Dephosphorylation of Translational Initiation Factors and 40S Ribosomal Subunits by Phosphoprotein Phosphatases from Rabbit Reticulocytes†

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ABSTRACT: Three major phosphoprotein phosphatase activities (I–III) were resolved from the postribosomal supernatant fraction by DEAE-cellulose chromatography. The three activities were fractionated further by chromatography on Sephacryl S-200. The major activity in each fraction had an apparent molecular weight of 270 000 and was designated B. The B forms had similar although not identical properties and constituted ~80% of the total phosphatase activity. A second major form (C) with an apparent molecular weight of 140 000 was observed in fractions I and II and comprised 15% of the phosphatase activity. Fraction III contained a third form of phosphatase (D) with an apparent molecular weight of 180 000 and properties similar to those of B. A fourth activity (A) with a molecular weight greater than 500 000 was observed in minor amounts in all three fractions. The activities dephosphorylated histone phosphorylated by the cyclic adenosine 3′,5′-monophosphate (cAMP)-regulated protein kinases from reticulocytes and casein phosphorylated by casein kinase II, a cyclic nucleotide independent protein kinase. The phosphatase activities were examined by using phosphorylated 40S ribosomal subunits and initiation factors 2 (eIF-2) and 3 (eIF-3). The B form dephosphorylated 40S ribosomal protein S6 and eIF-3 phosphorylated by the cAMP-regulated protein kinases, eIF-3 phosphorylated by casein kinase II, and eIF-2 phosphorylated by the hemin-controlled repressor, casein kinase II, or the activated form of protease-activated kinase II. The C form dephosphorylated all of the substrates except eIF-2α. Dephosphorylation of the translational components by the B form was shown to be affected by compounds which regulate protein synthesis. High concentrations of guanosine 5′-triphosphate (4 mM) inhibited the phosphatase activity with all of the translational components tested. Adenosine 5′-triphosphate (4 mM), glucose 6-phosphate (4 mM), and hemin (0.025 mM) specifically inhibited the dephosphorylation of S6 and eIF-2α, while cAMP (4 mM) stimulated the dephosphorylation of eIF-2α by 1.5- to 2-fold.

A number of components involved in protein synthesis are readily phosphorylated and dephosphorylated in intact reticulocytes and reticulocyte lysates. These include 40S ribosomal protein S6[1] (Kabat, 1970; Traugh & Porter, 1976), eIF-2α[2] (M_r 38 000) and eIF-2β (M_r 53 000) (Barrieux & Rosenfeld, 1977; Farrell et al., 1978; Floyd et al., 1979; Benne et al., 1978), and eIF-3 and eIF-4B (Floyd et al., 1979; Benne et al., 1978). Purified components of the protein-synthesizing complex from reticulocytes have been phosphorylated in vitro with highly purified protein kinases and ATP or GTP. The 40S ribosomal subunits and eIF-3 (130 000-dalton subunit) are phosphorylated by the cAMP-regulated protein kinases (Traugh et al., 1973; Traugh & Porter, 1976; Traugh & Lundak, 1978). eIF-2α has been shown to be phosphorylated by a cyclic nucleotide independent protein kinase activity which copurifies with the hemin-controlled repressor (Kramer et al., 1976; Farrell et al., 1977; Levin et al., 1976; Gross & Mendelewski, 1977; Tahara et al., 1978; Trachsel et al., 1978). Two different cyclic nucleotide independent protein kinases, casein kinase II and protease-activated kinase II, phosphorylate eIF-2β (Traugh et al., 1976; Issinger et al., 1976; Tahara et al., 1978; Hathaway et al., 1979; Traugh et al., 1979). In addition, eIF-3 and eIF-5 are multiply phosphorylated in vitro (Traugh et al., 1976; Hathaway et al., 1979; Traugh et al., 1979).

The phosphorylation of eIF-2α has been shown to have a role in regulation of protein synthesis. eIF-2 forms a ternary complex with GTP and Met-tRNA$_f$ which binds to 40S ribosomal subunits (Dettman & Stanley, 1972; Schreier & Staehelin, 1973; Gupta et al., 1973; Levin et al., 1973; Safer et al., 1975a; Smith & Henshaw, 1975; Ranu & Wool, 1976). During hemin deficiency a translational repressor is formed in reticulocyte lysates which phosphorylates eIF-2 (Kramer et al., 1976; Farrell et al., 1977; Levin et al., 1976; Gross & Mendelewski, 1977). This phosphorylation event has been correlated with the inhibition of protein synthesis in hemin-deficient lysates (Farrell et al., 1977; Pinphanichakarn et al., 1976; Kramer et al., 1977). The inhibition of protein synthesis produced under conditions of hemin deprivation has been shown to be reversed by high concentrations (10 mM) of cAMP, GTP, and 2-aminopurine while ATP potentiates the inhibition (Legon et al., 1974; Balkow et al., 1975; Ernst et al., 1976). In addition, it has been observed that sugars and sugar phosphates, especially glucose 6-phosphate, can relieve inhibition of protein synthesis caused by hemin deficiency (Ernst et al., 1978).

Although the protein kinases which phosphorylate these components have been studied in some detail, the phosphatases involved in these events have not been described. Thus, we have partially purified the phosphoprotein phosphatases from rabbit reticulocytes and examined the dephosphorylation of

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[1] Ribosomal proteins have been identified according to the uniform nomenclature proposed by McConkey et al. (1979). S6 was previously called S13 in rabbit reticulocytes according to the nomenclature of Howard et al. (1975).

[2] Abbreviations used: cAMP, cyclic adenosine 3′,5′-monophosphate; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; eIF, eucaryotic initiation factor; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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40S ribosomal subunits, eIF-2, and eIF-3.

Materials and Methods

Materials. Salt-washed 40S ribosomal subunits from rabbit reticulocytes were prepared as described by Traugh & Porter (1976) and stored at −70 °C. Initiation factors 2 and 3 from rabbit reticulocytes were generously supplied by Dr. W. C. Merrick, Department of Biochemistry, Case Western Reserve University. eIF-2 was prepared as described by Safer et al. (1975b) and was greater than 95% pure when analyzed by gel electrophoresis. eIF-3 was prepared as described by Safer et al. (1976) and ranged in purity from 75 to 95%.

Acrylamide and *N,N'*-methylenebis(acrylamide) were from Eastman, and the latter was recrystallized from acetone before use. Sodium dodecyl sulfate was from British Drug House. *N,N,N',N'*-tetramethylethylenediamine and Coomassie Brilliant Blue R were from Sigma. [γ-^{32}P]ATP was prepared as described elsewhere (Hathaway et al., 1979).

Preparation of Protein Kinases. Preparation of protein kinases from rabbit reticulocytes by chromatography on DEAE-cellulose and phosphocellulose has been described previously (Hathaway et al., 1979; Hathaway & Traugh, 1979). The protein kinases used in these studies included the type I and type II cAMP-regulated protein kinases and three cyclic nucleotide independent protein kinases. The latter consisted of the hemin-controlled repressor, the activated form of protease-activated kinase II, and casein kinase II. One unit of protein kinase incorporated 1 pmol of phosphate/min into the substrate at 30 °C. The cAMP-regulated enzymes were quantitated with histone IIA (Sigma), and casein kinase II was quantitated with casein (Hathaway et al., 1979).

Phosphorylation of Substrates. Radiolabeled phosphohistone and phosphocasein were prepared by incubation of the substrate with protein kinase and [γ-^{32}P]ATP. Reaction mixtures for the phosphorylation of histone contained the following components: 50 mM Mops, pH 7.0; 10 mM $MgCl_2$; 1.4 μM cAMP; 0.14 mM [γ-^{32}P]ATP; 10 mg/mL histone IIA; and 1000–2500 units/mL cAMP-regulated protein kinase. Reaction mixtures for the phosphorylation of casein contained the following: 50 mM Mops, pH 7.0; 10 mM $MgCl_2$; 140 mM KCl; 0.14 mM [γ-^{32}P]ATP; 10 mg/mL casein; and 1000–2500 units/mL CK II. Reaction volumes varied from 4 to 18 mL.

