-labelled with 8-azido cyclic [^{32}P]AMP. After labelling, proteins were separated by SDS/PAGE, and detected by autoradiography. Cytosol protein photoaffinity-labelled in the presence of 10 μM non-radioactive cyclic AMP (lane 1) and without added non-radioactive cyclic AMP (lane 2). (*b*) Nuclear 0.35 M-NaCl non-histone-protein extracts from both unstimulated and dibutyryl cyclic AMP-treated H4IIE hepatoma cells were photoaffinity-labelled. After labelling, identical amounts of protein (about 55 μg) were separated by SDS/PAGE and detected by autoradiography. Non-histone nuclear protein from unstimulated H4IIE hepatoma cells photoaffinity-labelled in the absence of non-radioactive cyclic AMP (lane 1); non-histone nuclear protein from dibutyryl cyclic AMP-stimulated H4IIE hepatoma cells photoaffinity-labelled without added non-radioactive cyclic AMP (lane 2) and in the presence of 10 μM non-radioactive cyclic AMP (lane 3).

^{32}P content was determined or the nuclear C subunit activity was compared with that of control nuclei. Less than 0.04% of the total input radioactivity or C-subunit activity was recovered with the nuclear pellet, indicating negligible artifactual binding of the exogenously added subunits. No differences in these results were observed regardless of whether nuclei from unstimulated or stimulated cells were used.

Effects of dibutyryl cyclic AMP on protein kinase activity in nuclear non-histone-protein extracts

C-subunit activity was determined in nuclear protein extracts from hepatoma cells that had been stimulated with dibutyryl cyclic AMP for various time periods. The data are shown in Fig. 1. When total nuclear protein kinase activity is expressed as ^{32}P c.p.m. incorporated per μg of protein or per number of nuclei, marked increases in protein kinase activity are observed 5–10 min and again at 60–120 min after addition of dibutyryl cyclic AMP.

To determine the kinase activity contributed by the C subunit, nuclear kinase activity was assayed without and with the heat-stable protein kinase inhibitor from rabbit skeletal muscle (Ashby & Walsh, 1974). In the presence of the inhibitor, protein kinase actvity in nuclear extracts from cells either unstimulated or stimulated for 10 min was nearly completely inhibited (Table 1). In contrast, nuclear protein kinase activity from cells stimulated for 120 min was only partially (about 60%) inhibited, demonstrating an increase not only in C subunit but also in cyclic AMP-independent protein kinase activity. This latter activity was not further investigated. In agreement with data obtained by previous investigators (Jungmann & Kranias, 1977), addition of cyclic AMP (10 μM) to the assays had no stimulatory effect on nuclear protein kinase activity (results not shown), suggesting that the nuclear C subunit was present in its dissociated form and not as the holoenzyme.

At 10 min after dibutyryl cyclic AMP treatment, total nuclear protein kinase activity increased approx. 3-fold

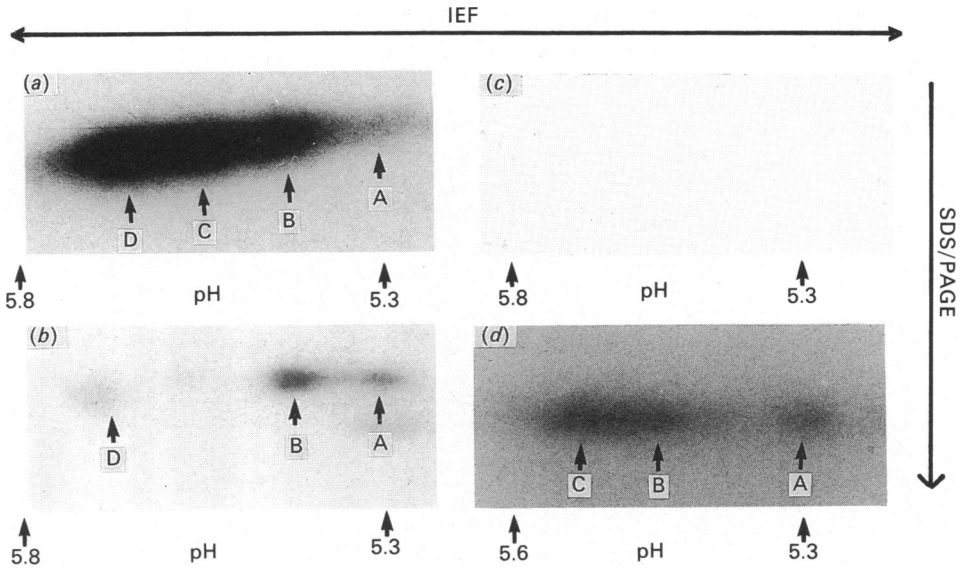

Fig. 3. Photoaffinity labelling and two-dimensional SDS/PAGE of hepatoma-cell nuclear and cytosolic RII

