MOLECULAR AND CELLULAR BIOLOGY, May 1993, p. 2822–2834
0270-7306/93/052822-13$02.00/0

Vol. 13, No. 5

Nuclear Protein Phosphatase 2A Dephosphorylates Protein Kinase A-Phosphorylated CREB and Regulates CREB Transcriptional Stimulation

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Received 2 November 1992/Returned for modification 6 January 1993/Accepted 22 February 1993

Cyclic AMP (cAMP)-dependent protein kinase A (PKA) stimulates the transcription of many eucaryotic genes by catalyzing the phosphorylation of the cAMP-regulatory element binding protein (CREB). Conversely, the attenuation or inhibition of cAMP-stimulated gene transcription would require the dephosphorylation of CREB by a nuclear protein phosphatase. In HepG2 cells treated with the protein serine/threonine (Ser/Thr) phosphatase inhibitor okadaic acid, dibutyryl-cAMP-stimulated transcription from the phosphoenolpyruvate carboxykinase (PEPCK) promoter was enhanced over the level of PEPCK gene transcription observed in cells treated with dibutyryl-cAMP alone. This process was mediated, at least in part, by a region of the PEPCK promoter that binds CREB. Likewise, okadaic acid prevents the dephosphorylation of PKA-phosphorylated CREB in rat liver nuclear extracts and enhances the ability of PKA to stimulate transcription from the PEPCK promoter in cell-free reactions. The ability of okadaic acid to enhance PKA-stimulated transcription in vitro was entirely dependent on the presence of CREB in the reactions. The phospho-CREB (P-CREB) phosphatase activity present in nuclear extracts coelutes with protein Ser/Thr phosphatase type 2A (PP2A) on Mono Q, amino-hexyl Sepharose, and heparin agarose columns and was chromatographically resolved from nuclear protein Ser/Thr-phosphatase type 1 (PP1). Furthermore, P-CREB phosphatase activity in nuclear extracts was unaffected by the heat-stable protein inhibitor-2, which is a potent and selective inhibitor of PP1. Nuclear PP2A dephosphorylated P-CREB 30-fold more efficiently than did nuclear PP1. Finally, when PKA-phosphorylated CREB was treated with immunopurified PP2A and PP1, the PP2A-treated CREB did not stimulate transcription from the PEPCK promoter in vitro, whereas the PP1-treated CREB retained the ability to stimulate transcription. Nuclear PP2A appears to be the primary phosphatase that dephosphorylates PKA-phosphorylated CREB.

The activation of signal transduction pathways by hormonal and developmental stimuli ultimately leads to changes in the expression of specific genes. These changes often occur at the level of gene transcription and are mediated by rapid changes in the phosphorylation state of specific transcription factors. For example, the transcriptional transactivation function of the cyclic AMP (cAMP)-regulatory element binding protein (CREB) is stimulated by phosphorylation (15, 28, 45). CREB phosphorylation by the cAMP-dependent protein kinase A (PKA) is sufficient for the transcriptional transactivation in response to elevated intracellular cAMP levels (15, 18, 28, 45).

The stimulation of transcription of certain genes by cAMP is generally characterized by a rapid increase in the rate of transcription to a maximal level, followed by a slow decline in transcription to basal levels (29, 38). The decrease in the rate of transcription from stimulated levels occurs even under conditions where PKA activity is elevated, and it is refractory to the addition of agents that elevate cAMP levels and PKA activity for several hours (29, 38). These data indicate that the ability of cAMP and PKA to stimulate transcription is actively attenuated or reversed during the period of decline in the rate of RNA synthesis from stimulated levels. One regulatory mechanism for the control of cAMP-stimulated transcription would be the dephosphorylation of phospho-CREB (P-CREB) by nuclear protein phosphatases.

Recently, Hagiwara et al. (17) reported that the phosphorylation of CREB by PKA in whole cells or nuclear extracts was enhanced by okadaic acid, an inhibitor of protein Ser/Thr phosphatase types 1 (PP1) and 2A (PP2A) (4). Likewise, the dephosphorylation of PKA-phosphorylated CREB in nuclear extracts from PC12 cells was inhibited by okadaic acid. PP1 and PP2A catalytic subunits purified from cytosolic extracts of skeletal muscle were both tested for their ability to dephosphorylate P-CREB, and PP1 was found to be more efficient in dephosphorylating P-CREB than PP2A. To demonstrate that PP1 catalytic subunit could inhibit cAMP-stimulated gene expression, the catalytic subunit of this enzyme in the absence of a regulatory subunit was introduced into cells either by microinjection or by using a eucaryotic PP1 expression vector. In both experiments the data were interpreted as indicating that the PP1 catalytic subunit inhibited the expression of reporter proteins from a cAMP-responsive promoter. These results were used to suggest that PP1 plays an important role in controlling the expression of cAMP-responsive genes, even though it was

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never directly shown that a nuclear PP1 could catalyze P-CREB dephosphorylation.

In this report we demonstrate that cAMP- and PKA-stimulated transcription from the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter is also enhanced by okadaic acid in live cells or in cell-free transcription reactions. The ability of okadaic acid to enhance transcription from the PEPCK promoter in vitro correlates directly with its ability to protect P-CREB from dephosphorylation in nuclear extracts prepared from rat liver. The P-CREB phosphatase activity in rat liver nuclear extracts copurified with PP2A and was clearly resolved from PP1 on Mono Q, amino-hexyl Sepharose, and heparin agarose columns. Nuclear PP2A dephosphorylated P-CREB approximately 30-fold more efficiently than nuclear PP1. Finally, PP2A-dephosphorylated P-CREB fails to stimulate transcription from the PEPCK promoter in cell-free reactions. These results directly identify the P-CREB phosphatase as nuclear PP2A, not the nuclear PP1 enzyme, and also show that nuclear PP2A is responsible for the attenuation of cAMP-stimulated gene transcription in liver nuclei.

MATERIALS AND METHODS

Purification of recombinant CREB. The CREB bacterial expression system, recombinant ATF-1 protein, and recombinant ATF-2 protein were gifts from James P. Hoeffler, University of Colorado Medical School, Denver. Human placental CREB cDNA inserted in pET-3b was used for expression of the CREB protein in *Escherichia coli* BL21/DE3 (42). Bacteria were grown to an optical density at 600 nm of 0.5 to 0.6, and CREB expression was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation 3 h after IPTG induction, washed, and suspended in 10 mM Tris-HCl (pH 7.8)–1 mM EDTA (TE). The bacteria were lysed by boiling for 8 min followed by cooling on ice. The lysate was then centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant was collected and applied to a 1-ml cAMP-regulatory element (CRE) affinity column prepared as follows. Two oligonucleotides, CTAGATTGTGTTACGTCA GTTC and CTAGGAACTGACGTAACACAAT, were annealed to produce a double-stranded CRE DNA having 4-bp overhangs. The annealed oligonucleotides were phosphorylated with T4 polynucleotide kinase and ligated with T4 DNA ligase as previously described (22). The overhangs on the ends of the ligated oligonucleotides were then filled with dATP, dCTP, dGTP, biotin-14-dTTP, and Klenow fragment as described by Arias and Dynan (2). The CRE DNA was then linked to streptavidin agarose. Unbound protein was eluted with sequential washes of the column with 10 ml of TE and 10 ml of TE containing 0.3 M NaCl. CREB was eluted with 3 ml of 1.0 M NaCl in TE. The eluate containing the CREB protein was dialyzed against 1.5 liter of TE and stored in aliquots at −20°C.

Bacterial expression of PP1 and PP2A. A cDNA encoding the catalytic subunit of human protein phosphatase 1α, obtained by reverse transcriptase-polymerase chain reaction cloning from HL-60 total RNA (unpublished data), and a cDNA encoding the catalytic subunit of the human liver protein phosphatase 2A were ligated into pRSET expression plasmids (Invitrogen, San Diego, Calif.). *E. coli* BL21(DE3/pLysS) cells transformed with these plasmids were grown to an optical density of 0.6 (A_{600}), and expression of the phosphatase protein was induced with 0.4 mM IPTG for 2 h. Cells were harvested by centrifugation and solubilized in Laemmli sample buffer.

Generation of anti-CREB, anti-phosphatase 2A, and anti-phosphatase 1 catalytic subunit antisera. Synthetic peptides corresponding to residues 110 to 124 of the human placenta CREB (RREILSRRPSYRKIL [18]), 298 to 309 of phosphatase 2A catalytic subunit (PHVTRRTPDYFL), and 298 to 311 of phosphatase 1 catalytic subunit (RPITPPRN SAKAKK) were coupled to keyhole limpet hemocyanin at pH 7.4 by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, according to the method of Kris et al. (27). The peptide-keyhole limpet hemocyanin (250 μg) conjugates were injected subcutaneously into rabbits first in complete Freund adjuvant and then as two boosts in incomplete adjuvant. Blood was collected 1 week after the second boost. Serum was collected and characterized for specific immunoreactivity towards CREB, phosphatase 2A, or phosphatase 1 catalytic subunits.

Immunoaffinity purification of antibodies. Antipeptide antibodies specific for CREB, PP1, and PP2A were purified from rabbit antisera by affinity chromatography. The synthetic peptides used for preparing antisera were covalently attached to amino-hexyl Sepharose (Pharmacia, Uppsala, Sweden) by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Bio-Rad, Richmond, Calif.). Rabbit serum was mixed with the appropriate peptide-Sepharose conjugate for 24 h at 4°C. The slurry was transferred to a column and washed extensively with 10 mM Tris, pH 7.5, followed by 10 mM Tris (pH 7.5)–500 mM NaCl. The bound antibodies were eluted by sequential addition of 100 mM glycine, pH 2.0, followed by 100 mM triethylamine, pH 11.5. The eluates were pooled and concentrated by ammonium sulfate precipitation (50% saturated solution, final concentration). The antibodies were pelleted by centrifugation, resuspended in phosphate-buffered saline (PBS), and dialyzed against PBS overnight at 4°C.