The reaction mixtures were incubated for 1 h at 30 °C and then placed on ice; subsequent steps were carried out at 4 °C. Noncovalently bound ^{32}P was removed by repeated acid precipitation of the protein. Trichloroacetic acid was added to a final concentration of 25%; the precipitate was collected by centrifugation at 3000*g* for 10 min and redissolved in a volume of 0.5 N NaOH equal to the initial reaction volume. This procedure was repeated 2 additional times, and the final precipitate was dissolved in one-sixth the initial volume of 0.1 N NaOH. The phosphorylated proteins were dialyzed extensively against 50 mM Tris-HCl, pH 7.4, and stored at −20 °C. For determination of the specific activity of the phosphoproteins, 0.005 mL of the phosphorylated substrate was precipitated in 5 mL of 10% trichloroacetic acid. The precipitate was collected on a glass fiber filter and washed 3 times with 5 mL of 5% trichloroacetic acid and once with 95% ethanol. The filter was dried and counted in toluene scintillation fluid. The amount of ^{32}P incorporated into protein was determined from the specific activity of the [γ-^{32}P]ATP. Typical values for ^{32}P incorporation into histone and casein were 25 and 5 nmol/mg, respectively.

Assay for Phosphoprotein Phosphatase Activity. Phosphoprotein phosphatase activities were determined by monitoring the release of radioactive phosphate from phosphorylated histone or casein using the method of Parvin & Smith (1969) as modified by Kato & Bishop (1972). The phosphatase assays were performed in a final volume of 0.08 mL. Assays with histone contained the following: 50 mM Mops, pH 7.0; 5 mM $MnCl_2$; 20 mM 2-mercaptoethanol; phosphorylated histone containing 1 nmol of ^{32}P; and the phosphatase activity. Reaction mixtures for the dephosphorylation of casein contained the following: 50 mM Mes, pH 5.4, or 50 mM Mops, pH 7.0; 5 mM $MnCl_2$, where indicated; 20 mM 2-mercaptoethanol; phosphorylated casein containing 1 nmol of ^{32}P; and the phosphatase activity. The final pH of the reaction mixtures containing Mes was raised to 5.6 by the addition of the phosphatase solution. Sufficient enzyme was used to release between 10 and 20% of the radioactive phosphate during the 15-min incubation period at 30 °C. Under these conditions, phosphate release was linear with time and with the amount of added enzyme. The reaction was terminated by the addition of 0.09 mL of silicotungstic acid solution (60 mM silicotungstic acid in 0.06 N H_2SO_4), and the precipitate was removed by centrifugation in a Beckman microfuge. To 0.2 mL of butanol was added 0.1 mL of the supernatant solution followed by 0.1 mL of ammonium metavanadate–molybdate reagent. The samples were vortexed immediately and the aqueous and butanol phases allowed to separate. An aliquot (0.1 mL) of the butanol phase containing the molybdovanadophosphate complex was removed and spotted on a 2 × 2 cm square of Whatman ET31 filter paper. The filter paper was dried under a heat lamp and the radioactivity determined by scintillation counting. One enzyme unit of phosphatase activity was defined as that amount of enzyme which released 1 pmol of phosphate/min from phosphohistone at 30 °C.

Purification of Phosphoprotein Phosphatase Activities. The ribosome-free supernatant fraction (300 mL) was prepared as previously described (Traugh & Sharp, 1979), dialyzed against two changes of TEM (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 10 mM 2-mercaptoethanol), and mixed with 30 g (dry weight) of DEAE-cellulose (DE-52, Whatman) equilibrated in TEM. These and subsequent steps were carried out at 4 °C. The slurry was stirred slowly for 45 min and transferred to a Büchner funnel. The DEAE-cellulose was washed 6 times with 200-mL portions of TEM to remove hemoglobin and other loosely associated proteins. The washed DEAE-cellulose was suspended in 2.5 volumes of TEM and poured into a 3.7 × 22.5 cm column. The column was washed with TEM until the absorbance of the effluent at 280 nm had returned to base line. The protein was eluted with a linear gradient of 1.5 L ranging from 0 to 0.5 M NaCl in the same buffer.

Column fractions (15 mL) were analyzed for phosphatase activity with histone and casein as substrate. Phosphatase activities I, II, and III were identified, pooled separately, and concentrated by the addition of solid ammonium sulfate to 80% saturation. During the addition of ammonium sulfate, the pH was maintained at 7.2 by the dropwise addition of 2 N ammonium hydroxide. The precipitate was collected by centrifugation at 15000*g* for 30 min, resuspended in a small volume of TEMN (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM 2-mercaptoethanol, and 0.1 M NaCl), and dialyzed against the same buffer.

The three phosphatase fractions obtained by DEAE-cellulose chromatography were purified further by chromatography on Sephacryl S-200 (Pharmacia). A slurry of Sephacryl S-200 was prepared by mixing TEMN with the preswollen gel. The slurry was poured into a column (2.5 × 80 cm) fitted with a

buffer reservoir and packed at a flow rate of 90 mL/h. The column was washed with 3 volumes of TEMN. Between 4 and 12 mL of concentrated, dialyzed phosphatase activity (6.8–7.5 mg of protein/mL) from the DEAE-cellulose step was applied to the column, and the column was developed with TEMN at a flow rate of 16 mL/h. Absorbance was monitored at 280 nm. The individual phosphatase fractions from the DEAE-cellulose step were chromatographed separately, but on the same S-200 resin. The column fractions (6 mL) were assayed for phosphatase activity with histone and casein, and the phosphatase activities were pooled separately and dialyzed against TEM or concentrated by ultrafiltration using an Amicon PM-10 membrane prior to dialysis.

Estimation of Molecular Weights by Gel Filtration. The apparent molecular weights of the phosphoprotein phosphatases were determined by gel filtration on Sephacryl S-200. The column (2.5 × 80 cm) was calibrated as described by Andrews (1965) with the following molecular weight standards: carbonic anhydrase (M_r 30000), bovine serum albumin (M_r 67000), lactate dehydrogenase (M_r 130000), catalase (M_r 240000), phosphorylase *a* (M_r 370000), and β-galactosidase (M_r 520000). The column was developed with TEMN at a flow rate of 16 mL/h. The elution volumes of the molecular weight standards were determined to the nearest milliliter by monitoring the absorbance at 280 nm. Elution volumes for the phosphatase activities were determined by assaying the column fractions (6 mL) for phosphate release from histone and casein.

Phosphorylation of 40S Ribosomal Subunits. 40S ribosomal subunits were phosphorylated by the type II cAMP-regulated protein kinase. The components in the reaction mixture of 0.2 mL included the following: 50 mM Tris-HCl, pH 7.4; 5 mM $MgCl_2$; 1.4 μM cAMP; 0.28 mM [γ-^{32}P]ATP; 0.4 mg of 40S subunits; and 250 units of cAMP-regulated protein kinase. The mixture was incubated for 30 min at 30 °C; a 15-fold excess of nonlabeled ATP was added, and the mixture was incubated for an additional 5 min to decrease nonspecific binding of the radiolabel. The mixture was rapidly cooled to 4 °C and dialyzed against 50 mM Tris-HCl, pH 7.4, and 3 mM $MgCl_2$. Activated charcoal was present in a separate dialysis bag to adsorb the ATP. The phosphorylated subunits were used immediately.