Nuclear non-histone-protein extracts (55 μg of protein each) from dibutyryl cyclic AMP-stimulated (10 min) (*a*) and unstimulated (*b*) hepatoma cells and cytosol (50 μg of protein) from unstimulated cells (*d*) were photoaffinity-labelled and analysed by two-dimensional SDS/PAGE before autoradiography. Panel (*c*) shows the autoradiograph of nuclear protein from stimulated cells after photoaffinity labelling in the presence of 10 μM non-radioactive cyclic AMP. Abbreviation: IEF, isoelectric focusing.

when the activity is expressed per unit number of nuclei, but only 2.4-fold when the activity is expressed per μg of nuclear protein. This apparent discrepancy is most probably due to the observed increase (from 2.9 to 3.8 mg of protein/10^8 nuclei) of the protein content per unit number of nuclei after stimulation with dibutyryl cyclic AMP. The increased protein content obscures quantitative changes of nuclear protein kinase when its specific activity is expressed per weight of nuclear protein. A similar general increase in nuclear protein was previously observed in the regenerating rat liver (Laks *et al.*, 1981) and in the isoprenaline-stimulated rat parotid gland (Schwoch & Freimann, 1986).

Photoaffinity labelling of cytosolic and nuclear RI and RII subunits with 8-azido cyclic [^{32}P]AMP

To identify the RI and RII subunits, nuclear extracts and cytosol were affinity-labelled with 8-azido cyclic [^{32}P]AMP. The labelled proteins were subjected to SDS/PAGE. After autoradiography, both the RI and RII subunits were identified in the cytosol (Fig. 2*a*, lane 2). RI appears as a single band with an apparent M_r of 49000. The cytosolic RII is separated into two bands: one band of higher electrophoretic mobility (M_r 54000), representing the dephospho form of RII, and a slower-migrating band (M_r 56000), consisting of the phospho form of RII as reported previously (Rangel-Aldao *et al.*, 1979). Photoaffinity labelling of nuclear extracts resulted in the identification of only the ^{32}P-labelled phospho form of RII (M_r 56000) (Fig. 2*b*, lanes 1 and 2). The binding of 8-azido cyclic [^{32}P]AMP to RI and RII was inhibited in the presence of 10 μM non-radioactive cyclic AMP (Fig. 2*a*, lane 1, and Fig. 2*b*, lane 3), demonstrating the specificity of cyclic nucleotide binding.

A comparison of the autoradiograph band densities by densitometry (results not shown) allows a semi-quantitative estimate of the relative RII levels and shows that, on the basis of a comparison of the relative band densities (Fig. 2*b*, cf. lanes 1 and 2), nuclear RII increased approx. 2-fold after dibutyryl cyclic AMP stimulation (10 min) as

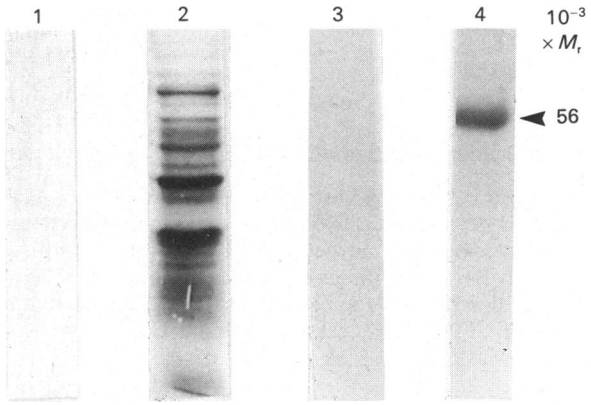

Fig. 4. Assessment of the efficiency of protein transfer from the polyacrylamide gel to nitrocellulose during blotting