Preparation of phosphorylated substrates. Purified, bacterially expressed CREB (approximately 1 μg, 28.5 pmol) was phosphorylated in reaction mixtures containing 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, 100 μM [^{32}P]ATP (4.38×10^6cpm/nmol) and 0.1 μg of PKA catalytic subunit (Sigma, St. Louis, Mo.). The reaction mixtures were incubated for 1 h at 37°C and then passed over a Sephadex G-25 spin column prepared in a 1-ml syringe barrel. The calculated specific activity of [^{32}P]CREB was 0.954 pmol of $^{32}P_i$ per pmol of CREB.

Labeled phosphorylase *a* was prepared by a modification of a previously described method (1). Phosphorylase *b* (1 mg) was incubated in a 400-μl reaction mixture containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM $CaCl_2$, 50 μg of phosphorylase kinase, and 100 μM [^{32}P]ATP (14,000 cpm/pmol). The reaction mixture was incubated for 2 h at 37°C. Proteins were precipitated with an equal volume of saturated ammonium sulfate and collected by centrifugation. The protein pellets were washed with 50 mM Tris-HCl (pH 7.5)–1 mM dithiothreitol–1 mM EDTA (buffer A) containing 40% saturated ammonium sulfate. The pellet was resuspended in 1 ml of buffer A and dialyzed overnight against 500 ml of buffer A with one change of dialysis buffer.

Labeled histone H1 was prepared by a modification of the method of Jakes and Schlender (20). Histone H1 (500 μg) was incubated at 37°C for 1.5 h in a 1-ml reaction mixture containing 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 20 μg of phosphatidyl-

serine, 1 μg of diacylglycerol, 100 nM phorbol myristate acetate, 100 μM [^{32}P]ATP (13,000 cpm/pmol), and 110 ng of protein kinase C (Calbiochem, San Diego, Calif.). Proteins were precipitated by using ice-cold trichloroacetic acid (20% final concentration) followed by washing to remove unincorporated ATP (20). The precipitation and washing steps were repeated once, and the final protein pellet was dried and resuspended in 1 ml of distilled water.

Preparation of rat liver nuclear extracts. Rat liver nuclear extracts were prepared by a modification of the method described by Gorski et al. (16). Nuclei were collected by centrifugation through a 2 M sucrose–10% glycerol cushion and resuspended in 25 mM Na-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9)–1.5 mM $MgCl_2$–25% glycerol–1 mM dithiothreitol–100 mM KCl. The suspension was stirred gently while 1.2 M KCl was added in the same buffer to yield 0.3 M KCl. The suspension was rocked for 30 min at 4°C and then centrifuged at 35,000 rpm in a 70Ti rotor for 30 min at 4°C. The supernatant was collected, and solid $(NH_4)_2SO_4$ added to 0.3 g/ml. The precipitate was collected by centrifugation and resuspended in 0.8 ml of 25 mM Na-HEPES (pH 7.6)–10% glycerol–40 mM KCl–1 mM dithiothreitol–0.1 mM EDTA (dialysis buffer) per 15 g of starting liver tissue. The solution was dialyzed for 4 h against 500 ml of dialysis buffer which was changed once after the first 2 h. Precipitated material was removed by centrifugation for 5 min at 10,000 × *g*. The resulting supernatant was used as nuclear extract and stored in aliquots (5 to 8 mg of protein per ml) at −70°C. The nuclear extracts were free of cytosolic and microsomal factors as determined by the absence of the following marker proteins: hexokinase, cytochrome *c*, succinate dehydrogenase, and hydroxymethylglutaryl coenzyme A reductase.

Immunodepletion of CREB from rat liver nuclear extracts. One hundred microliters of CREB-specific antisera was mixed with a 100-μl packed volume of protein A-Sepharose beads for 1 h at 4°C. The beads were recovered by centrifugation and washed five times with 1 ml of dialysis buffer used for nuclear extract preparation. A 100-μl portion of rat liver nuclear extract was added to the packed beads and gently mixed for 1 h at 4°C. The beads were pelleted by centrifugation, and the CREB-depleted nuclear extract was used immediately for in vitro transcription analysis. No CREB was detected in the depleted extracts by Western blot (immunoblot) analysis or by gel retardation analysis with a ^{32}P-phosphate-labeled CRE oligonucleotide.

In vitro transcription analysis. In vitro transcription reactions (20 μl of reaction mixture) and primer extension analysis were performed as previously described (26). Some reaction mixtures contained Walsh peptide (PKA inhibitor peptide [10]), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7), and *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W7) or other additions as noted in the figure legends.

Changes in the phosphorylation state of CREB that occurred during the incubation of the in vitro transcription reactions were determined by comparing the phosphorylation state of CREB in 5-μl aliquots taken from the reaction mixtures before and after the mixtures were incubated. CREB was recovered from the aliquots by immunoprecipitation with CREB-specific antibodies linked to protein A-Sepharose. The immunoprecipitates were separated on 10% polyacrylamide–sodium dodecyl sulfate (SDS) gels and transferred to nitrocellulose sheets. The blots were subjected to Western blot analysis with the anti-CREB antibodies to ensure that equal amounts of CREB were recovered from the reactions. Changes in CREB phosphorylation were determined from autoradiograms of the ^{32}P-labeled CREB.

Chromatographic resolution PP1 and PP2A from rat liver nuclear extracts. Rat liver nuclear extracts (5 mg of protein) were applied to a Mono Q column (Pharmacia, Piscataway, N.J.) equilibrated in buffer B (25 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA). The column was developed over a 30-min period with a linear gradient of 0 to 400 mM NaCl in buffer B (flow rate of 1 ml/min). One-milliliter fractions were collected and assayed for P-CREB phosphatase activity as described below. Fractions containing PP1 or PP2A were identified by Western blot analysis using anti-PP1 and anti-PP2A antibodies.

P-CREB phosphatase-containing fractions from the Mono Q column were pooled and applied to either an amino-hexyl Sepharose column (Pharmacia) or a heparin agarose column (GIBCO BRL, Gaithersberg, Md.) equilibrated with buffer A. Both columns were eluted with 0 to 1 M NaCl step gradients (0.1 M NaCl increase per step, 1 ml per step). Aliquots from fractions were assayed for P-CREB phosphatase activity or for the presence of PP1 and PP2A.

Western blot analysis of immunoprecipitated CREB. CREB-specific antiserum was covalently cross-linked to protein A-Sepharose beads with dimethyl pimelimidate (Sigma). Immunoprecipitations were performed by incubating 100-μl portions of rat liver nuclear extract with 100 μl (packed volume) of the cross-linked antibody-protein A-Sepharose beads for 1 h at 4°C. Beads were recovered by centrifugation, and the supernatants were transferred to fresh microcentrifuge tubes. The beads were washed five times with 1-ml volumes of PBS.

The supernatant and immunoprecipitated proteins were resolved on 10% polyacrylamide–SDS gels and transferred to nitrocellulose. The nitrocellulose blots were blocked in PBS containing 1% dried milk and then treated with the CREB-specific antibody followed by goat anti-rabbit immunoglobulin G (heavy plus light chains)–alkaline phosphatase conjugate (Bio-Rad, Hercules, Calif.). After the blots were washed, immunocomplexes were visualized with bromochloro-indoyl-phosphate and Nitro Blue Tetrazolium.

Protein phosphatase assays. Samples (as indicated in the figure legends) were incubated in reaction mixtures containing 25 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM dithiothreitol, 15 μg of bovine serum albumin, and the indicated [^{32}P]phosphate-labeled substrates for 10 to 20 min at 37°C. The reactions were terminated by the addition of cold trichloroacetic acid (20% final concentration). Proteins were pelleted by centrifugation at 10,000 × *g* for 10 min, and the supernatant $^{32}P_i$ was quantitated by scintillation counting. The relative activities of PP1 and PP2A in rat liver nuclear extracts were determined as previously described (9, 12, 40).

Cell transfections. HepG2 cells were transfected by calcium phosphate-DNA coprecipitation, and chloramphenicol acetyltransferase (CAT) assays were performed as described by Parks et al. (35). Luciferase assays were performed as described by DeWet et al. (13). Transfection efficiencies were normalized by cotransfecting the cells with a Rous sarcoma virus β-galactosidase gene, and β-galactosidase activities were measured in the cell lysates.

Purification of PP2A complexes and I-2. The heterotrimeric (ABC) and heterodimeric (AC) forms of PP2A were isolated from rat skeletal muscle as previously described (21, 34). Free PP2A catalytic subunit was purified as described by others (31). Inhibitor-2 (I-2) was purified as described by Yang et al. (47).

Tryptic phosphopeptide mapping. Tryptic phosphopeptide

mapping was performed as described by Kazlauskas and Cooper (24).