Phosphorylation of eIF-2. eIF-2 is composed of three subunits (Safer et al., 1976; Trachsel et al., 1977; Benne et al., 1976) designated α, β, and γ. The molecular weights of the subunits were determined to be 38000, 53000, and 57000, respectively, by gel electrophoresis in sodium dodecyl sulfate (Traugh et al., 1976). Reaction mixtures for the phosphorylation of eIF-2α contained the following: 50 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 0.28 mM [γ-^{32}P]ATP; 6.8 μg of the hemin-controlled repressor; and 54 μg of purified eIF-2 in a final volume of 0.45 mL. Reaction mixtures for the phosphorylation of eIF-2β contained the following: 50 mM Mops, pH 7.0; 10 mM $MgCl_2$; 140 mM KCl; 0.28 mM [γ-^{32}P]ATP; either 1125 units of casein kinase II or 67 μg of the activated form of protease-activated kinase II; and 54 μg of purified eIF-2 in a final volume of 0.45 mL. The mixtures were incubated for 30 min at 30 °C and for an additional 5 min after the addition of a 15-fold excess of nonradioactive ATP. The mixtures were brought rapidly to 4 °C and dialyzed against 50 mM Tris-HCl, pH 7.4, in the presence of activated charcoal. The phosphorylated factor was used immediately or stored at −70 °C.

Phosphorylation of eIF-3. eIF-3, a large molecular weight complex composed of ~10 different polypeptide chains (Safer et al., 1976; Sundkvist & Staehelin, 1975; Benne & Hershey, 1976), has been shown to be differentially phosphorylated. The cAMP-regulated protein kinases have been shown to phosphorylate the 130000 molecular weight subunit (Traugh & Lundak, 1978). Highly purified preparations of casein kinase II phosphorylated the 130000, 69000, and 35000 molecular weight subunits (Traugh et al., 1979). Reaction mixtures for phosphorylation of eIF-3 by the type II cAMP-regulated protein kinase contained the following in a final volume of 0.2 mL: 50 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 1.4 μM cAMP; 0.28 mM [γ-^{32}P]ATP; 500 units of protein kinase; and 0.134 mg of eIF-3. Reaction conditions for phosphorylation of eIF-3 by casein kinase II were identical with those described for the phosphorylation of eIF-2β, except that eIF-3 was used. Incubation and dialysis conditions were identical with those described for eIF-2. Phosphorylated eIF-3 was used immediately or stored at −70 °C.

Dephosphorylation of Translational Components. To discriminate between individual dephosphorylation events on multisubunit initiation factors, we monitored dephosphorylation by polyacrylamide gel electrophoresis followed by autoradiography. The 40S subunits and initiation factors were dephosphorylated in a final volume of 0.045 mL. Dephosphorylation mixtures for 40S subunits contained the following: 50 mM Mops, pH 7.0; 5 mM $MnCl_2$; 20 mM 2-mercaptoethanol; 30 μg of phosphorylated 40S subunits; and 1.5 units of phosphatase activity. Duplicate control mixtures lacking the phosphatase activity were also included in each experiment. Dephosphorylation of initiation factors was performed under the same conditions as used for 40S subunits, except that 1.5–2.0 μg of eIF-2 and 10–12 μg of eIF-3 were used. The amount of phosphorylated factor used in the assay was determined by the number of subunits and was calculated to give visible bands when the gel was stained for protein. When initiation factors were used as substrate, 0.5–2.0 units of phosphatase was added to the incubation mixtures. After incubation for 45 min at 30 °C, the reactions were terminated by the addition of 0.02 mL of sample buffer (see below) and analyzed by polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in 0.75-mm thick slab gels with the buffer system described by Laemmli (1970) as modified by Hathaway et al. (1979). The stacking gel (3 cm long) contained a final acrylamide concentration of 4.7%, and the resolving gel (8 cm long) had a final acrylamide concentration of 10%. In both stacking and resolving gels the ratio of acrylamide to bis(acrylamide) was 37:1 and the concentration of sodium dodecyl sulfate was 1%. Prior to electrophoresis, 0.02 mL of sample buffer (25 mM Tris-HCl, pH 7.8, 2.1 M 2-mercaptoethanol, 6% sodium dodecyl sulfate, 30% glycerol, and 0.1% bromphenol blue) was added to the dephosphorylation reaction mixtures. The samples were heated to 65 °C for 10 min and loaded into sample wells in the stacking gel. The voltage was set at 100 V until the dye front had moved into the resolving gel and then was increased to 150 V. Electrophoresis was terminated when the dye front was within 5 mm of the bottom of the gel. The gel was removed and stained in 0.1% Coomassie Brilliant Blue in 50% methanol and 7.5% acetic acid for 15–20 min. After destaining in 50% methanol and 7.5% acetic acid, the gel was mounted on Whatman 3 MM filter paper and dried. Autoradiograms of the dried gels were obtained with Kodak No-Screen X-ray film.

Quantitation of Phosphatase Activity with Translational Components. The radioactive phosphate bound to protein was quantitated by either of two methods. In the first, the ra-

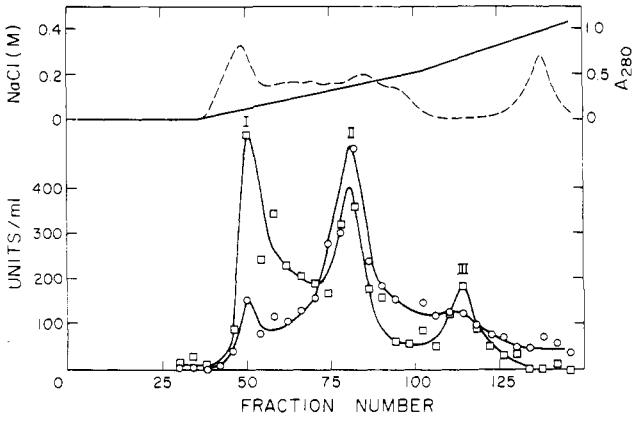

FIGURE 1: Identification of multiple forms of phosphoprotein phosphatase activity by chromatography of the postribosomal supernate on DEAE-cellulose. The postribosomal supernatant fraction from reticulocytes was chromatographed on DEAE-cellulose as described under Materials and Methods. Fractions of 15 mL were collected and aliquots (0.015 mL) were analyzed for phosphatase activity with histone at pH 7.0 (○) and casein at pH 5.6 (□). Absorbance (---) was monitored continuously at 280 nm. NaCl concentration (—) was monitored by conductivity.

dioactive protein bands were excised from the dried gels and counted in 5 mL of toluene scintillation fluid. In the second method, radioactive phosphate was quantitated by scanning the autoradiogram. Care was taken to ensure that exposure was linearly related to the amount of radioactivity. Peak areas were determined by excision and weighing.

When the phosphatases were analyzed with phosphorylated initiation factors, substrate was of necessity limiting. The amount of labeled phosphate added to incubations varied between the different phosphoprotein substrates. Under these conditions it is impossible to draw conclusions about relative rates of dephosphorylation. Instead, each of the phosphorylated translational components was incubated with the same amount of phosphatase for the same period of time. This method gave a clear indication of which components were dephosphorylated by the different phosphatase activities.

Results

Chromatography of the Postribosomal Supernatant Fraction on DEAE-cellulose. Phosphoprotein phosphatase activity in the postribosomal supernatant fraction from rabbit reticulocytes was resolved into multiple forms by chromatography on DEAE-cellulose. Three peaks of phosphoprotein phosphatase activity were observed when the column fractions were assayed with phosphohistone and phosphocasein (Figure 1). The phosphatase activities were designated I, II, and III in order of elution. Fraction I eluted between 0.05 and 0.10 M NaCl, fraction II eluted between 0.10 and 0.20 M NaCl, and fraction III eluted between 0.25 and 0.30 M NaCl.