H4IIE hepatoma cells were incubated for 24 h with 50 μCi of [^{35}S]methionine. A nuclear 0.35 M-NaCl non-histone-protein extract was prepared and resolved by SDS/PAGE. Proteins were transferred for 24 h on to nitrocellulose by passive diffusion. The gel and nitrocellulose membrane were dried and placed on Kodak X-Omat XRP-5 film for 3 days to obtain autoradiographs. The ^{35}S-labelled proteins transferred on to nitrocellulose are shown in lane 2. Labelled proteins remaining in the gel are shown in lane 1. Lanes 3 and 4 show autoradiographs of 8-azido cyclic [^{32}P]AMP-labelled nuclear 0.35 M-NaCl protein extracts from H4IIE hepatoma cells resolved by PAGE and blotted on to a nitrocellulose membrane. Transfer of the ^{32}P label covalently incorporated into RII from the polyacrylamide gel (lane 3) to the membrane (lane 4) was judged to be quantitative.

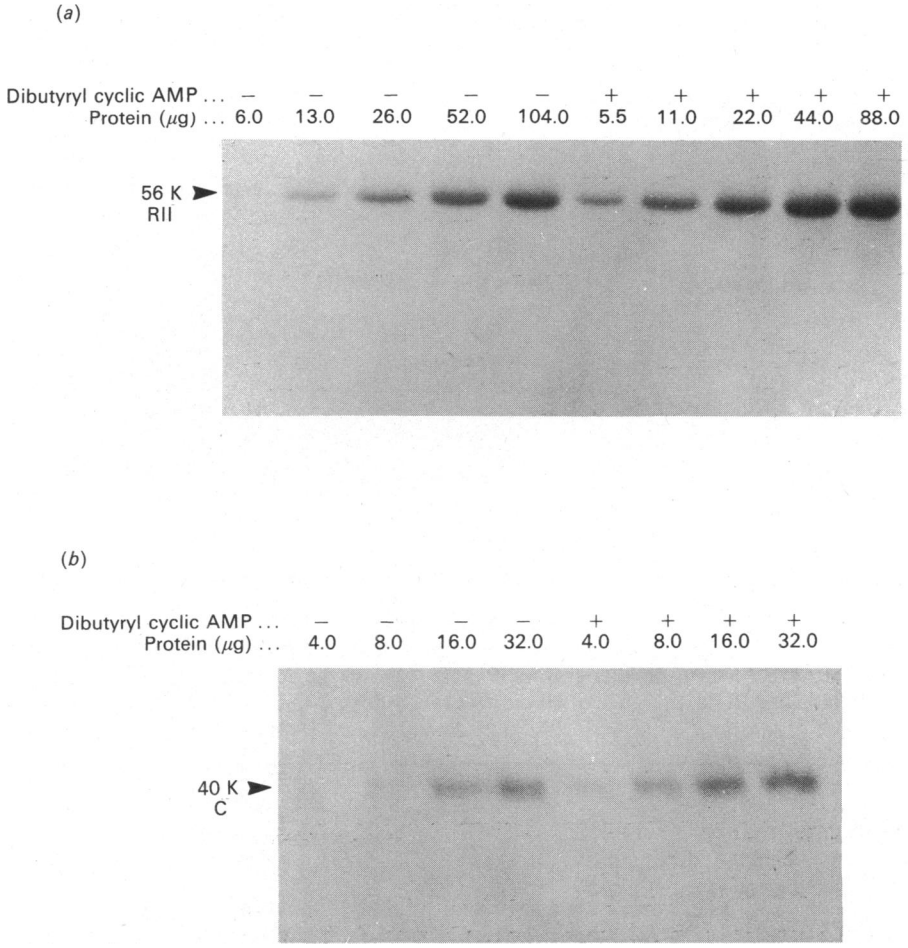

Fig. 5. Immunodetection of the RII subunit and C subunit in nuclear non-histone-protein extracts by immunoblotting

Nuclear 0.35 M-NaCl non-histone-protein extracts were prepared from untreated and dibutyryl cyclic AMP-stimulated (10 min) H4IIE hepatoma cells. Samples containing increasing amounts of nuclear non-histone protein were separated by SDS/PAGE and transferred to nitrocellulose by passive diffusion. The nitrocellulose membranes were incubated with either a 1:100 dilution of anti-RII antiserum or a 1:250 dilution of anti-C antiserum. Immunocomplexes were detected with the BRL immunodetection kit as described in the Experimental section. Arrows indicate the position of the M_r-56000 (56K) regulatory subunit RII (*a*) or the M_r-40000 (40K) catalytic subunit C (*b*).

compared with nuclei from untreated hepatoma cells. However, since the photoaffinity labelling of the regulatory subunits with 8-azido cyclic [^{32}P]AMP can be rather inefficient, a more precise determination by immunochemical methods was carried out (see below).