RESULTS

An okadaic acid-sensitive protein Ser/Thr phosphatase attenuates cAMP-stimulated PEPCK gene transcription. Comparison of PEPCK gene promoter-regulated transcription in the presence or absence of phosphatase inhibitors indicated that a protein Ser/Thr phosphatase is involved in regulating cAMP-stimulated transcription. Human hepatocarcinoma (HepG2) cells were transfected with a plasmid containing the cAMP-responsive, full-length (bp −490 to +73 relative to the start site of transcription) PEPCK gene promoter linked to the CAT reporter gene. The cells were then treated with dibutyryl-cAMP (Bt_2cAMP) and/or okadaic acid. The effects of these agents on CAT activity in the cells are shown in Fig. 1A. As expected, the addition of Bt_2cAMP alone stimulated CAT expression from the PEPCK promoter five- to sixfold in 4 h, after which CAT activity began to decline. Interestingly, when both Bt_2cAMP and okadaic acid were added together, CAT activity was stimulated 10- to 12-fold in the same time period and remained elevated for the duration of the experiment. No significant change in CAT activity was observed in untreated cells or in cells treated with okadaic acid alone. These data suggested that an okadaic acid-sensitive protein Ser/Thr phosphatase(s) was attenuating the effects of Bt_2cAMP on PEPCK gene transcription.

The ability of Bt_2cAMP and okadaic acid to stimulate transcription was observed with the full-length PEPCK promoter (Fig. 1B), as well as with a 5′ deletion mutant of the promoter (−109 to +73) that contains just the proximal CRE, CRE-1 (−91-TTACGTCA-−84), and the TATA site of the PEPCK gene promoter (37, 41). However, Bt_2cAMP and okadaic acid had no effect on transcription from the deletion mutant that contains just the TATA site (−68 to +73) of the PEPCK gene promoter. These data indicate that the proximal CRE-1 sequence is required for the stimulation of PEPCK gene transcription in response to Bt_2cAMP and okadaic acid. Furthermore, since purified CREB has been shown to bind the CRE-1 site (35), these data suggested that P-CREB may be a target of an okadaic acid-sensitive nuclear protein Ser/Thr phosphatase.

Cell-free transcription analysis provided further evidence that a nuclear protein phosphatase(s) could inhibit cAMP- or PKA-stimulated transcription from the PEPCK promoter. It was previously shown that the addition of purified PKA catalytic subunit, the only known mediator of cAMP action in eucaryotic cells, to in vitro transcription reactions stimulated transcription from the PEPCK gene promoter and required the CRE-1 site (26, 36). Experiments were performed with the −109 to +73 PEPCK promoter deletion that contains only the CRE-1 and TATA sites to avoid potential interference from other regulatory sequences in the full-length promoter. As previously reported (26), the addition of PKA catalytic subunit to these reactions stimulated RNA synthesis from the PEPCK gene promoter five- to sixfold relative to that in control reactions to which no PKA was added (Fig. 2A). When both okadaic acid and PKA catalytic subunit were added to the reactions, transcription was further enhanced, with levels approaching 9- to 10-fold the level of transcription in control reactions. Similar data were obtained in experiments performed with the full-length PEPCK gene promoter (data not shown). Okadaic acid by itself stimulated transcription from the PEPCK promoter deletion three- to fourfold in the in vitro assays. The stimulation of transcription by okadaic acid in the cell-free system is probably due to phosphorylation of CREB and/or other factors by endogenous PKA and potentially other kinases that are protected by okadaic acid inhibition of nuclear phosphatases in the extracts. Cumulatively, the results indi-

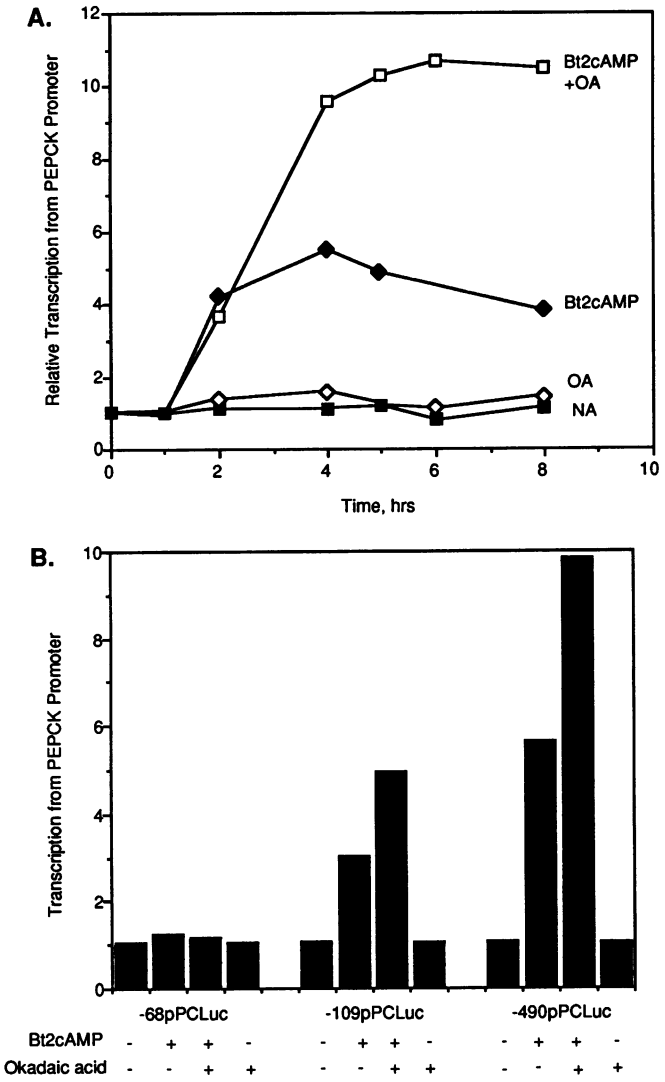


FIG. 1. Okadaic acid enhances Bt_2cAMP-stimulated transcription from the PEPCK promoter in HepG2 cells. (A) HepG2 cells were transfected with a plasmid containing the full-length PEPCK promoter (−490 to +73) linked to the CAT reporter gene. Eighteen hours later the cells were treated with 500 μM Bt_2cAMP, 1 μM okadaic acid (OA), or both agents together. No additions (NA) were made to control cells. At the times indicated, the cells were harvested and CAT activity was measured in cell lysates as described in Materials and Methods. Levels of transcription are shown relative to the levels of CAT activity measured at zero time. (B) HepG2 cells were transfected with plasmids containing the full-length (−490pPCLuc) PEPCK promoter, or promoter deletions truncated at −68 or −109 bp 5′ to the transcription start site, linked to the luciferase reporter gene. Eighteen hours posttransfection, the cells were treated with Bt_2cAMP, okadaic acid, or both agents as indicated. Four hours later, the cells were harvested and luciferase activity was measured in cell lysates as described in Materials and Methods. Levels of luciferase activity (transcription) are shown relative to the level of luciferase activity produced in untreated control cells. Results are averages from three experiments.

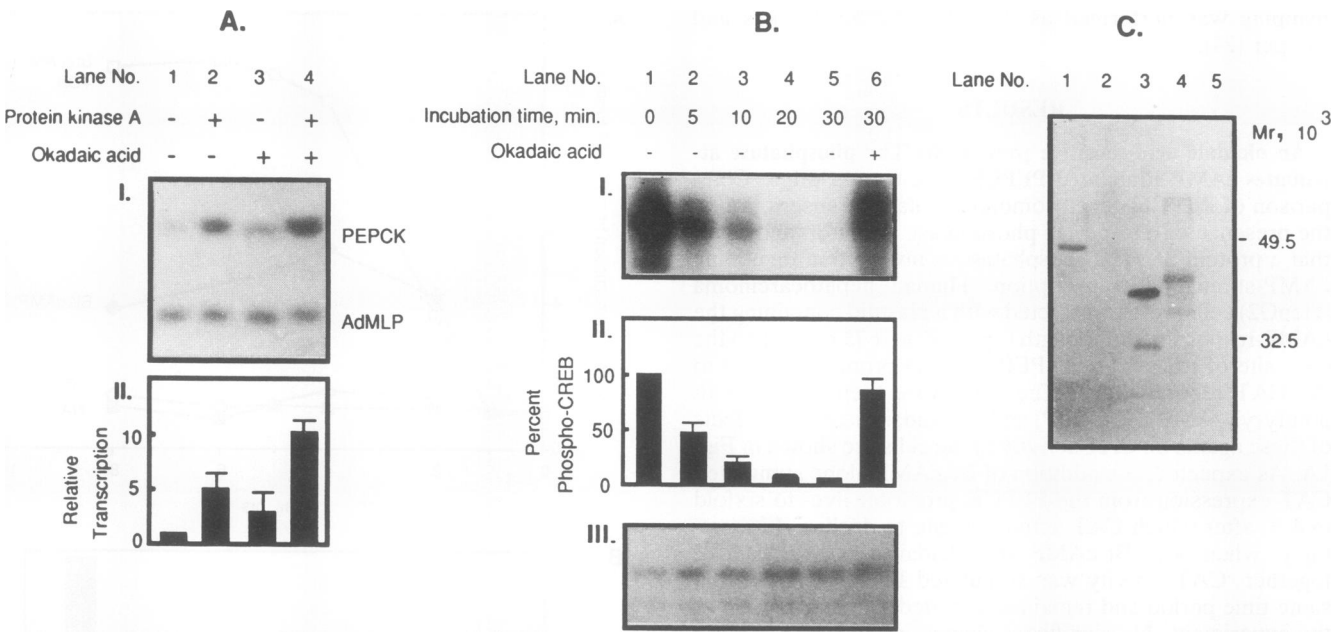


FIG. 2. Effect of okadaic acid on PKA-dependent transcription from the PEPCK promoter and P-CREB dephosphorylation in rat liver nuclear extracts. (A) Transcription from the PEPCK promoter was measured as described in Materials and Methods. Reaction mixtures contained 1.0 ng of purified PKA catalytic subunit and/or 200 nM okadaic acid as indicated. The autoradiogram (I) shows the primer extension products corresponding to RNA transcripts from the PEPCK promoter and the internal control, adenovirus major late promoter (AdMLP). The levels of transcription from the PEPCK promoter were corrected for transcription from the AdMLP control and are shown relative to the control lane in the bar graph (II). Data for the bar graph are the averages from three independent experiments. (B) Purified, bacterially expressed CREB was phosphorylated with [^{32}P]ATP and PKA. The P-CREB was incubated with nuclear extract under the same conditions used for in vitro transcription reactions for the times indicated. The reaction mixture for lane 6 also contained 200 nM okadaic acid. CREB was recovered from the reaction mixtures by immunoprecipitation in the presence of 200 nM okadaic acid and 25 μg of Walsh peptide per ml. Immunoprecipitates were then separated on 10% polyacrylamide–SDS gels and transferred to nitrocellulose. (I) Autoradiogram of the blot, showing the intensity of the 45-kDa CREB band; (II) amount of P-CREB relative to that in the zero time reaction; (III) amount of CREB recovered from the reaction mixtures as determined by Western blot analysis. (C) Specificity of CREB-specific antibodies by Western blot analysis. Lane 1, rat liver nuclear extract; lane 2, rat liver cytosolic extract; lane 3, recombinant CREB; lane 4, recombinant ATF1 protein; lane 5, recombinant ATF-2 protein.

cate that nuclei contain an okadaic acid-sensitive protein Ser/Thr phosphatase(s) that inhibits PKA-stimulated transcription from the PEPCK gene promoter.