All three fractions were observed to dephosphorylate both histone phosphorylated by the cAMP-regulated protein kinases and casein phosphorylated by casein kinase II. Initial studies on the three phosphatase fractions identified the optimal pH values for these activities with histone and casein as substrate. A broad pH optimum between pH 6.5 and 7.5 was observed with all three fractions with histone (data not shown). When casein was used as substrate, all three fractions had a sharp pH optimum at 5.6. A second optimum was observed between pH 7.0 and 7.5. At low pH values (5.5 and below) casein was observed to precipitate; this did not have a major effect on the phosphatase activity since optimal phosphate release was sometimes observed in assays which contained precipitated material. The phosphatase profiles shown in Figure 1 were

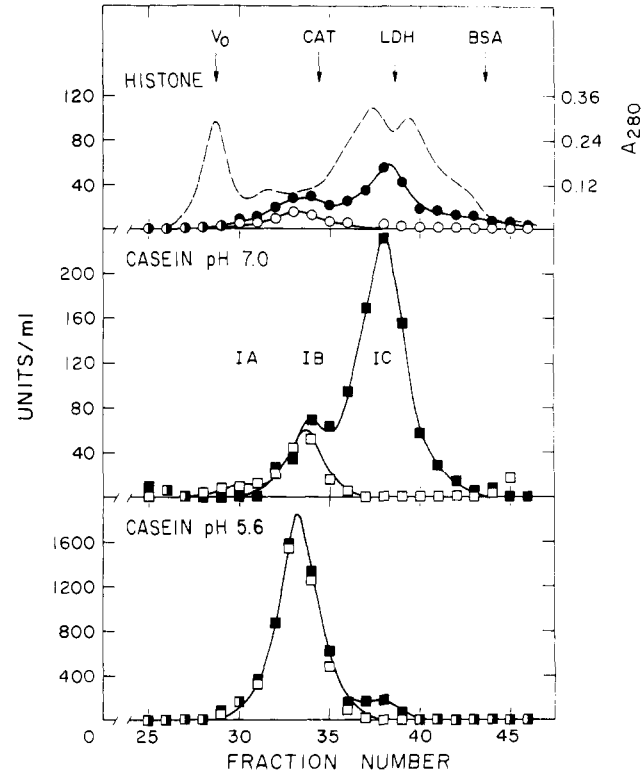

FIGURE 2: Chromatography of DEAE-cellulose fraction I on Sephacryl S-200. Fraction I from DEAE-cellulose was chromatographed on Sephacryl S-200 as described under Materials and Methods. A sample (4.0 mL) containing 8000 units of phosphatase activity with histone (assayed in the presence of 5 mM $MnCl_2$) and 10000 units of phosphatase activity with casein (assayed at pH 5.6 in the absence of $MnCl_2$) was applied to the column. Phosphatase activity was monitored with histone (upper panel), casein at pH 7.0 (middle panel), and casein at pH 5.6 (lower panel). Assays were conducted in the absence (open symbols) and presence (closed symbols) of 5 mM $MnCl_2$. Arrows indicate the void volume (V_0) and the elution volumes of catalase (CAT), lactate dehydrogenase (LDH), and bovine serum albumin (BSA).

analyzed with histone at pH 7.0 and with casein at pH 5.6. Total phosphatase activity in all three fractions was reduced with casein when assayed at pH 7.0.

Chromatography of DEAE-cellulose Fractions on Sephacryl S-200. The phosphoprotein phosphatase activities in fraction I were resolved further by chromatography on Sephacryl S-200. Phosphatase activity was assayed with histone at pH 7.0 and with casein at pH 7.0 and 5.6 in the presence and absence of $MnCl_2$. Two major peaks of phosphatase activity were resolved and designated as IB and IC in order of elution (Figure 2). A minor peak of activity, designated as IA, was usually present in small amounts (less than 2% of the activity in fraction I) but varied with the preparation. All fractions dephosphorylated both histone and casein.

Two major phosphatase activities were also observed when DEAE-cellulose fraction II was chromatographed on Sephacryl S-200 (Figure 3). These major activities chromatographed at the same elution volumes as IB and IC and were identified as IIB and IIC. In addition, a minor activity was observed which had the same elution volume as IA and was designated as IIA.

Fraction III from DEAE-cellulose was also resolved into two major activities following chromatography on Sephacryl S-200. The first peak of activity had the same elution volume as IB and IIB and was designated as IIIB (Figure 4). The second major activity had an elution volume between those of B and C forms and was designated as IIID. A minor peak

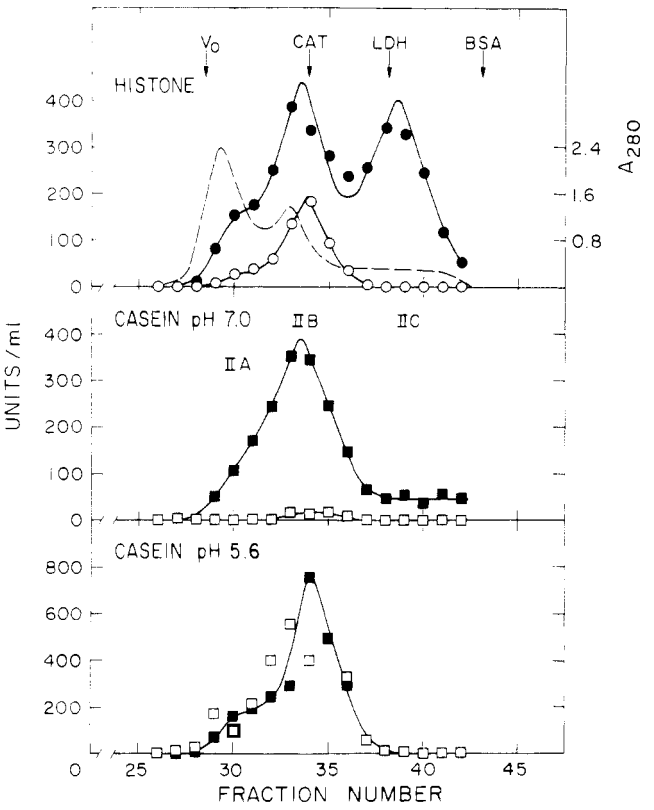

FIGURE 3: Chromatography of DEAE-cellulose fraction II on Sephacryl S-200. Fraction II from DEAE-cellulose was chromatographed on Sephacryl S-200 as described under Materials and Methods. A sample of 12 mL, which contained 95 000 units of phosphatase activity with histone (assayed with 5 mM $MnCl_2$) and 68 000 units of phosphatase activity with casein (assayed at pH 5.6 in the absence of $MnCl_2$), was applied to the column. Phosphatase activity was monitored with histone (upper panel), casein at pH 7.0 (middle panel), and casein at pH 5.6 (lower panel). Assays were conducted in the absence (open symbols) and presence (closed symbols) of 5 mM $MnCl_2$.