To analyse the heterogeneity of nuclear RII variants and to determine whether unique RII variants (charge variants, isoforms) became associated with nuclei as the consequence of dibutyryl cyclic AMP stimulation, 8-azido cyclic [^{32}P]AMP-labelled nuclear and cytosolic RII samples were analysed by two-dimensional gel electrophoresis (Fig. 3). Cytosolic RII was resolved as three spots (Fig. 3*d*, spots A, B, C) with pI 5.30 (form A), 5.47 (form B) and 5.55 (form C). The form A and B variants were also observed at relatively low levels in nuclei from unstimulated cells (see Fig. 3*b*) together with a trace amount of a form D variant (pI 5.65). As shown in Fig. 3(*a*), RII from nuclei of dibutyryl cyclic AMP-stimulated cells was resolved as four isoelectric variants, forms A–D. Even though we have not been able to obtain a better resolution of the spots, one can note that variants C and D are present in relatively high abundance in nuclei from stimulated, but not from unstimulated, cells. Also, the slightly more basic RII variant D was not detected in hepatoma-cell cytosol. Studies are needed to analyse the structural similarities and relationships of the nuclear and cytosolic variants.

As shown in Fig. 3(*c*), the presence of 10 μM non-radioactive cyclic AMP during photoaffinity labelling of nuclear proteins with 8-azido cyclic [^{32}P]AMP decreased ^{32}P labelling of RII to undetectable levels, indicating the specificity of 8-azido cyclic AMP binding. Photoaffinity labelling of cytosolic RII was similarly prevented by the presence of excess unlabelled cyclic AMP (autoradiograph not shown).

Immunoblot analysis of nuclear non-histone-protein extracts

An immunoblotting procedure was used to determine whether the observed dibutyryl cyclic AMP-mediated modulation of nuclear C and RII subunit levels was due to a net decrease in RII and C antigens. Before immunoblot analysis of RII and C was attempted, the efficiency of protein transfer during blotting was quantitatively

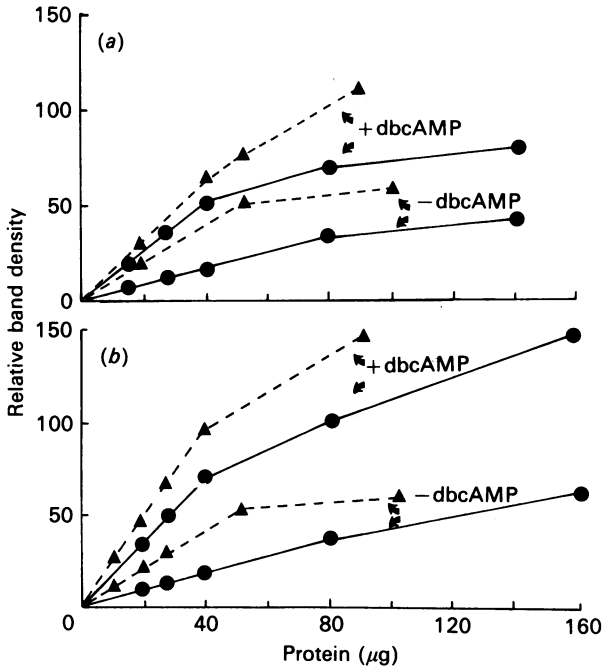

Fig. 6. Densitometric quantification of RII and C subunits in immunoblots of nuclear non-histone protein fractions from unstimulated and dibutyryl cyclic AMP-stimulated H4IIE cells

The relative band densities of immunoblotted RII and C subunits were determined by scanning a 'positive' negative of the blot with a Zeineh soft-laser-beam densitometer. The densities were plotted as a function of the amount of nuclear non-histone protein present in each electrophoresis well. (*a*) RII and C subunits from 5 min-stimulated cells; (*b*) RII and C subunits from 10 min-stimulated cells. ●, RII subunit; ▲, catalytic subunit. Abbreviation: dbcAMP, dibutyryl cyclic AMP.

