Characterization of the Primary Gene Product of Rat Incisor α-Phosphophoryn†

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ABSTRACT: The nature of the primary gene product for the α-phosphophoryn component of rat incisor dentin has been examined by cell-free translation of the total RNA and poly(A+) mRNA from rat maxillary incisors, including pulp cells and odontoblasts. The RNA was extracted by the guanidinium thiocyanate method and translated in a rabbit reticulocyte system. The translated proteins were analyzed by gradient gel electrophoresis, and α-phosphophoryn was identified by isolation on an anti-rat α-phosphophoryn antibody coupled Sepharose column and dot-blot procedures. The major protein identified as α-phosphophoryn had a molecular weight of 153 000 (±5000) and had chromatographic properties similar to those of α-phosphophoryn. Since tissue-isolated rat phosphophoryn has a molecular weight of only ~90 000 when fully phosphorylated, it appears that the primary gene product is a prepro-α-phosphophoryn. Thus, α-phosphophoryn in the extracellular space of rat incisor dentin must be the product of one or more posttranslational proteolytic processing steps.

Phosphophoryns, proteins rich in aspartic acid, serine, and phosphoserine, are found, uniquely, in dentin. Because of their very anionic character they have been implicated as important participants in the mineralization processes in dentin (Maier et al., 1983; Stetler-Stevenson & Veis, 1983) and as models for other mineralization systems (Veis, 1982).

Phosphophoryns from rat incisors (Butler et al., 1981; Dimuzio & Veis, 1978a,b), bovine molars (Stetler-Stevenson & Veis, 1983), and human teeth (Takagi & Veis, 1984) have been isolated and characterized. The bovine molar phosphophoryn is the best characterized and, as determined from both gradient electrophoretic gels and primary physical techniques, has a molecular weight of 155 000 (Stetler-Stevenson & Veis, 1983). The rat incisor contains three distinct phosphoproteins (Butler et al., 1981), two of which are typical phosphophoryns, α and β (Dimuzio & Veis, 1978a). Thus far it has not been possible to determine whether these phosphoproteins are completely independent molecules or are related as degradative products. The rat incisor α- and β-phosphophoryns have molecular weights in the range of 90 000, as determined by gradient gel electrophoresis, substantially less than the weight of the bovine molar phosphophoryn. Clarification of the relationship between the rat incisor phosphophoryns has been complicated by the problems of degradation in situ or during isolation and possible heterogeneity of phosphorylation (Stetler-Stevenson & Veis, 1983; Jontell et al., 1982).

In order to clarify these questions, and in parallel with the development of an antibody to rat α-phosphophoryn, we decided to produce the apoprotein in a cell-free system from isolated odontoblast mRNA. This approach should permit an investigation of the primary gene product. Initially, since bovine molar phosphophoryn appears to be a single protein, we investigated the translation of bovine molar odontoblast mRNA, but we were more successful with the mRNA from maxillary incisors from young, growing rats. We report here the nature of the α-phosphophoryn produced by the translation of rat incisor odontoblast mRNA.

Materials and Methods

Preparation of RNA from Rat Incisors. The maxillary incisors of young male Sprague-Dawley rats, 30–40 g, were removed directly after carbon dioxide suffocation. The teeth were freed of extraneous material and frozen in liquid N_2 within 4 min after death. The frozen teeth were ground under liquid N_2 by hand for 15 min, and the resultant powder was stored at −70 °C.

A variation of the method of Monson (Monson, 1982) was used with sterile solutions and equipment throughout. Tooth powder (1.2 g) was homogenized (37 °C) for 120 s at top speed in a Polytron probe in the presence of 10 mL of Monson solution A: 295.9 g of guanidinium thiocyanate, 4.5 g of ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 4.7 g of ethylenediaminetetraacetic acid (EDTA), and 10 g of *N*-laurylsarcosine, all in 500 mL of H_2O. Just before use solution A was made 0.1% (w/v) in mercaptoethanol and 0.3% (w/v) in antifoam A. After homogenization 10 mL more of solution A (37 °C) was added. The supernatant from centrifugation at 27000*g* (25 °C) for 15 min in an SS34 rotor received 8 mL more of solution A and then 7 mL of Monson solution B (5.7 M CsCl). The resultant solution layered upon a 5-mL CsCl cushion (solution B) completely filled the volume of a Beckman ("Ultraclear") 1 in. × $3^1/_2$ in. centrifuge tube. The subsequent centrifugation (20 °C) was 22 h in a Beckman L70 centrifuge at 26 000 rpm by using an SW-27 rotor. After centrifugation the upper brownish layer and the debris-containing interface plus about half of the cushion were removed. The walls of the tube and the surface of the residual cushion were washed 3 times with Monson solution C (7.5 M guanidine hydrochloride). Excision of the bottom of the tube with a scalpel after $^1/_2$ h of inverted draining made the brownish pellet easily accessible. The surface of the pellet was washed with 20 μL of cold H_2O, then 2 mL of cold H_2O was added, and the pellet was broken up. After the pellet was heated (65 °C) for 2 min and subsequently chilled, 0.2 mL of 1.65 M lithium acetate, pH 5.5, and 4.4 mL of 95% ethanol (−20 °C) were added. Subsequent centrifugation (−10 °C) for 20 min at 10 000 rpm (Corex tubes, HB4 rotor, Sorvall Model RC-5B) gave a precipitate which was taken up in 10 mL of 10 mM EDTA, pH 8. The resultant solution was extracted with 6 mL of chloroform–butanol (4:1 v/v). The organic phase, after separation, was extracted 2

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more times, each with 2 mL of 10 mM EDTA solution.