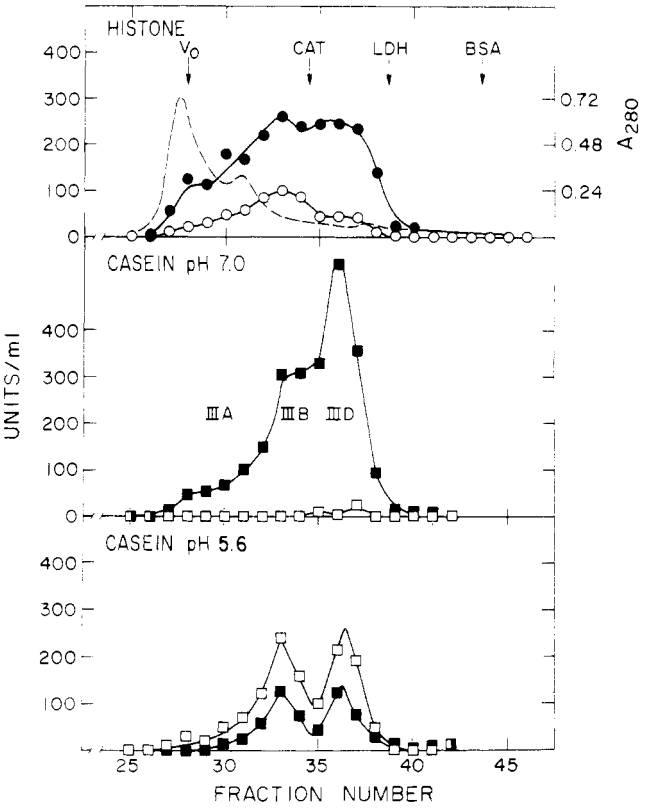

FIGURE 4: Chromatography of DEAE-cellulose fraction III on Sephacryl S-200. The phosphatase activity in fraction III from DEAE-cellulose was chromatgraphed on Sephacryl S-200 as described under Materials and Methods. A sample of 3.8 mL, which contained 32 000 units of phosphatase activity with histone (assayed with 5 mM $MnCl_2$) and 36 000 units of phosphatase activity with casein (assayed at pH 5.6 in the absence of $MnCl_2$), was applied to the column. Phosphatase activity was monitored with histone (upper panel), casein at pH 7.0 (middle panel), and casein at pH 5.6 (lower panel). Assays were conducted in the absence (open symbols) and presence (closed symbols) of 5 mM $MnCl_2$.

of activity with the same elution volume as IA and IIA was identified and designated as IIIA. IIIB had properties very similar to those of IIB, and both enzymatic activities had approximately the same rates of dephosphorylation of histone and casein.

Estimation of Molecular Weights. The molecular weights of the phosphoprotein phosphatase activities were estimated with Sephacryl S-200. Elution volumes for the phosphatases were determined from the initial chromatography of the DEAE-cellulose fraction on S-200 and by rechromatography of the purified activities. The A forms had an apparent molecular weight of greater than 500 000. The B forms eluted just ahead of catalase with an apparent molecular weight of 270 000. A molecular weight of 140 000 was determined for the C forms, and IIID had an apparent molecular weight of 180 000.

Dephosphorylation of 40S Subunits. The cAMP-regulated protein kinases have been shown to specifically phosphorylate a single protein in the 40S ribosomal subunit, S6, previously known as S13 (Traugh & Porter, 1976). There are four to five different sites on S6 which are phosphorylateable. With purified 40S ribosomal subunits, two to three of these sites are phosphorylated by the cAMP-regulated protein kinases (Del Grande & Traugh, 1979). Thus, 40S ribosomal subunits phosphorylated by the cAMP-regulated protein kinases were examined as substrate for the partially purified phosphoprotein phosphatases. Analyses of the phosphorylated ribosome preparations by gel electrophoresis in sodium dodecyl sulfate showed a single phosphorylated protein which corresponded to S6 (Figure 5). Phosphate release was monitored by gel electrophoresis followed by autoradiography and quantitated by excising the phosphorylated bands from the gel and counting them. The amount of radiolabeled phosphate released by each enzyme was determined by comparing the amount of radioactive phosphate present in samples incubated in the presence of the phosphatases to control samples incubated without enzyme. Phosphatase fractions IB, IC, IIB, and IIIB released radioactive phosphate from S6. IIID had essentially no activity with the ribosomal subunits. Dephosphorylation of IC was the highest (40% of the radioactive phosphate was removed in 45 min), while IIB and IIIB removed ~25% of the phosphate. At high concentrations of phosphatase, all the radioactive phosphate was removed.

Dephosphorylation of eIF-2. The α subunit (M_r 38 000) of purified eIF-2 was phosphorylated with [γ-^{32}P]ATP by the hemin-controlled repressor, and the β subunit (M_r 53 000) was phosphorylated by protease-activated kinase II or casein kinase II (Figure 5). After dialysis the phosphorylated eIF-2 was incubated with the various phosphatases and the amount of radioactive phosphate released was monitored. A degree of specificity was observed in the dephosphorylation of eIF-2α; phosphatases IIB and IIIB released 26 and 30% of the phosphate, respectively, during the 45-min incubation period (Table I). The other phosphatase fractions did not release a significant amount of phosphate from this subunit. A lack of specificity was observed in the dephosphorylation of eIF-2β.

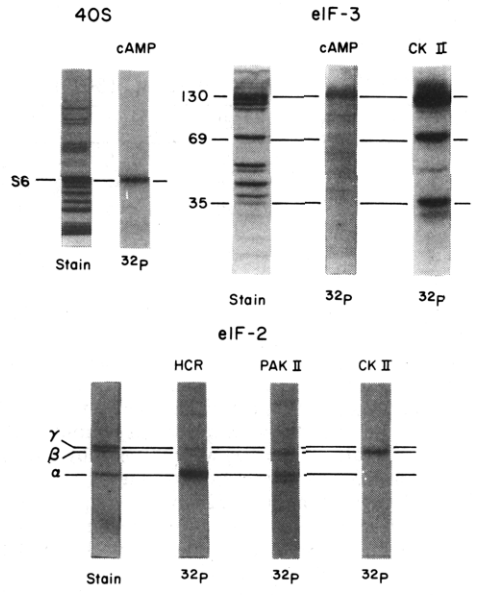

FIGURE 5: Analysis of phosphorylated translational components by polyacrylamide gel electrophoresis. 40S ribosomal subunits were phosphorylated by the type II cAMP-regulated protein kinase (cAMP), eIF-2 was phosphorylated by the hemin-controlled repressor (HCR), the activated form of protease-activated kinase II (PAK II), and casein kinase II (CK II), and eIF-3 was phosphorylated by type II cAMP-regulated protein kinase and casein kinase II. The reactions were conducted as described under Materials and Methods, and an aliquot (0.015 mL) of each reaction mixture was analyzed by polyacrylamide gel electrophoresis as described under Materials and Methods. The protein patterns (stain) and corresponding autoradiograms (^{32}P) are shown for 40S ribosomal subunits (30 μg), eIF-2 (1.8 μg), and eIF-3 (10 μg).

Table I: Examination of Phosphatase Activities with eIF-2[a]

phosphatase fraction	$^{32}P_i$ released (%)		
	eIF-2α[b]	eIF-2β[c]	eIF-2β[d]
IB	5	60	39
IC	9	40	44
IIB	26	75	50
IIIB	30	59	24
IIID	6	nd[e]	nd

[a] Dephosphorylation of eIF-2 by the individual phosphatase fractions was examined by gel electrophoresis followed by autoradiography as described under Materials and Methods. The percent of radioactive phosphate released from eIF-2 phosphorylated by each of the three protein kinase activities was quantitated by excising and counting the radioactive protein band or by scanning the autoradiogram. Incubations with eIF-2 phosphorylated by the hemin-controlled repressor contained 2.0 units of phosphatase activity. Incubations with eIF-2 phosphorylated by casein kinase II or protease-activated kinase II contained 0.5 unit of phosphatase activity. [b] Total $^{32}P_i$ incorporated into eIF-2α by the hemin-controlled repressor was 3.5 pmol (1720 cpm), 0.2 mol of $^{32}P_i$/mol of eIF-2. [c] Total $^{32}P_i$ incorporated into eIF-2β by the activated form of protease-activated kinase II was monitored by spectrophotometric scanning of the autoradiogram. [d] Total $^{32}P_i$ incorporated into eIF-2β by casein kinase II was 0.7 pmol (173 cpm), 0.1 mol of $^{32}P_i$/mol of eIF-2. [e] nd = not determined.

With eIF-2β phosphorylated by the protease-activated kinase II, all of the phosphatase activities tested were active in the dephosphorylation of this substrate (Table I). Forty to seventy-five percent of the phosphate was released by the various enzymes. Similar results were seen when eIF-2β phosphorylated by casein kinase II was used as substrate, except that 24–50% of the phosphate was released.