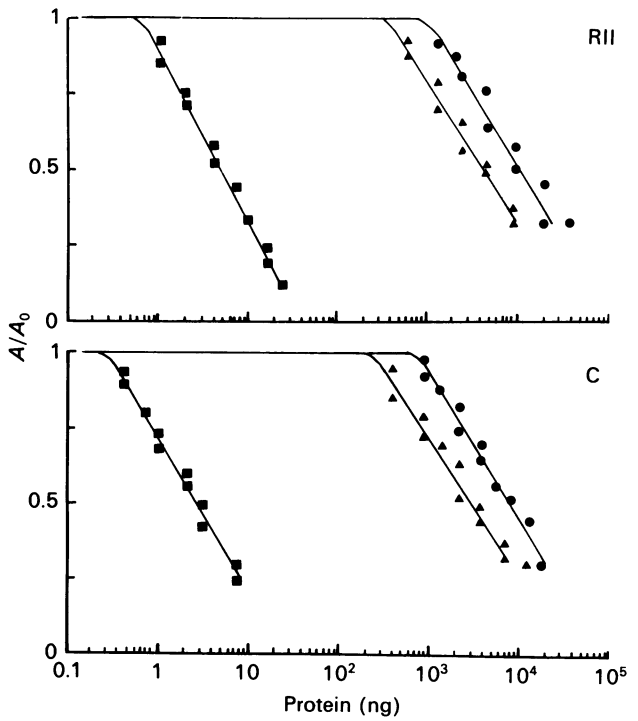

Fig. 7. E.l.i.s.a. of the RII and C subunits of cyclic AMP-dependent protein kinase in nuclei isolated from rat H4IIE hepatoma cells which had been stimulated for 10 min with dibutyryl cyclic AMP or from unstimulated cells

A/A_0 = absorbance (414 nm) measured in the presence of competing antigen relative to that measured in the absence of competing antigen (i.e. 100% absorption). ●, nuclear extract from unstimulated cells; ▲, nuclear extract from stimulated cells; ■, standard purified RII or C subunit respectively.

evaluated. To do this, electrophoretically separated [^{35}S]methionine-labelled nuclear proteins as well as 8-azido cyclic [^{32}P]AMP-labelled RII were blotted, and the efficiency of transfer was determined by comparison of the autoradiographs obtained from the gel and the nitrocellulose filter. The autoradiographs shown in Fig. 4 (cf. lane 1 with 2 and lane 3 with 4) indicate that the transfer of ^{32}P-labelled RII as well as of all ^{35}S-labelled nuclear proteins was quantitative.

By using affinity-purified polyclonal antibodies against RII and C, the relative nuclear levels of RII and C were determined. The immunoblots of nuclear proteins from unstimulated and stimulated cells are shown in Fig. 5. A single band corresponding to either the RII subunit (Fig. 5*a*) or the C subunit (Fig. 5*b*) is detected. Densitometric scanning of the blots indicated a linear relationship between the band density of the antigen and nuclear non-histone protein (in the range of 10–50 μg of protein applied to the gel) (Fig. 6). After a 10 min stimulation period, the relative nuclear concentrations of RII and C were about 2–3-fold higher than in untreated cells (see Fig. 6*b*). Immunoblots prepared from nuclear extracts from hepatoma cells stimulated for only 5 min (Fig. 6*a*) also showed a relative increase in RII and C, but not to the same extent as in cells stimulated for 10 min. We were unable to detect the presence of RI in H4IIE hepatoma-cell nuclei by this immunoblotting procedure.

Table 2. Quantification of nuclear cyclic AMP-dependent protein kinase subunits in H4IIE hepatoma cells by e.l.i.s.a.

H4IIE hepatoma cells were stimulated for the indicated time periods with 0.5 mM-dibutyryl cyclic AMP as described in the Experimental section. Results are means ± S.E.M. for four experiments; N.D., not detected.

Subunit	Stimulation time (min)	Subunit concn. (pmol/10^8 nuclei)	Stimulated/control
RI	0	N.D.	–
	10	N.D.	–
	120	N.D.	–
RII	0	5.1 ± 0.32	–
	10	13.3 ± 1.52	2.6
	120	10.5 ± 1.33	2.0
C	0	16.4 ± 2.14	–
	10	34.1 ± 3.92	2.1
	120	27.9 ± 2.68	1.8

Quantification of protein kinase subunits in nuclear non-histone-protein extracts by e.l.i.s.a.