To examine the possibility that P-CREB was the target of a nuclear protein Ser/Thr phosphatase(s), bacterially expressed CREB was phosphorylated with PKA catalytic subunit and [^{32}P]ATP. The P-CREB was purified to remove PKA and free nucleotides and incubated with rat liver nuclear extract under the same conditions used for in vitro transcription analysis. The reactions were terminated at various times with a cocktail of protein kinase-phosphatase inhibitors (Walsh peptide, H7, W7, okadaic acid, and β-glycerolphosphate) to prevent further changes in the phosphorylation state of CREB. CREB was then recovered from the reactions by immunoprecipitation with CREB-specific antibodies. Analysis of the recovered CREB by SDS-polyacrylamide gel electrophoresis and autoradiography showed that the P-CREB protein was rapidly lost over time in the absence of phosphatase inhibitors, with a half-life of approximately 5 min (Fig. 2B, panel I). No detectable P-CREB was detected following incubation with nuclear extract for 20 min. However, virtually all the P-CREB was recovered from reaction mixtures containing 200 nM okadaic acid, even after 30 min of incubation (Fig. 2B, panel I, lane 6). Western analysis of the immunoprecipitates (Fig. 2B, panel III) showed that approximately equal amounts of total CREB protein were recovered from the reactions, indicating that the loss of P-CREB was not due to proteolytic degradation or failure to recover equal amounts of CREB from the reactions. These findings indicate that PKA-phosphorylated CREB is dephosphorylated by an okadaic acid-sensitive protein Ser/Thr phosphatase present in rat liver nuclear extracts.

The specificity of the anti-CREB antibodies is demonstrated by the Western blot shown in Fig. 2C. The CREB-specific antibodies recognize recombinant CREB and ATF-1 proteins, as well as the 47-kDa endogenous CREB in rat liver nuclear extracts. No other bands are detected in nuclear extracts, even corresponding to ATF-1 (~43 kDa) or the CRE modulator (26 kDa). These antibodies do not recognize recombinant ATF-2 protein.

The site in CREB phosphorylated by PKA and dephosphorylated in nuclear extracts was identified by tryptic phosphopeptide mapping (Fig. 3). A single phosphopeptide was detected on maps of CREB phosphorylated by purified PKA catalytic subunit (Fig. 3A). This peptide comigrated with a PKA-labeled, synthetic peptide, RPSYR (Fig. 3B and C), which corresponds to amino acids 117 to 121 of human placental CREB. Therefore, PKA phosphorylates CREB on serine 119.

Dephosphorylation of P-CREB inhibits PKA-stimulated transcription from the PEPCK gene promoter in vitro. Okadaic acid enhanced PKA-stimulated transcription from the promoter of the PEPCK gene in cell-free transcription

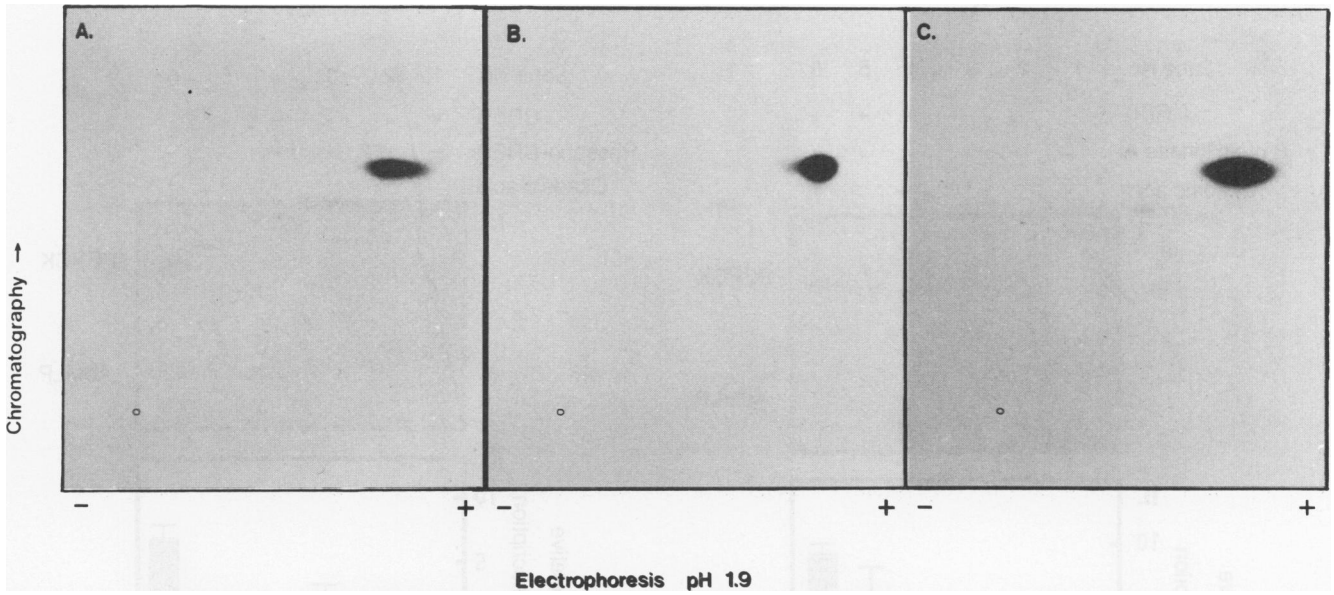


FIG. 3. Tryptic phosphopeptide mapping of PKA-phosphorylated CREB mapping was performed as described in Materials and Methods. Peptides were separated on cellulose thin-layer plates by electrophoresis at pH 1.9 from left to right (anode at left), followed by chromatography from bottom to top. The origin where peptides were spotted is indicated (O). (A) PKA-labeled CREB; (B) PKA-labeled synthetic peptide (RPSYR); (C) mixture of PKA-labeled CREB and the PKA-labeled synthetic peptide.

reactions and inhibited the dephosphorylation of P-CREB in the nuclear extracts used for the transcription reactions. To determine whether CREB is also required for the effects of PKA and okadaic acid on PEPCK promoter-driven transcription in vitro, reactions were performed using nuclear extracts depleted of endogenous CREB by immunoprecipitation with CREB-specific antibodies. No CREB was detectable in these extracts either by gel retardation analysis with a labeled oligonucleotide containing the CRE sequence or by Western blot analysis (data not shown).

The CREB-depleted nuclear extracts were used to assess the ability of PKA and/or okadaic acid to stimulate transcription from the PEPCK promoter in the absence or presence of bacterially expressed CREB (Fig. 4A). PKA and okadaic acid were unable to stimulate transcription from the PEPCK promoter in the absence of added recombinant CREB protein (Fig. 4A, panels I and II, lanes 1 to 3). However, in reactions to which CREB was added, PKA stimulated RNA synthesis from the PEPCK promoter approximately 5-fold and PKA plus okadaic acid stimulated transcription almost 10-fold (Fig. 4A, panels I and II, lanes 4 to 6). The small but detectable increase in transcription in the absence of added PKA may be related to the ability of CREB to function as a basal transcription factor as well as an inducible factor. This hypothesis is supported by previous findings showing that deletion or mutation of the CRE-1 site in the PEPCK promoter greatly diminishes basal transcription from the PEPCK promoter (26, 41).

To correlate the changes in PEPCK promoter-driven transcription with the phosphorylation state of CREB, reactions were performed in the presence of [^{32}P]ATP, making it possible to detect CREB phosphorylation during the incubation. Aliquots were removed from the transcription reactions, and CREB was immunoprecipitated in the presence of the protein kinase-phosphatase inhibitor cocktail to prevent further changes in the phosphorylation state of the CREB protein. The immunoprecipitates were resolved on 10% polyacrylamide–SDS gels and analyzed by Western blot analysis and autoradiography (Fig. 4A, panel III). CREB added to reactions alone (lane 4) was slightly phosphorylated during the course of the transcription reaction, possibly accounting for the slight increase in transcription from the PEPCK promoter compared with that in the control reaction (lane 4 relative to lane 1). Addition of PKA catalytic subunit resulted in a two- to threefold increase in CREB phosphorylation, whereas CREB recovered from reaction mixtures containing both PKA and okadaic acid was phosphorylated approximately eightfold more compared with the CREB recovered from reaction mixtures with no added PKA and okadaic acid (Fig. 4A, panel III, lanes 5 and 6 versus lane 4). Equal amounts of CREB were recovered from these reactions as determined by Western blot analysis using anti-CREB antibodies (data not shown). We conclude from these results that CREB is required for PKA and okadaic acid to stimulate transcription from the PEPCK gene promoter. Furthermore, the phosphorylation of CREB by PKA and the enhanced phosphorylation of CREB by PKA and okadaic acid correlate directly with the stimulated levels of transcription from the PEPCK promoter.