The aqueous washings were combined, and 1 mL of the lithium acetate solution was added and then 22 mL of 95% ethanol (−20 °C). Recentrifugation of these combined solutions as above in the HB4 rotor gave a pellet which was taken up in 500 μL of H_2O. Aliquots were frozen at −140 °C.

In some cases, the isolated rat incisor RNA was passed over an oligo(dT) column, and the poly(A+) mRNA was collected as described below, with the addition of vanadium ribonucleosidate as an RNase inhibitor.

Oligo(dT) Column Purification of RNA. A preparation of [^{3}H]poly(A) (New England Nuclear, Boston, MA) was used to calibrate an oligo(dT) column (~0.6 mL, type T-2; Collaborative Research, Waltham, MA). The column was first treated with 0.1 M NaOH, then with ~1% diethyl pyrocarbonate (DEP), and then with H_2O. Fifty milliliters of binding buffer [4.5 mL of 100 mM EDTA, 4.5 mL of 1 M tris(hydroxymethyl)aminomethane (Tris), pH 7.4, 27.5 mL of 10% w/v sodium dodecyl sulfate (SDS), 45 mL of 4 M NaCl, and 375 mL of H_2O] equilibrated the column, and then 50 μL of sample in binding buffer was washed onto the column. Fifty milliliters more of binding buffer was ultimately run through, during which time the material in the first several eluate tubes was recycled through the column. Then a minimal amount of elution buffer (4.5 mL of 100 mM EDTA, 4.5 mL of 1 M Tris, pH 7.4, 4.5 mL of 10% w/v SDS, and 436.5 mL of H_2O) was passed through. The radioactivity of eluate tubes was monitored in 3a70B cocktail (Research Products International, Mt. Prospect, IL), and these labeled eluates were combined, heated 5 min at 70 °C, chilled immediately to room temperature, made 0.4 M with respect to NaCl, and readded to the column which had continued to run with 30 mL of binding buffer. The column was rewashed with 2 mL of binding buffer and then run with 50 mL more of binding buffer and then elution buffer. Once again the eluate radioactivity was monitored, and appropriate fractions were combined. The [^{3}H]poly(A) eluted as a single peak, and its original radioactivity was conserved.

Cell-Free Protein Synthesis. Amersham nuclease-treated, amino acid depleted rabbit reticulocyte lysate (lots 7, 9, and 13; Amersham Corp., Arlington Heights, IL) was optimized for K^+ and Mg^{2+} and rat incisor RNA as described by the manufacturer. Standard K^+, Mg^{2+}, and unlabeled amino acid mixtures were also from Amersham. The final reaction mixture (usually a multiple of 500 μL) contained 50 μM concentrations of the unlabeled amino acids (in each case depleted of the amino acid used for labeling), 80 mM potassium acetate, 160 μL/mL of appropriately diluted RNA (or H_2O blank), and 700 μL/mL of reticulocyte lysate. The solution was 500 μCi/mL in generally labeled [^{3}H]serine, generally labeled [^{3}H]proline, or [^{35}S]methionine (all from New England Nuclear, Boston, MA). The RNA preparations were heated 1 min at 70 °C and then quickly chilled before addition to the reaction mixture. Incubations were at 28 °C, and aliquots were taken at timed intervals within a 2-h period. Each aliquot was added to 1 mL of 0.01% (w/v) bovine serum albumin plus 0.5 mL of a stock solution of cold serine (100 mg), NaOH (5 g), and 2.4% H_2O_2, all in 100 mL of H_2O. The treated aliquots were incubated 15 min at 37 °C, cooled for 15 min, mixed with 1 mL of 24% (w/v) trichloroacetic acid (TCA), and then stored overnight at 4 °C. The resultant precipitates were collected on 0.45-μm pore size Millipore filters, washed several times with 8% TCA, and the cpm values were read in 10 mL of liquifluor cocktail (New England Nuclear) in a Beckman LS 6800 scintillation counter.