An e.l.i.s.a. was used to quantify changes of the subunits (RI, RII and C) in nuclear non-histone-protein

extracts. Competition experiments revealed a concentration-dependent inhibition of antibody binding to the coated antigens by competing RII and C (Fig. 7). Parallel inhibition curves were obtained with nuclear non-histone-protein extracts from untreated and dibutyryl cyclic AMP-treated hepatoma cells (representative examples are shown for RII and C from dibutyryl cyclic AMP-treated and untreated cells; Fig. 7). The amount of antigen was determined by comparison with the standard curves generated by using known amounts of RII or C as competing antigen. The data in Table 2 show that, in dibutyryl cyclic AMP-stimulated cells, amounts of nuclear RII and C subunit were markedly increased 10 min as well as 120 min after cyclic nucleotide addition. The amounts of nuclear RI were below the level of sensitivity of the assay.

DISCUSSION

It has been our working hypothesis that cyclic AMP, through activation of nuclear cyclic AMP-dependent protein kinase, may regulate the transcription of cyclic AMP-inducible eukaryotic genes by an as yet undefined mechanism. This mechanism may involve the phosphorylative modification of chromosomal proteins (Jungmann & Kranias, 1977) and/or a direct/indirect interaction of the cyclic AMP-binding regulatory subunits (RI or RII) with *cis*-regulatory DNA sequences. Either mechanism requires modulation of nuclear cyclic AMP-dependent protein kinase subunits in order to serve the cell in a regulatory capacity. Therefore, we have quantified cyclic AMP-mediated alterations of nuclear cyclic AMP-dependent protein kinase subunit levels in H4IIE hepatoma cells during a time period preceding and coinciding with the induction of phosphoenolpyruvate carboxykinase and tyrosine aminotransferase gene transcription (Gunn *et al.*, 1976; Lamers *et al.*, 1982; Culpepper & Liu, 1983; Granner *et al.*, 1983; Sasaki *et al.*, 1984). For the quantitative measurements, we have applied both biochemical and immunochemical techniques. Each experimental approach demonstrated a temporally limited 2–3-fold increase in nuclear C and RII subunit levels as the consequence of dibutyryl cyclic AMP stimulation. Additionally, through analysis of nuclear RII subunit by two-dimensional SDS/PAGE, we have detected the presence of a unique variant form of RII which was not present in hepatoma-cell cytosol. The precise molecular nature of this nuclear variant is unknown, and requires further investigation.

The mechanism of the modulation of nuclear RII and C subunit levels is interesting to contemplate. Although an orthodox explanation would evoke an increase in nuclear RII and C subunits through a cyclic AMP-mediated dissociation of resident nuclear holoenzyme, which is suggested by some previous studies (Murray *et al.*, 1985), most of our data are not consistent with such a mechanism. First addition of cyclic AMP to isolated intact H4IIE hepatoma-cell nuclei did not result in any detectable activation of nuclear protein kinase activity (results not shown), consistent with many similar observations (for review, see Jungmann & Kranias, 1977). Second, evaluation of nuclear subunit levels by several independent immunochemical methods convincingly demonstrated a net increase in immunoreactive subunit molecules after dibutyryl cyclic AMP stimulation. Immunoreactivity of the antigens is not altered by dibutyryl cyclic AMP treatment. Additionally, the antisera interact equally well with either the free dissociated subunit or with the subunit in the non-dissociated holoenzyme form (J. Kwast-Welfeld & R. A. Jungmann, unpublished work). Therefore an apparent change of nuclear subunit level owing to a selective interaction of the antibody with either the dissociated or the undissociated subunit can be ruled out.

Whether the nuclear subunit modulation observed is caused by intracellular translocation of the subunits or by differences in the nuclear turnover of RII and C before and after stimulation cannot be decided at present. However, we have previously suggested and provided supporting evidence that a subunit modulation of the type observed in these studies may be the consequence of a cyclic AMP-mediated translocation of subunits from the extranuclear cellular space into the nuclear compartment (Jungmann & Kranias, 1977). Such molecular events constitute a relatively selective mechanism whereby the hormonal signal at the cell membrane is transduced to nuclear sites, resulting in the functional interaction of the subunits with nuclear components and subsequent physiological response. Similar translocation phenomena have been observed in several other cell systems (Corbin *et al.*, 1977; Jungmann & Kranias, 1977; Schwoch & Hilz, 1978; Cho-Chung, 1980; Schwartz & Costa, 1980; Cho-Chung *et al.*, 1981; Nesterova *et al.*, 1981; Mednieks & Jungmann, 1982; Kuettel *et al.*, 1984; Nigg *et al.*, 1985*a*; Squinto *et al.*, 1985; Schwoch & Freimann, 1986; Kwast-Welfeld & Jungmann, 1988).