Dephosphorylation of eIF-3. eIF-3 has been shown to be phosphorylated in vitro by several protein kinase activities from reticulocytes (Issinger et al., 1976; Traugh & Lundak, 1978; Hathaway et al., 1979). The cAMP-regulated protein kinases phosphorylated the 130 000 molecular weight subunit while the casein kinase II phosphorylated subunits with molecular weights of 130 000, 69 000, and 35 000 (Figure 5).

Table II: Examination of Phosphatase Activities with eIF-3[a]

phosphatase fraction	$^{32}P_i$ released (%)		
	eIF-3[b] (130 000)	eIF-3[c] (130 000)	eIF-3[d] (69 000)
IB	64	18	77
IC	72	nd[e]	21
IIB	60	22	82
IIIB	53	8	78
IIID	65	nd	nd

[a] Dephosphorylation of eIF-3 by the phosphatase activities was examined by gel electrophoresis followed by autoradiography and quantitated by excision of the protein band or by scanning the autoradiogram as described under Materials and Methods. Radioactivity released by phosphatases IB, IC, IIB, IIIB, and IIID from the 130 000 molecular weight subunit of eIF-3, phosphorylated by the cAMP-regulated protein kinase, and from the 130 000–110 000 and 69 000 molecular weight subunits, phosphorylated by casein kinase II, was determined. Incubation mixtures with eIF-3 phosphorylated by cAMP-regulated protein kinase contained 1.5 units of phosphatase activity, and reaction mixtures with eIF-3 phosphorylated by casein kinase II contained 2.0 units of phosphatase activity. [b] Total $^{32}P_i$ incorporated into the 130 000 molecular weight subunit of eIF-3 by the cAMP-regulated protein kinase was 2.5 pmol (850 cpm), 0.2 mol of $^{32}P_i$/mol of eIF-3. [c] Total $^{32}P_i$ incorporated into the 130 000-110 000 molecular weight subunits of eIF-3 by protein kinase activities copurifying with casein kinase II was monitored by spectrophotometric scanning of the autoradiogram. [d] Total $^{32}P_i$ incorporated into the 69 000 molecular weight subunit of eIF-3 by casein kinase II was monitored by spectrophotometric scanning of the autoradiogram. [e] nd = not determined.

All of the phosphatase fractions tested were found to release phosphate from eIF-3 phosphorylated by the cAMP-regulated protein kinases. The amount of phosphate released varied from 53 to 72% (Table II). When dephosphorylation of eIF-3 phosphorylated by casein kinase II was examined, IB and IIB released ~20% of the phosphate from the 130 000 molecular weight subunit and IIIB released 8% (Table II); the 69 000 dalton subunit was dephosphorylated by all of the phosphatases tested. The B forms released ~80% of the bound radioactivity, and phosphatase IC released 21%.

Effects of Small Molecular Weight Compounds on Dephosphorylation of Translational Components. The dephosphorylation of 40S subunits by IIB and IIIB was examined in the presence of small molecular weight compounds which have been shown to affect protein synthesis. Each of the compounds tested had similar effects on both phosphatases (Table III). ATP (4 mM) inhibited the activity of IIB by 65% and the activity of IIIB by 87%. At high concentrations (4 mM), GTP was the most inhibitory compound; the activities of IIB and IIIB were decreased by 91 and 87%, respectively. Glucose 6-phosphate inhibited both phosphatases by ~60%, NaF by ~50%, and hemin (0.025 mM) by ~45%. Manganese was required for the reactions to occur, and dephosphorylation of 40S subunits was inhibited when $MnCl_2$ was omitted from the assays.

The individual compounds had similar effects on the dephosphorylation of eIF-2α by phosphatases IIB and IIIB. Glucose 6-phosphate inhibited the B forms 84–97% and ATP inhibited 72–100%, while GTP inhibited 69–81%. Inhibition by hemin was 59–69%, while NaF inhibited the phosphatase activities by ~30%. cAMP was observed to stimulate the activity of IIB and IIIB by 113 and 56%, respectively. As observed with the dephosphorylation of 40S subunits, de-

Table III: Effects of Regulatory Compounds on Dephosphorylation of Translational Components[a]

addn	concn (mM)	40S ribosomal subunits[b]		eIF-2α[c]		eIF-2β[d]		eIF-3 (M_r 130 000)		
		IIB	IIIB	IIB	IIIB	IIB	IIIB	IIB[e]	IIIB[f]	IIID[f]
none		100	100	100	100	100	100	100	100	100
hemin	0.025	65	46	31	41	53	57	86	104	106
G-6-P	4.0	39	42	3	16	82	85	86	118	100
ATP	4.0	35	13	0	28	82	80	64	82	75
GTP	4.0	9	13	19	31	28	0	25	38	60
cAMP	4.0	100	100	213	156	105	113	89	91	113
NaF	4.0	48	50	66	72	97	108	79	69	nd[g]
$MnCl_2$	omitted	0	0	0	0	0	0	0	0	25

[a] Phosphatase activities IIB and IIIB were examined with 40S ribosomal subunits, eIF-2, and eIF-3, and eIF-3 was examined with IIID in the presence of small molecular weight compounds. Dephosphorylation was monitored by gel electrophoresis followed by autoradiography and quantitated by excising the gel slice or by spectrophotometric scanning of the autoradiogram. Activity is expressed relative to that with no additions. [b] 40S ribosomal protein S6 contained 15 pmol of radioactivity (30 400 cpm), 0.7 mol of $^{32}P_i$/40S ribosomal subunit. All assays contained 1.5 units of phosphatase activity. In the absence of regulatory compounds, 23 and 24% of the $^{32}P_i$ were released by IIB and IIIB, respectively. [c] Total $^{32}P_i$ incorporated into eIF-2α by the hemin-controlled repressor was monitored by spectrophotometric scanning of the autoradiogram. Assays contained 2 units of phosphatase activity. In the absence of regulatory compounds, 32% of the $^{32}P_i$ was released. [d] Total $^{32}P_i$ incorporated into eIF-2β by casein kinase II was monitored by spectrophotometric scanning of the autoradiogram. Assays contained 0.5 unit of phosphatase activity. In the absence of regulatory compounds, 87 and 79% of the $^{32}P_i$ were released by IIB and IIIB, respectively. [e] Total $^{32}P_i$ incorporated into the 130 000-dalton subunit of eIF-3 by cAMP-regulated protein kinase was 3.8 pmol (1000 cpm), 0.2 mol of ^{32}P/mol of eIF-3. Assays contained 1.5 units of phosphatase activity. In the absence of regulatory compounds, 56% of the $^{32}P_i$ was released. [f] Total $^{32}P_i$ incorporated into the 130 000-dalton subunit of eIF-3 by cAMP-regulated protein kinase was 2.8 pmol (680 cpm), 0.3 mol of $^{32}P_i$/mol of eIF-3. Assays contained 1.5 units of phosphatase activity. In the absence of regulatory compounds, 45 and 48% of the $^{32}P_i$ were released by IIB and IIID, respectively. [g] nd = not determined.

phosphorylation of eIF-2α by the B forms required Mn^{2+}.

The effects of the same small molecular weight compounds on the dephosphorylation of eIF-2β were also examined. In contrast to results obtained with the α subunit, glucose 6-phosphate and ATP had little effect on the dephosphorylation of the β subunit by phosphatase B (Table III). The activities were inhibited 15–20% by both glucose 6-phosphate and ATP. Addition of 4 mM GTP, on the other hand, resulted in a 72–100% inhibition. cAMP was slightly stimulatory, whereas NaF had little or no effect on the phosphatase activities. Hemin inhibited the dephosphorylation of eIF-2β by ~45%.