CREB is required for the PKA and okadaic acid-mediated stimulation of transcription from the PEPCK promoter; however, it is possible that other factors, in addition to CREB, also play a role in modulating transcription from the PEPCK promoter in response to differential phosphorylation and dephosphorylation. To demonstrate that the phosphorylation state of CREB regulates transcription from the PEPCK promoter, either unphosphorylated CREB or PKA-phosphorylated CREB was added directly to CREB-depleted in vitro transcription reaction mixtures in the absence or presence of okadaic acid (Fig. 4B). The use of [^{32}P]CREB made it possible to monitor any CREB dephosphorylation during the incubation of the transcription reaction mixtures. Likewise, transcription reactions were performed with unphosphorylated CREB in the presence of [^{32}P]ATP so that phosphorylation of

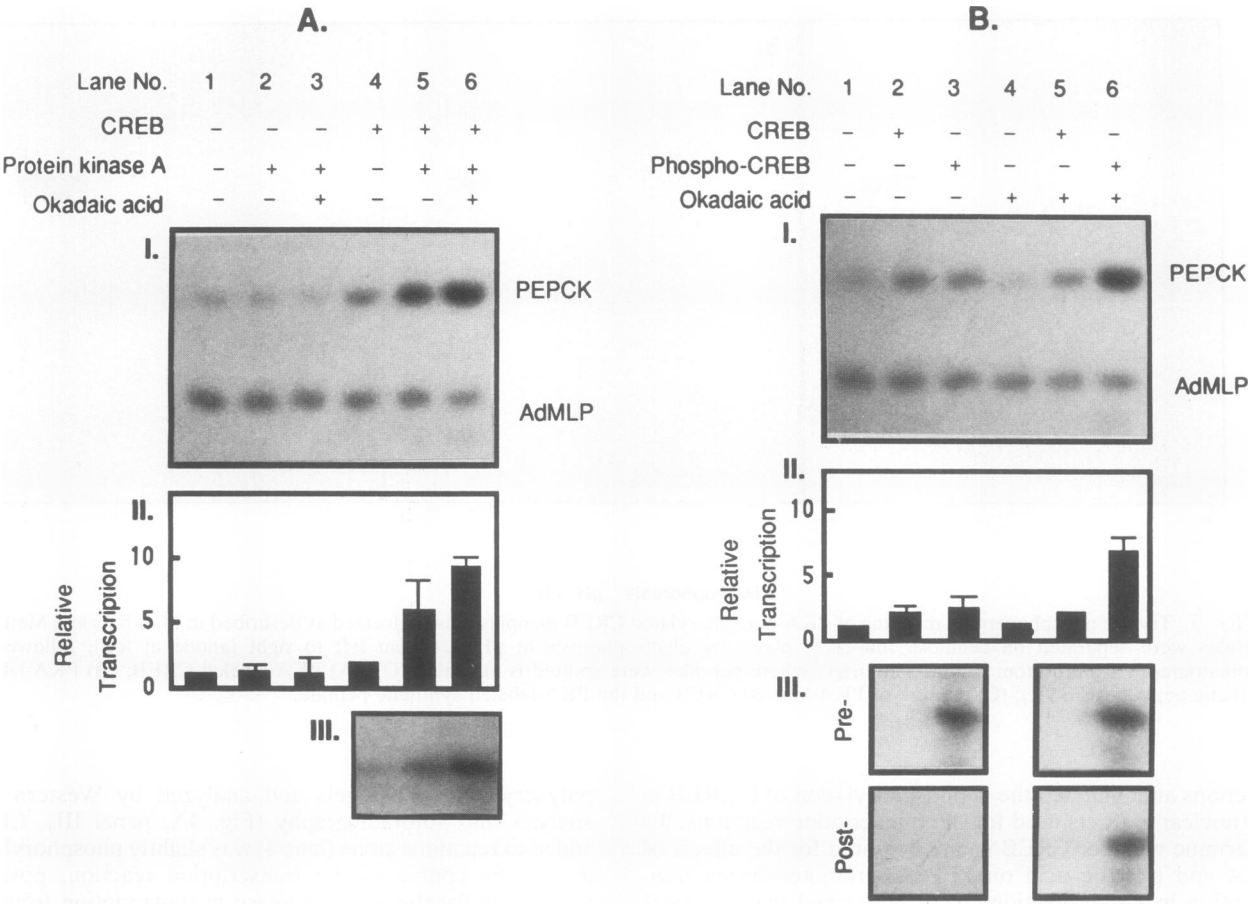


FIG. 4. CREB mediates the effects of PKA and okadaic acid on transcription from the PEPCK promoter in vitro. In vitro transcription reactions were performed with plasmids containing the −109 PEPCK promoter deletion and the adenovirus major late promoter (AdMLP) as templates and rat liver nuclear extract depleted of CREB by immunoprecipitation. (A) Ten nanograms of purified, bacterially expressed CREB, 10 ng of PKA catalytic subunit, and/or 200 nM okadaic acid was added to the indicated reaction mixtures. (I) Primer extension products from RNAs synthesized from the PEPCK promoter and AdMLP; (II) relative levels of transcription from the PEPCK promoter after correction for differences in transcription from the AdMLP. Levels of transcription are shown relative to the level in lane 1 and are averaged from three separate experiments. CREB was recovered from 5 μl of the reaction mixtures shown in lanes 4, 5, and 6 by immunoprecipitation and separated on a 10% polyacrylamide–SDS gel. [^{32}P]ATP was included in these reactions. (III) Autoradiogram showing the relative levels of CREB phosphorylation in these reactions. (B) Cell-free transcription reactions were performed as described in Materials and Methods using CREB-depleted nuclear extracts. Ten nanograms of unphosphorylated or phosphorylated CREB and/or 200 nM okadaic acid was added as indicated. All reaction mixtures contained 25 μg of Walsh peptide per ml, 50 μM H7, and 50 μM W7. The autoradiogram (I) shows the relative intensity of primer extension products corresponding to RNA transcripts from the PEPCK promoter and AdMLP. The bar graph (II) shows the levels of transcription from the PEPCK promoter corrected for transcription from the AdMLP control and relative to the level in the first lane. Bar graph data are averages from three experiments. (III) CREB was recovered from 5-μl aliquots of the transcription reactions before (pre-) and after (post-) the reaction mixtures were incubated. Mixtures for reactions performed with unphosphorylated CREB contained [^{32}P]ATP (lanes 2 and 5). [^{32}P]CREB was added to the indicated reactions (lanes 3 and 6). Panels show autoradiograms of CREB recovered from the reactions and separated on 10% polyacrylamide–SDS gels.

the protein during the reaction could be monitored. The reaction mixtures all contained a protein kinase inhibitor mixture (Walsh peptide, H7, and W7) to minimize phosphorylation of the CREB or other proteins in the reactions. The addition of unphosphorylated CREB to reactions, in either the absence or presence of okadaic acid, resulted in a small increase in RNA synthesis (Fig. 4B, panels I and II, lanes 2 and 5 versus lanes 1 and 4). The addition of P-CREB to reactions in the absence of okadaic acid also showed a twofold increase in transcription (lane 3), but in reactions in the presence of okadaic acid and P-CREB, transcription was stimulated five- to sixfold compared with that in reactions containing unphosphorylated CREB (lane 6 versus lane 5). Analysis of 5-μl aliquots removed from the reaction mixtures before and after in vitro transcription showed that unphosphorylated CREB was not detectably phosphorylated during incubation of the reaction mixtures (panel III, lanes 2 and 5). As expected, P-CREB added in the absence of okadaic acid was completely dephosphorylated during the course of the reaction (panel III, lane 3). However, in reactions containing okadaic acid, no dephosphorylation of the P-CREB was detected (panel III, lane 6). Western analysis of CREB recovered from the reactions showed no loss of CREB protein during the reaction (data not shown). These data clearly demonstrate that the phosphorylation of CREB by PKA and its dephosphorylation by a protein Ser/Thr phosphatase(s) present in our nuclear extracts regulate the rate of RNA synthesis from the PEPCK promoter.

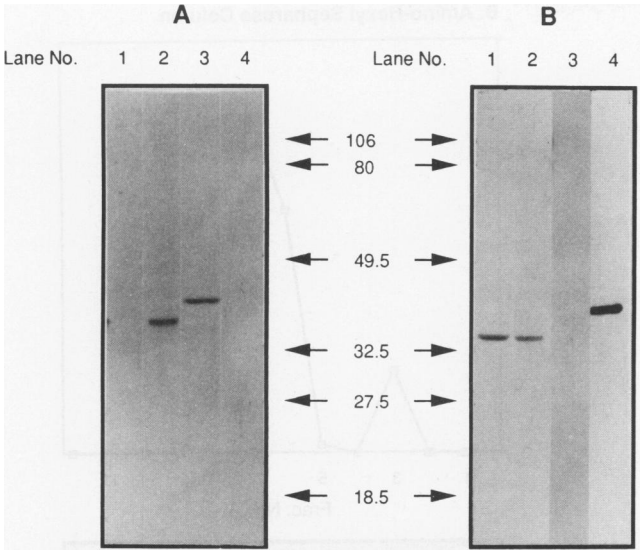


FIG. 5. Characterization of PP1 and PP2A antibodies. Western blot analysis was performed as described in Materials and Methods. Panel A was probed with affinity-purified rabbit anti-PP1, and panel B was probed with affinity-purified rabbit anti-PP2A. Samples included rat liver cytosolic fraction (60 μg) (lanes 1), rat liver nuclear extract (60 μg) (lanes 2), bacterial lysate containing PP1-fusion protein (50 μg) (lanes 3), and bacterial lysate containing PP2A-fusion protein (50 μg) (lanes 4).