The most novel result of the present study is the demonstration of a modulation of nuclear RII levels. Although several groups have presented biochemical and immunocytochemical evidence suggesting the occurrence of C-subunit translocation (Corbin *et al.*, 1977; Schoch & Hilz, 1978; Cho-Chung, 1980; Schwartz & Costa, 1980; Cho-Chung *et al.*, 1981; Laks *et al.*, 1981; Nesterova *et al.*, 1981; Mednieks & Jungmann, 1982; Kuettel *et al.*, 1984; Nigg *et al.*, 1985*b*; Squinto *et al.*, 1985; Schwoch & Freimann, 1986; Kwast-Welfeld & Jungmann, 1988), translocation of regulatory subunits was either not detected (Kuettel *et al.*, 1984; Nigg *et al.*, 1985*b*) or occurred only in combination of C with RI (Laks *et al.*, 1981; Squinto *et al.*, 1985; Schwoch & Freimann, 1986) or C with RII (Kwast-Welfeld & Jungmann, 1988). Therefore, it seems possible that cyclic AMP regulation of certain nuclear events might be mediated by a change of only the C-subunit concentration in the nucleus, whereas other events may require concomitant changes in the amount of R subunit.

The amounts of the C and RII subunits in hepatoma-cell nuclei increased after dibutyryl cyclic AMP stimulation without an accompanying change in the amount of RI subunit, although hepatoma-cell cytosol contains both RII as well as RI in the ratio approx. 4:1, implying a functional role for C and RII but not for RI under these experimental conditions. The accumulation of type II protein kinase subunits in the nucleus is likely to be of considerable importance for the mediation of physiological responses elicited by cyclic AMP. As a direct consequence of C-subunit action, nuclear proteins are structurally and possibly functionally altered. Nuclear phosphoproteins whose degree of phosphorylation is altered by the C subunit include histone H1 in the

glucagon-stimulated rat liver (Langan, 1969), histones H1-1, H1-2, H1-3 and H3 as well as RNA polymerase II subunits in rat C6 glioma cells (Harrison *et al.*, 1982; Lee *et al.*, 1984), a M_r-19000 protein in forskolin-treated pituitary cells (Waterman *et al.*, 1985), and a M_r-76000 protein during dibutyryl cyclic AMP-induced regression of mammary-tumour growth (Cho-Chung *et al.*, 1979). Although the functional consequences of these phosphorylations are still to be determined, more recent studies have established a direct functional link between the type II cyclic AMP-dependent protein kinase as well as the C subunit and protein induction (Reisine *et al.*, 1985; Grove *et al.*, 1987; Montminy & Bilezikjian, 1987). Of specific interest is the demonstration that in PC12 cells cyclic AMP causes the phosphorylation of a M_r-43000 nuclear protein which exhibits specific binding affinity for the cyclic AMP-responsive element of the somatostatin promoter (Montminy & Bilezikjian, 1987).

Although the modulation and association of the C subunit with the nucleus is thus of considerable importance for determining the degree of phosphorylation of specific nuclear proteins, no function of the regulatory subunits at the nuclear level has so far been identified. We have previously reported that topoisomerase I activity is associated with the rat liver RII subunit (Constantinou *et al.*, 1985). However, more recently Shabb & Granner (1988) and our own laboratory were able to separate topoisomerase activity from RII. Our own experimental findings showed the presence in rat ovary nuclei of a M_r-56000 peptide (identical with rat ovary RII), exhibiting active topoisomerase activity, which co-purifies with RII (J. Kwast-Welfeld & R. A. Jungmann, unpublished work). Thus further studies are needed to identify the nuclear role of RII. In the rat H4IIE hepatoma cells, cyclic AMP-mediated modulation of nuclear RII and C levels occurs concomitantly with or just before the onset of cyclic AMP-induced transcription of the phosphoenolpyruvate carboxykinase gene (Lamers *et al.*, 1982; Sasaki *et al.*, 1984). Based on an evaluation of all pertinent experimental findings, the rapidity and coincidence of these molecular events in H4IIE hepatoma cells very strongly supports the notion that they are mechanistically linked.

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