The effects of regulatory compounds on dephosphorylation of the 130 000-dalton subunit of eIF-3 phosphorylated by the cAMP-regulated protein kinase were examined with phosphatase fractions IIB, IIIB, and IIID. The three phosphatase activities reacted similarly to each of the compounds (Table III). Hemin, glucose 6-phosphate, and cAMP had little or no effect on the phosphatases. ATP was slightly inhibitory (18–36%), while GTP inhibited the B forms of the phosphatases 62–75% and the D form 40%. NaF reduced the activity of the B forms by 21–31%. $MnCl_2$ was an absolute requirement for the dephosphorylation of eIF-3 by IIB and IIIB, but IIID had activity in the absence of manganese.

Discussion

Multiple forms of phosphoprotein phosphatase activity were identified in the postribosomal supernate of rabbit reticulocytes by chromatography on DEAE-cellulose and Sephacryl S-200. Three chromatographically different phosphatase activities from DEAE-cellulose were resolved further by gel filtration. A major form of phosphatase with an apparent molecular weight of 270 000 was present in all three fractions and was designated as IB, IIB, and IIIB, respectively. The properties of IIB and IIIB were nearly identical. The activity of IB was similar in many respects to that IIB and IIIB. These three forms constituted ~80% of the total phosphatase activity in the postribosomal supernate of reticulocytes.

Three other forms of phosphatase activity had apparent molecular weights of greater than 500 000 (A), 180 000 (D), and 140 000 (C). The A form was present in low amounts in all three fractions, constituted less than 2% of the total activity, and was probably a high molecular weight aggregate of the other forms. The D form was present only in fraction III, and the properties of this enzyme were similar to those of IIB and IIIB. The C form, present in fractions I and II from DEAE-cellulose, constituted ~15% of the total activity. Multiple forms of phosphoprotein phosphatase similar to the forms identified in reticulocytes have been resolved from rabbit liver (Kobayashi et al., 1975), rabbit skeletal muscle (Kobayashi & Kato, 1977), and canine heart (Li, 1975). A single molecular weight form of phosphorylase phosphatase (M_r 260 000) was recovered from liver extracts prepared by a gentle homogenization procedure (Lee et al., 1976, 1978); however, when harsher conditions were utilized, a number of lower molecular weight species were observed.

All of the translational components tested were dephosphorylated by phosphoprotein phosphatases from reticulocytes. The B form appeared to be a universal phosphatase which dephosphorylated 40S ribosomal subunits and eIF-3 phosphorylated by the cAMP-regulated protein kinase, eIF-3 phosphorylated by casein kinase II, and eIF-2 phosphorylated by the hemin-controlled repressor, casein kinase II, and protease-activated kinase II. Dephosphorylation of all of the translational components by the same phosphatase activity is vastly different from the phosphorylation reactions where a number of different protein kinases recognize unique primary sequences adjacent to a serine or threonine residue. The C form (~15% of the total activity) dephosphorylated all the components tested except eIF-2α.

The effects of possible regulatory compounds on the major phosphatase activity have been examined. High concentrations of GTP inhibited the activity of the B form with all of the phosphorylated substrates. The same concentrations of ATP and glucose 6-phosphate inhibited the dephosphorylation of 40S ribosomal subunits and eIF-2α but had little or no effect on the phosphatase activities with eIF-2β and eIF-3. Hemin and NaF were also found to inhibit dephosphorylation of 40S ribosomal subunits and eIF-2α; little effect on dephosphorylation of other substrates was observed. No inhibition was observed with cAMP; however, a significant stimulation of dephosphorylation was shown to occur with eIF-2α. Thus, a number of small molecular weight compounds which are

involved in protein synthesis differentially inhibit or stimulate phosphatase activity B depending on the substrate examined. Glucose 6-phosphate, ATP, and hemin inhibit dephosphorylation of two different components, 40S ribosomal subunits and eIF-2α, each of which is phosphorylated by a different protein kinase. The compounds have no effect on dephosphorylation of the other phosphorylated substrates. The differential regulation of phosphatase activity B suggests that these compounds are affecting dephosphorylation by interacting with the substrate and altering the availability of the phosphorylated site.

The effects of the regulatory compounds on the dephosphorylation of eIF-2α are particularly interesting because of the role of this phosphorylation event in the regulation of protein synthesis. Under conditions of hemin deprivation, increased phosphorylation of eIF-2 occurs prior to inhibition of protein synthesis (Floyd & Traugh, 1978; Farrell et al., 1978). The inhibition is reversed by addition of hemin, GTP, or phosphorylated sugars (Gross, 1974; Balkow et al., 1975; Ernst et al., 1976, 1978). From our studies it can be concluded that these compounds do not reverse the inhibition by acting on phosphtases IIB and IIIB since these compounds result in an inhibition of the phosphatases rather than a stimulation. However, high concentrations of cAMP also reverse the inhibition (Legon et al., 1974; Balkow et al., 1975; Ernst et al., 1976), and at 4 mM concentrations of this nucleotide dephosphorylation of eIF-2α was stimulated 1.5- to 2-fold. Little or no stimulation by cAMP was observed with the other substrates. Concentrations of ATP similar to those used in these studies have been shown to potentiate the inhibition observed by hemin deprivation (Balkow et al., 1975; Ernst et al., 1976). The ATP could be inhibiting the phosphatase activity since this nucleotide inhibited the dephosphorylation of eIF-2α by phosphatase B. In contrast to the effect of GTP, the inhibition by ATP appeared to be relatively specific and only dephosphorylation of eIF-2α and S6 was affected.

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References

Andrews, P. (1965) *Biochem. J. 96*, 595–606.

Balkow, K., Hunt, T., & Jackson, R. J. (1975) *Biochem. Biophys. Res. Commun. 67*, 366–375.

Barrieux, A., & Rosenfeld, M. G. (1977) *J. Biol. Chem. 252*, 3843–3847.

Benne, R., & Hershey, J. W. B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3005–3009.

Benne, R., Wong, C., Luedi, M., & Hershey, J. W. B. (1976) *J. Biol. Chem. 251*, 7675–7681.

Benne, R., Edman, J., Traut, R. R. & Hershey, J. W. B. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 108–112.

Del Grande, R., & Traugh, J. A. (1979) *J. Supramol. Struct., Suppl. 3*, 28.

Dettman, G. L., & Stanley, W. M. (1972) *Biochim. Biophys. Acta 287*, 124–133.

Ernst, V., Levin, D. H., Ranu, R. S., & London, I. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3112–3116.

Ernst, V., Levin, D. H., & London, I. M. (1978) *J. Biol. Chem. 253*, 7163–7172.

Farrell, P. J., Balkow, K., Hunt, T., Jackson, J., & Trachsel, H. (1977) *Cell 11*, 187–200.

Farrell, P. J., Hunt, T., & Jackson, R. J. (1978) *Eur. J. Biochem. 89*, 517–521.

Floyd, G. A., & Traugh, J. A. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1792.

Floyd, G. A., Merrick, W. C., & Traugh, J. A. (1979) *Eur. J. Biochem. 96*, 277–286.

Gross, M. (1974) *Biochim. Biophys. Acta 366*, 319–332.

Gross, M., & Mendelewski, J. (1977) *Biochem. Biophys. Res. Commun. 74*, 559–569.

Gupta, N. K., Woodley, C. L, Chen, Y. C., & Bose, K. K. (1973) *J. Biol. Chem. 248*, 4500–4511.

Hathaway, G. M., & Traugh, J. A. (1979) *J. Biol. Chem. 254*, 762–768.

Hathaway, G. M., Lundak, T. S., Tahara, S. M., & Traugh, J. A. (1979) *Methods Enzymol. 60*, 495–511.