Nuclear protein phosphatase 2A dephosphorylates CREB and inhibits PKA-stimulated CREB transcription activity. We have shown that okadaic acid inhibits the dephosphorylation of PKA-phosphorylated CREB in rat liver nuclear extracts and enhances PKA-stimulated, CREB-mediated transcription from the promoter of the PEPCK gene. The inhibition of P-CREB phosphatase activity by okadaic acid suggested either PP1 or PP2A as a candidate phosphatase enzyme. Resolution of these two phosphatases from nuclear extracts and subsequent biochemical studies were facilitated by antipeptide antibodies specific for PP1 and PP2A. The specificity of these antibodies was determined by Western blot analysis as shown in Fig. 5. Affinity-purified rabbit anti-PP1 detected the PP1 catalytic subunit (38 kDa) in nuclear extracts, as well as a bacterially expressed PP1 fusion protein (42 kDa) (Fig. 5A). However, the rabbit anti-PP1 did not detect a bacterially expressed PP2A fusion protein or a band corresponding to PP2A catalytic subunit in nuclear extracts. Likewise, the affinity-purified rabbit anti-PP2A readily detects the PP2A catalytic subunit (36 kDa) in nuclear extracts and the PP2A fusion protein (42 kDa) but does not detect the PP1 fusion protein (Fig. 5B). Furthermore, the binding of the affinity-purified antipeptide antibodies to their respective phosphatase catalytic subunits could be completely blocked with the synthetic peptides used in the preparation of the antisera (data not shown). Fractionation of rat liver nuclear extracts on a Mono Q anion-exchange column demonstrated that the P-CREB phosphatase activity (3.478 fmol of $^{32}P_i$ released from CREB per fmol of PP2A per min) coeluted with PP2A catalytic subunit detected by Western blot analysis (Fig. 6A). The nuclear PP1 enzyme was clearly resolved from the peak of nuclear extract P-CREB phosphatase activity.

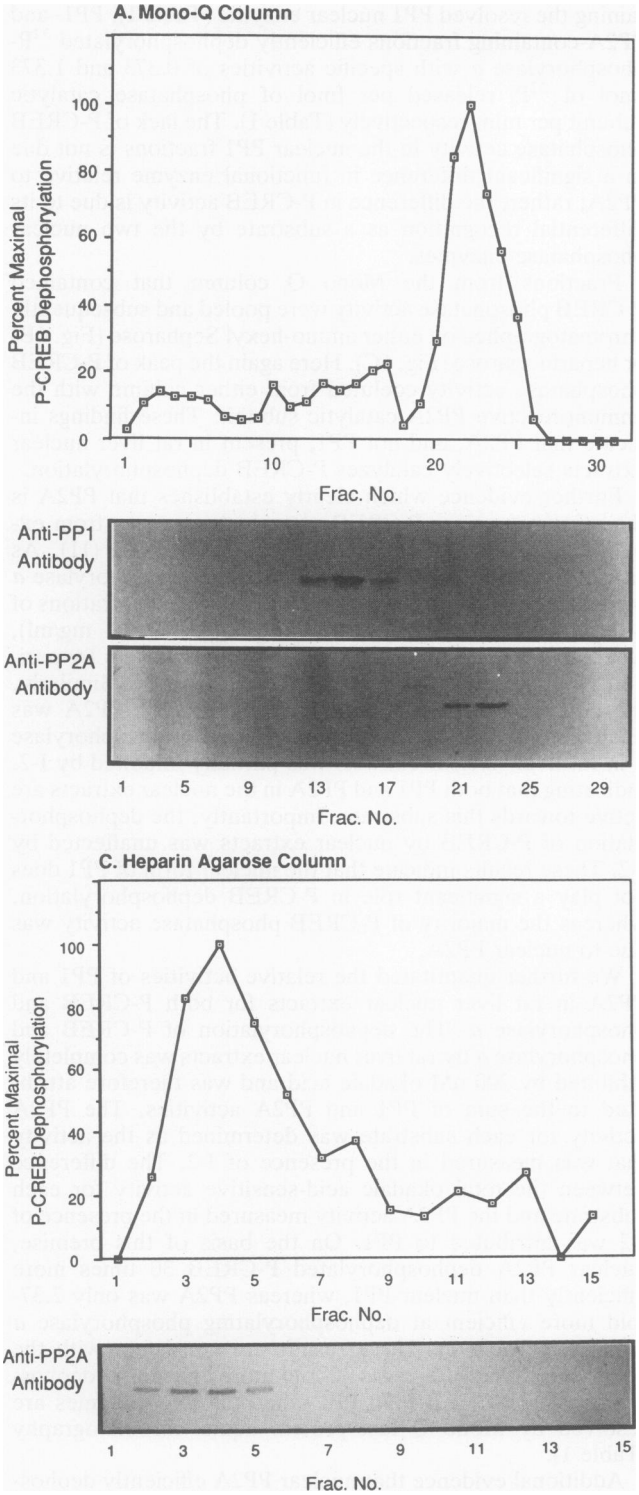
The Mono Q fractions containing PP2A dephosphorylated P-CREB approximately 30 times more than did those containing the resolved PP1 nuclear enzyme (Table 1). PP1- and PP2A-containing fractions efficiently dephosphorylated ^{32}P-phosphorylase *a* with specific activities of 0.373 and 1.373 fmol of $^{32}P_i$ released per fmol of phosphatase catalytic subunit per min, respectively (Table 1). The lack of P-CREB phosphatase activity in the nuclear PP1 fractions is not due to a significant difference in functional enzyme relative to PP2A; rather, the difference in P-CREB activity is due to its differential recognition as a substrate by the two nuclear phosphatase enzymes.

Fractions from the Mono Q column that contained P-CREB phosphatase activity were pooled and subsequently chromatographed on either amino-hexyl Sepharose (Fig. 6B) or heparin agarose (Fig. 6C). Here again the peak of P-CREB phosphatase activity coeluted from either column with the immunoreactive PP2A catalytic subunit. These findings indicate that PP2A, and not PP1, present in rat liver nuclear extracts selectively catalyzes P-CREB dephosphorylation.

Further evidence which clearly establishes that PP2A is the primary nuclear P-CREB phosphatase comes from experiments using the potent inhibitor of PP1, I-2 (11). As shown in Fig. 7, the dephosphorylation of phosphorylase *a* by nuclear PP1 was inhibited by increasing concentrations of I-2 (50% inhibitory concentration [IC_{50}], ~0.001 mg/ml), whereas the dephosphorylation of phosphorylase *a* by purified PP2A was not sensitive to the addition of I-2. Similarly, the dephosphorylation of P-CREB by purified PP2A was unaffected by I-2. The dephosphorylation of phosphorylase *a* in rat liver nuclear extracts was partially inhibited by I-2, indicating that both PP1 and PP2A in the nuclear extracts are active towards this substrate. Importantly, the dephosphorylation of P-CREB by nuclear extracts was unaffected by I-2. These results indicate that the nuclear form of PP1 does not play a significant role in P-CREB dephosphorylation, whereas the majority of P-CREB phosphatase activity was due to nuclear PP2A.

We further quantitated the relative activities of PP1 and PP2A in rat liver nuclear extracts for both P-CREB and phosphorylase *a*. The dephosphorylation of P-CREB and phosphorylase *a* by rat liver nuclear extracts was completely inhibited by 200 nM okadaic acid and was therefore attributed to the sum of PP1 and PP2A activities. The PP2A activity for each substrate was determined as the activity that was measured in the presence of I-2. The difference between the total okadaic acid-sensitive activity for each substrate and the PP2A activity measured in the presence of I-2 was attributed to PP1. On the basis of this premise, nuclear PP2A dephosphorylated P-CREB 30 times more efficiently than nuclear PP1, whereas PP2A was only 2.37-fold more efficient at dephosphorylating phosphorylase *a* than PP1 (Table 1). These results are consistent with the observation that PP2A was 30-fold more efficient at dephosphorylating P-CREB than PP1 when the two enzymes are resolved by Mono Q fast protein liquid chromatography (Table 1).

Additional evidence that nuclear PP2A efficiently dephosphorylates P-CREB came from experiments in which PP2A and PP1 were immunoprecipitated directly from rat liver nuclear extracts and tested for their ability to dephosphorylate P-CREB. Quantitative immunoprecipitation of PP1 from nuclear extracts did not decrease the P-CREB phosphatase activity in the extracts. Quantitative removal of PP2A from rat liver nuclear extracts was not possible, since the PP2A antiserum is less efficient in immunoprecipitating the PP2A enzyme. However, direct measurement of P-CREB dephosphorylation by immunoprecipitated PP1 and PP2A showed



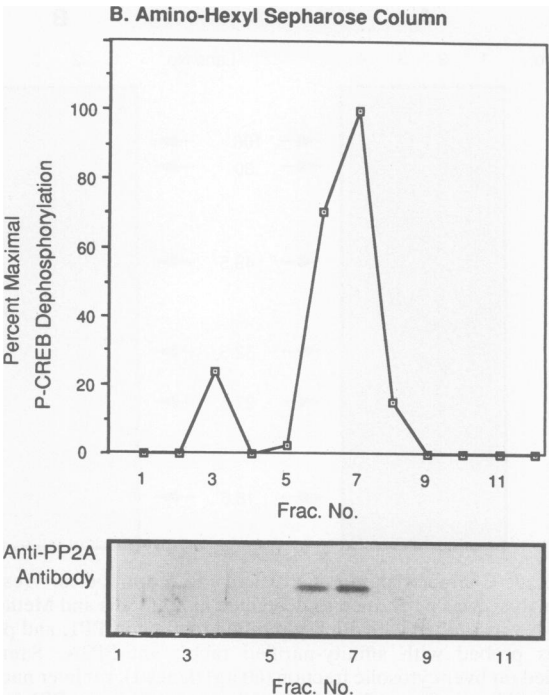


FIG. 6. Chromatographic resolution of P-CREB phosphatase from rat liver nuclear extracts. Five milligrams of rat liver nuclear protein was fractionated on a Mono Q column (A) developed with a linear 0 to 400 mM NaCl gradient as described in Materials and Methods. A 25-μl portion of each fraction was assayed for P-CREB phosphatase activity (top), and 75-μl portions of alternate fractions were analyzed by Western blotting (bottom) using antibodies specific for PP1 and PP2A as indicated. The peak of P-CREB phosphatase activity from the Mono Q column was further resolved on either an amino-hexyl Sepharose (B) or a heparin agarose (C) column. Each of these columns was developed with a 0 to 1 M NaCl step gradient (0.1 M increase per step). Aliquots of each fraction were assayed for P-CREB phosphatase activity (top) or analyzed by Western blot (bottom) for PP2A as described above.

that PP2A dephosphorylated P-CREB approximately 63-fold more efficiently than PP1. The specific activities of P-CREB dephosphorylation by immunoprecipitated PP2A and PP1 were 156 and 2.49 fmol of $^{32}P_i$ released per pmol of catalytic subunit per min, respectively.

The results of biochemical fractionation and immunologi-

TABLE 1. Dephosphorylation of P-CREB and phosphorylase *a* by rat liver nuclear PP1 and PP2A[a]

Fraction and activity	Dephosphorylation of:	
	P-CREB	Phosphorylase
RLNE[b]		
PP1	0.017	0.996
PP2A	0.567	2.36
PP2A/PP1 ratio	33.3	2.37
Mono Q[c]		
PP1	0.114	0.373
PP2A	3.47	1.37
PP2A/PP1 ratio	30.5	3.68

[a] Dephosphorylation of P-CREB and phosphorylase *a* was measured as described in Materials and Methods. Reaction mixtures contained 4.2 pmol of P-CREB (4,383 cpm/pmol) or 3.8 pmol of phosphorylase *a* (13,593 cpm/pmol). Dephosphorylation is expressed as femtomoles of $^{32}P_i$ released per femtomole of phosphatase catalytic subunit per minute. Concentrations of PP1 and PP2A in the nuclear extract and Mono Q fractions were determined by comparison with known concentrations of purified PP1 and PP2A on Western blots.

[b] Total PP1 plus PP2A activity towards each substrate is the activity in the nuclear extract in the absence of okadaic acid minus the activity in the presence of 400 nM okadaic acid. PP2A activity is the amount of okadaic acid-sensitive activity that is not inhibited by I-2. The PP1 activity is the I-2-sensitive activity. RLNE, rat liver nuclear extract.

[c] Immunoreactive PP1 and PP2A fractions from Mono Q chromatography.

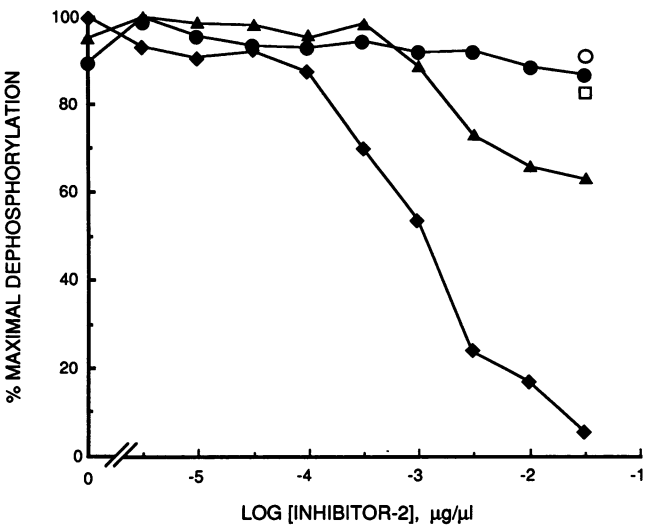


FIG. 7. Inhibition of the dephosphorylation of phosphorylase *a* and P-CREB by I-2. Shown are dephosphorylation of [^{32}P]phosphorylase *a* catalyzed by 50 ng of heterotrimeric PP2A (□), by 25 μg of rat liver nuclear protein (▲), and by 25 μl of nuclear PP1 (◆) obtained by Mono Q fractionation of rat liver nuclear extract as described in the legend to Fig. 6 and dephosphorylation of [^{32}P]CREB by heterotrimeric PP2A (○) and by rat liver nuclear protein (●). Reactions were performed as described in Materials and Methods and contained the indicated amounts of I-2.

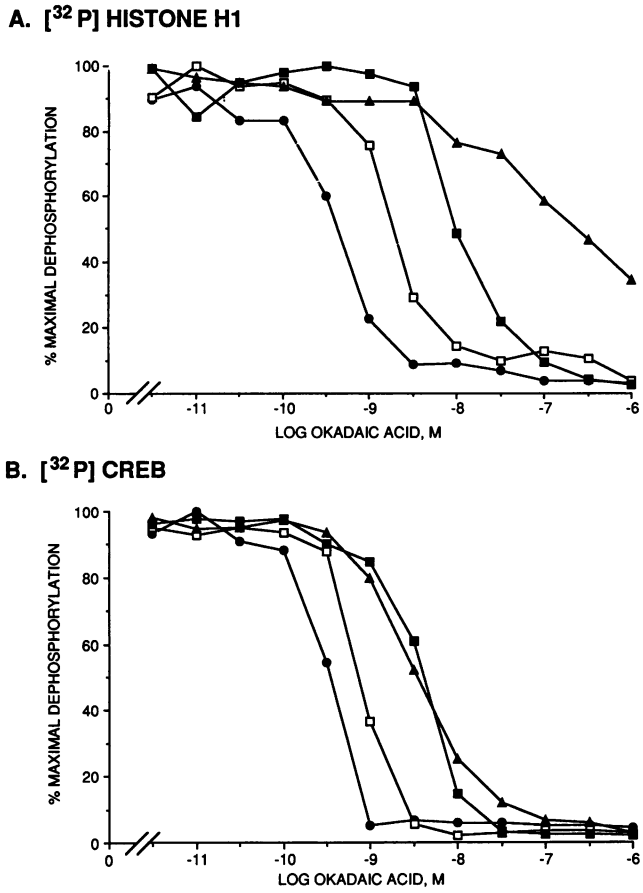


FIG. 8. Dose-response curves for the okadaic acid inhibition of P-histone H1 and P-CREB dephosphorylation. Dephosphorylation of [^{32}P]histone H1 (A) and [^{32}P]CREB (B) catalyzed by heterotrimeric PP2A (■), heterodimeric PP2A (□), PP2A catalytic subunit (●), or rat liver nuclear extracts (▲) was determined in the presence of the indicated concentrations of okadaic acid. Reactions were performed as described in Materials and Methods. The purified PP2A preparations contained approximately equal molar amounts of catalytic subunit as determined by comparative Western blotting. Twenty-five micrograms of rat liver nuclear protein was used in the indicated reactions. The results are the averages from two independent experiments.

cal analysis define nuclear PP2A as P-CREB phosphatase. The activity and substrate specificity of the 36-kDa PP2A catalytic subunit (C subunit) are controlled by two regulatory subunits: a 60- to 63-kDa A subunit and either a 55-kDa B, a 54-kDa B′, or a 74-kDa B″ subunit (11). In most tissues PP2A exists in a heterotrimeric (ABC) complex, but a heterodimeric (AC) form has also been purified from a number of tissues (43). In order to determine which oligomeric form of PP2A is responsible for P-CREB dephosphorylation in nuclear extracts, the okadaic acid sensitivity of nuclear extracts was compared with the sensitivity of heterotrimeric (ABC), heterodimeric (AC), and free catalytic (C) PP2A subunit. The okadaic acid sensitivity of each preparation was compared by using P-histone H1 (Fig. 8A) or P-CREB (Fig. 8B) as a substrate. The IC_{50}s of okadaic acid with free C-subunit, AC, and ABC forms of PP2A were 0.3, 2, and 10 nM, respectively, with P-histone H1 as the substrate. These values are similar to those reported previously in other studies using phosphorylated myosin light chain as a substrate (23). With rat liver nuclear extracts, the inhibition curve was much shallower and only partial inhibition of P-histone dephosphorylation was observed at 100 nM okadaic acid. This finding suggests that additional P-histone H1 phosphatases that are significantly less sensitive to inhibition by okadaic acid than PP2A or PP1 are present in the nuclear extracts. At present we have not identified these putative additional phosphatases and their regulatory properties.

Use of P-CREB as a substrate gave results with nuclear extracts significantly different from those observed with P-histone H1 (Fig. 8B). The IC_{50} for okadaic acid inhibition of P-CREB dephosphorylation with nuclear extracts was identical to that observed with the heterotrimeric (ABC) form of PP2A (approximately 3 nM). The free C and AC forms of PP2A also have some ability to dephosphorylate P-CREB and are inhibited by okadaic acid with IC_{50}s of approximately 0.3 and 0.7 nM, respectively. These results suggest that in rat liver nuclear extracts, the major P-CREB phosphatase activity is a function of the heterotrimeric ABC form of PP2A. In addition to exhibiting similar okadaic acid sensitivity as heterotrimeric PP2A, P-CREB phosphatase in nuclear extracts was found to fractionate on a Mono Q column identically to heterotrimeric PP2A (data not shown).