Howard, G. A., Traugh, J. A., Croser, E. A., & Traut, R. R. (1975) *J. Mol. Biol. 93*, 391–404.

Issinger, O.-G., Benne, R., Hershey, J. W. B., & Traut, R. R. (1976) *J. Biol. Chem. 251*, 6471–6474.

Kabat, D. (1970) *Biochemistry 9*, 4150–4175.

Kato, K., & Bishop, J. S. (1972) *J. Biol. Chem. 247*, 7420–7429.

Kobayashi, M., & Kato, K. (1977) *J. Biochem. (Tokyo) 81*, 93–97.

Kobayashi, M., Kato, K., & Sato, S. (1975) *Biochim. Biophys. Acta 377*, 343–355.

Kramer, G., Cimadevilla, J. M., & Hardesty, B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3078–3082.

Kramer, G., Henderson, A. B., Pinphanichakarn, P., Wallis, N. J., & Hardesty, B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1445–1449.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lee, E. Y. C., Brandt, H., Capulong, Z. L., & Killilea, S. D. (1976) *Adv. Enzyme Regul. 14*, 467–490.

Lee, E. Y. C., Mellgren, R. C., Killilea, S. D., & Aylward, J. H. (1978) *Fed. Eur. Biochem. Soc., Meet., 11th, 1978 42*, 327–346.

Legon, S., Brayley, A., Hunt, T., & Jackson, R. J. (1974) *Biochem. Biophys. Res. Commun. 56*, 745–752.

Levin, D. H., Kyner, D., & Acs, G. (1973) *J. Biol. Chem. 248*, 6416–6425.

Levin, D. H., Ranu, R. S., Ernst, V., & London, I. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3112–3116.

Li, H.-C. (1975) *FEBS Lett. 55*, 134–137.

McConkey, E. H., Bielka, H., Gordon, J., Lastick, S. M., Lin, A., Ogata, K., Reboud, J-P., Traugh, J. A., Traut, R. R., Warner, J. R., Welfle, H., & Wool, I. G. (1979) *Mol. Gen. Genet. 169*, 1–6.

Parvin, R., & Smith, R. A. (1969) *Anal. Biochem. 27*, 65–72.

Pinphanichakarn, P., Kramer, G., & Hardesty, B. (1976) *Biochem. Biophys. Res. Commun. 73*, 625–631.

Ranu, R. S., & Wool, I. G. (1976) *J. Biol. Chem. 251*, 1926–1935.

Safer, B., Adams, S. L., Anderson, W. F., & Merrick, W. C. (1975a) *J. Biol. Chem. 250*, 9083–9089.

Safer, B., Anderson, W. F., & Merrick, W. C. (1975b) *J. Biol. Chem. 250*, 9067–9075.

Safer, B., Adams, S. L., Kemper, M., Berry, K. W., Lloyd, M., & Merrick, W. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2584–2588.

Schreier, M. H., & Staehelin, T. (1973) *Nature (London), New Biol. 242*, 35–38.

Smith, K. E., & Henshaw, E. C. (1975) *Biochemistry 14*, 1060–1066.

Sundkvist, I. C., & Staehelin, T. (1975) *J. Mol. Biol. 99*, 401–418.
Tahara, S. M., Traugh, J. A., Sharp, S. B., Lundak, T. S., Safer, B., & Merrick, W. C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 789–793
Trachsel, H., Erni, B., Schreier, M. H., & Staehelin, T. (1977) *J. Mol. Biol. 11*, 755–767.
Trachsel, H., Ranu, R. S., & London, I. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3654–3658.
Traugh, J. A., & Porter, G. G. (1976) *Biochemistry 15*, 610–616.
Traugh, J. A., & Lundak, T. S. (1978) *Biochem. Biophys. Res. Commun. 83*, 379–384.
Traugh, J. A., & Sharp, S. B. (1979) *Methods Enzymol. 60*, 534–541.
Traugh, J. A., Mumby, M., & Traut, R. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 373–376.
Traugh, J. A., Tahara, S. M., Sharp, S. B., Safer, B., & Merrick, W. C. (1976) *Nature (London) 263*, 163–165.
Traugh, J. A., Hathaway, G. M., Tuazon, P. T., Tahara, S. M., Floyd, G. A., Del Grande, R. W., & Lundak, T. S. (1979) *ICN–UCLA Symp. Mol. Cell. Biol.* (in press).

Purification and the Histones of *Dictyostelium discoideum* Chromatin†

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ABSTRACT: *Dictyostelium* chromatin has been purified from nuclei in high yield by differential centrifugation and nuclease cleaving. Its chemical composition has been assayed, and its histones have been analyzed by gel electrophoresis, peptide fingerprints, amino acid composition, and ion-exchange chromatography. The mass ratios of DNA/RNA/histone/nonhistone are 1.0:0.18:0.98:1.02. There are four histones including one unusual histone, H7, which is the most abundant histone in the slime mold. The H4-like protein is the most conserved protein, while the other histones show both similarities and differences with mammalian histones.

One of the goals of biology is to understand the molecular basis of gene regulation. For this purpose the components of the genetic apparatus have been dissected and studied. In eucaryotes the components are known, but their functions are not completely understood. The histones apparently convey a particular structure to the DNA (Felsenfeld, 1978). The nucleosomes of higher eucaryotes have two molecules each of four well-defined histones (Kornberg, 1974). Our purpose was to study the categories of *Dictyostelium* histones and their relationship to the structure and composition of its chromatin.

The simple life cycle of the slime mold makes it ideal for studying the relationship of gene expression to development. The synthesis of specific enzymes, membrane proteins, and mRNA is developmentally regulated (Sussman & Sussman, 1969; Siu et al., 1976; Ma & Firtel, 1978). The composition and structure of slime mold chromatin are similar to those in higher eucaryotes (Pederson, 1977; Bakke & Bonner, 1978). A rapid, unique method to purify chromatin is described here using limited nuclease digestions to free the chromatin from contaminants. We have found that the slime mold contains only four histones. One of these is very similar, but not identical, to H4. Another is present in nearly twice the amount of the H4-like protein and may replace both H2a and H2b. Therefore, the typical chromatin structure can be maintained by different basic proteins, except for H4. Its role is so vital that it must be carefully conserved.

Experimental Procedure

Organism and Growth Conditions. Dictyostelium discoideum, strain Ax-3, was grown at 22 °C on a rotary shaker in a sterile liquid medium termed HL5 by Cocucci & Sussman (1970). The cells have a generation time of ~12 h and were grown to a density of 8×10^6 cells/mL before harvesting. Cells were harvested in a large swinging bucket rotor at 700*g* in 1-L bottles and washed once with cold 0.4% NaCl.

Isolation of Nuclei and Chromatin. Nuclei were isolated by a modification of the method of Firtel & Lodish (1973). The harvested and washed cells were suspended at a density of 2×10^8 cells/mL in cold 0.37 M sucrose, 40 mM KCl, 20 mM phosphate, pH 7.6, 0.1 mM EGTA,[1] 1 mM PMSF, and 0.5% Nonidet P-40 (NP-40) with 1.0 mM $Mg(OAc)_2$ added after the EGTA is dissolved. This suspension was shaken by hand for 45 s to lyse the cells, and lysis was monitored by phase-contrast microscopy. The solution was then pelleted at 1000*g* for 5 min in a swinging bucket rotor. This procedure was repeated once more except that the $Mg(OAc)_2$ was increased to 3 mM, the pellet was resuspended with a glass Teflon homogenizer to shear off cytoplasmic material adhering to the nuclei, and the suspension was sedimented at 2000*g* for 5 min. The nuclei were further purified by resuspending with

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[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; GdmCl, guanidinium chloride; OAc, acetate; PCA, perchloric acid; NADH, nicotinamide adenine dinucleotide (reduced form); BSA, bovine serum albumin; IgG, immunoglobulin.

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