PP2A was also found to directly inhibit the ability of PKA-phosphorylated CREB to stimulate transcription from the PEPCK promoter in vitro. Immunoprecipitated PP1 and PP2A were incubated with PKA-phosphorylated CREB for several hours. At the end of the incubation period, less than 5% of the PP2A-treated CREB was still phosphorylated, while over 90% of the PP1-treated CREB remained in the phosphorylated form (data not shown). The phosphatase-treated CREBs were recovered and added to cell-free transcription reactions with CREB-depleted nuclear extract (Fig. 9, lanes 3 and 4). Control reactions were performed

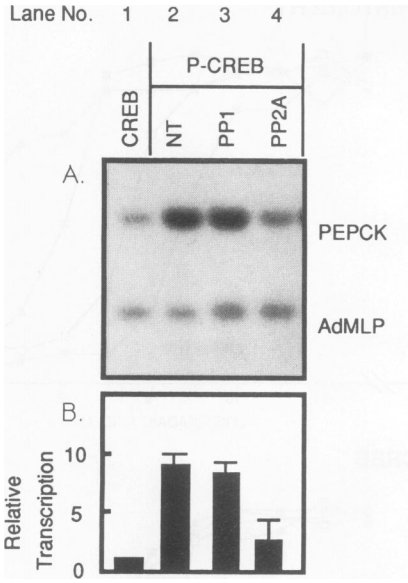


FIG. 9. PP2A inhibits P-CREB-dependent transcription from the PEPCK promoter in vitro. Transcription reactions were performed as described in Materials and Methods, using CREB-depleted nuclear extract. PKA-phosphorylated CREB (P-CREB) was untreated (NT) or treated with immunopurified protein phosphatase (PP1 or PP2A). The phosphatase-treated CREB and unphosphorylated CREB (CREB) were added to the transcription reactions as shown. All reaction mixtures contained 200 nM okadaic acid, 25 μg of Walsh peptide per ml, 50 μM H7, and 50 μM W7. (A) Intensities of PEPCK and adenovirus major late promoter (AdMLP) transcripts; (B) data corrected for AdMLP control. Bar graph data are averaged from three experiments.

with unphosphorylated CREB and PKA-phosphorylated CREB (Fig. 9, lanes 1 and 2, respectively) to establish the unstimulated and stimulated levels of transcription from the PEPCK promoter. All the reactions contained the protein kinase-phosphatase inhibitor mixture to prevent changes in CREB phosphorylation during incubation of the transcription reaction mixtures. Both untreated P-CREB and PP1-treated P-CREB stimulated transcription from the PEPCK promoter. However, the PP2A-treated P-CREB did not significantly stimulate PEPCK promoter-driven transcription. PP2A-treated CREB could be reactivated by phosphorylation with PKA and ATP (data not shown). Therefore, we conclude that PP2A efficiently dephosphorylates PKA-phosphorylated CREB and inhibits or attenuates cAMP- or PKA-stimulated transcription of the PEPCK gene.

DISCUSSION

The dephosphorylation of cytosolic and microsomal phosphoproteins by specific protein phosphatases is a primary mechanism for the rapid regulation of numerous metabolic systems (11). Recent studies suggest that nuclear protein phosphatases play equally important roles in regulating nuclear activities such as gene transcription (5, 8, 17, 32) and DNA synthesis (3, 7, 14, 25). The data presented here clearly demonstrate that nuclear PP2A dephosphorylates PKA-phosphorylated CREB and attenuates CREB-mediated transcription from the PEPCK promoter. This finding is significant because it is the first time a specific nuclear protein Ser/Thr phosphatase has been used to show specificity of dephosphorylation and regulation of the activity of a nuclear transcription factor. Previous studies that have examined nuclear protein dephosphorylation have used protein Ser/Thr phosphatases purified from the cytosol (5, 17, 32). This report is the first in which nuclear protein Ser/Thr phosphatases have been chromatographically resolved, immunologically identified, and tested for their ability to dephosphorylate a specific substrate. By these same techniques it should be possible to isolate and begin to define the roles of other nuclear protein Ser/Thr phosphatases in the regulation of nuclear processes.

We have identified the P-CREB phosphatase activity in our nuclear extracts as PP2A. This conclusion is based on the coelution of P-CREB phosphatase activity with the peak of immunoreactive PP2A by several chromatographic procedures. In these procedures, PP2A and P-CREB phosphatase are completely resolved from the peak of immunoreactive nuclear PP1. In addition, PP2A directly immunopurified from nuclear extracts dephosphorylates PKA-labeled CREB more efficiently than immunopurified nuclear PP1 and directly inhibits the ability of PKA-phosphorylated CREB to stimulate transcription from the PEPCK promoter in vitro.

Consistent with nuclear PP2A being the primary P-CREB phosphatase, studies show that the small-t antigen of simian virus 40 inhibits P-CREB dephosphorylation in nuclear extracts and enhances cAMP-stimulated PEPCK gene transcription in cells (44a). Simian virus 40 small-t antigen has been demonstrated to form complexes with the heterodimeric AC form of PP2A (33, 44, 46). The association of small-t antigen with the AC complex of PP2A effectively diminishes the level of heterotrimeric ABC PP2A complexes in the cell. Thus, small-t antigen association with phosphatase 2A AC complexes is predicted to result in a diminished P-CREB phosphatase activity, just as small-t antigen inhibits dephosphorylation of other substrates, such as large-T antigen and p53 (39). If true, the ability of P-CREB to activate transcription would be prolonged in cells expressing small-t antigen because the level of heterotrimeric phosphatase 2A would be diminished. If other transcription factors activated by phosphorylation are preferred substrates for the heterotrimeric ABC phosphatase 2A complex, a similar enhancement of specific gene transcription would be observed. Enhanced transcription of specific genes is, in fact, one consequence of small-t antigen expression (30). We are currently attempting to define the role of different phosphatase complexes in the regulation of specific transcription factors whose activities are controlled by phosphorylation and dephosphorylation. It will then be possible to determine whether the oligomeric state of phosphatase 2A plays a role in transcriptional regulation.

Our conclusion that PP2A is the primary phosphatase that dephosphorylates PKA-phosphorylated CREB is in direct contrast to the report of Hagiwara et al. (17) that identified PP1 as the primary P-CREB phosphatase. The differences between our findings and those of Hagiwara et al. (17) may be due to differences in protein phosphatase preparations used in each study. For example, some experiments presented by Hagiwara et al. (17) were performed with phosphatase catalytic subunits isolated from skeletal muscle cytosol, whereas our studies have focused on nuclear phosphatase holoenzymes. Several studies have reported that certain phosphatase catalytic subunits and holozymes differ in their substrate specificities (6, 11, 31, 46). Furthermore, we have taken great care in isolating protein phosphatases from nuclei free of cytosolic and microsomal marker proteins. Our purification of nuclear PP1 and PP2A indicates that the regulatory subunits of the nuclear enzymes are

different from their cytosolic counterparts (43a). Mono Q chromatography of cytosolic and nuclear PP1 isoforms shows dramatic differences in their elution profiles apparently related to differences in nuclear and cytosolic PP1 regulatory subunits found associated with the PP1 catalytic subunit. Similarly, evidence from our laboratories indicates that nuclear and cytosolic PP2A catalytic subunits may be complexed with immunologically distinct regulatory B subunits. Clearly, future analysis of nuclear protein dephosphorylation would benefit from the use of nuclear protein phosphatase holoenzymes rather than catalytic subunits of protein phosphatases.

In other experiments, Hagiwara et al. (17) demonstrated that expression of PP1 from a eucaryotic expression vector inhibited the cAMP-stimulated expression of a reporter protein from a CRE-containing promoter. Since these experiments measured the overall expression of a reporter protein, these results may indicate that expression of PP1 plays a role in regulating reporter protein expression at a posttranscriptional level, whereas we show here that nuclear PP2A directly dephosphorylates P-CREB and inhibits P-CREB-mediated transcription from the PEPCK promoter in cell-free reactions. Furthermore, expression and nuclear targeting of PP1 or other protein phosphatases using eucaryotic expression vectors have not been demonstrated, which makes interpretation of the results of Hagiwara et al. (17) difficult.

It is imperative to study the native forms of the protein phosphatases, in which the catalytic subunits are complexed with appropriate regulatory subunits, since the regulatory subunits appear to play important roles in determining normal substrate specificity, as well as catalytic activity (6, 11). In addition, the regulatory subunits of specific phosphatases determine cellular localization (6, 11, 19). Cumulatively, the involvement of the different phosphatase regulatory subunits in controlling enzyme function and localization emphasizes the importance of using the nuclear forms of PP1 and PP2A in studying the regulation of different transcription factors. The free phosphatase catalytic subunits cannot be used for unequivocal interpretation of the role of PP1 and PP2A in the control of phosphorylated nuclear proteins. Biochemical studies, in conjunction with cell-free transcription and DNA binding analysis, will allow us to further define the role of nuclear protein phosphatases in transcriptional regulation and to establish systems to study the regulation of these important enzymes in response to extracellular stimuli.

ACKNOWLEDGMENTS

This work was supported by NIH grant DK37871. B.E.W. is a recipient of a National Science Foundation postdoctoral fellowship.

B.E.W. and W.H.W. made equal contributions to this work.

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