Phosphophoryn Antibody Affinity Chromatography. A number of the translation products of total RNA, all from the 120-min time point, stored frozen, containing ^{3}H-labeled polypeptides, and ranging in volume from 50 to 200 μL, were pooled and diluted 1:1 v/v with PBS [0.9% NaCl (w/v), 2.5 mM NaH_2PO_4, and 7.2 mM Na_2HPO_4, pH 7.4; Fisher Chemical]. Blanks were treated similarly. The mixture was passed through a Millex-HA (0.45 μm pore) filter, and the filtrate was applied to a PBS-equilibrated anti-rat α-phosphophoryn rabbit IgG–Sephadex conjugated column (Tsay & Veis, 1985) and incubated for 2.5 h at 25 °C. The column was then washed with PBS until the effluent A_{230nm} returned to base line. The very small A_{230} peak corresponded to the elution of radioactivity from the column. The washed column was then eluted with PBS containing Millex-filtered 4 M guanidine hydrochloride ("ultrapure"; BRL), pH 7.4, until A_{230nm} returned to base line. The guanidine-eluted fraction was extensively dialyzed against deionized water at 4 °C by using dialysis tubing (Spectrapore 4, Spectrum) with a molecular weight cutoff of 12000–14000. The dialyzed material was lyophilized and then solubilized in gel electrophoresis running buffer [10% SDS (w/v), 60% sucrose (w/v), 0.1 M Tris-HCl, pH 8.8, and 0.1% bromophenol blue (w/v) (Bio-Rad)] and run on 5–8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels (Laemmli, 1970). Completed gels were treated for fluorography, using EN^{3}HANCE (New England Nuclear, Technical Bulletin HG-966); dried gels were exposed against Kodak X-Omat AR-5 film, preflashed, at −70 °C for 17–18 days.

Translated proteins from poly(A+) rat incisor mRNA were treated similarly. Results from total RNA and the poly(A+) mRNA were identical.

Dot-Blot Assays. The immuno dot blotting technique of Hawkes was used (Hawkes et al., 1982). Rabbit anti-rat α-phosphophoryn antibody and goat anti-rabbit IgG antibody conjugated to peroxidase (Tsay, 1984) were used to ascertain the presence of phosphophoryn. Fifteen microliters each of total RNA translated proteins and blank was spotted on nitrocellulose paper. Standard rat phosphophoryn and DEAE-cellulose eluate fractions were also spotted after lyophilization and redissolution at comparable concentrations. The antigen was applied for 60 min with subsequent back-coating of 1% phosphate-buffered bovine serum albumin (PBSA). The anti-phosphophoryn antibody was applied for 120 min at room temperature. Three 1% PBSA washes of 10 min each followed. The anti-rabbit antibody was then applied for 2 h at room temperature with three more washes. The color reagent was then applied: 10 mL of 0.01 M Tris, pH 7.4, was added to 50 mg of 3,5′-diaminobenzidine, which was then made up to 100 mL with Tris and 66 μL of 30% H_2O_2. Cocoa brown or purple-brown stains appeared within several minutes if phosphophoryn were present.

Gel Electrophoresis, Fluorography, and Gel Slicing. Polyacrylamide gradient (5–15%) gels, loaded with 175 and 75 μL of briefly boiled translated proteins, or other samples, were run for 180 min at 20 mA. High (45000–200000) and low (14400–92500) molecular weight protein standards (SDS–PAGE; Bio-Rad, Richmond, CA; 40 μL) were also run. Gels were stained in Coomassie Brilliant Blue (R-250; Bio-Rad, Richmond, CA) and photographed. Then, by use of a modification of the procedure of Bonner & Laskey (1974), the gels were soaked 60 min in dimethyl sulfoxide containing 2.2 g of 2,5-diphenyloxazole/100 mL, washed several times with H_2O, and then treated with 1% aqueous glycerol for 60 min. After the gel was dried, a fluorogram was made at −70 °C with 5

weeks of exposure (preflashed Kodak XRP-5 X-ray film).

Gradient gels were also sliced into 1-mm portions starting from the bottom where the dye front could serve as a marker. Each gel slice was put into a separate scintillation vial to which 1 mL of 9:1 (v/v) NCS tissue solubilizer (Amersham) was added. The material was heated 120 min at 50 °C after which the cpm values were read in 10 mL of liquifluor cocktail.

HPLC Fractionation of [^{3}H]Serine-Labeled Proteins. The mixture of total translated proteins was fractionated on an "Ultrapac" TSK-G3000 SW, 7.5 × 300 mm, column (LKB, Bromma, Sweden) equilibrated with 0.5 M KCl–10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer. These are nondenaturing conditions. The flow rate was 0.7 mL/min, and 0.4 mL was collected per tube. The translate and blank were run separately. Five hundred microliters of the former and 250 μL of the latter were applied to the column after Millex-HA (0.45 μm pore) filtration. The absorption of the eluate was monitored at 230 nm, and the radiolabeled proteins were measured by adding 50 μL from each tube to 10 mL of 3a70B cocktail. The contents of tubes 15–31 from the high molecular weight range of the elution pattern were combined as a fraction. Lysate hemoglobin (M_r 68 000) eluted in tubes 32–37 and acted as an internal molecular weight standard.

DEAE-cellulose Chromatography of High Molecular Weight High-Performance Liquid Chromatography (HPLC) Eluate. DEAE 52 ("preswollen microgranular"; Whatman; 7 g) was equilibrated with 0.05 M Tris, pH 8.2, and poured to give a column 12 × 1 cm. The combined HPLC eluate fractions 15–31 from several rat RNA-containing and also from RNA-deficient translations were fractionated on this column. Each sample for chromatography was frozen immediately after HPLC elution; a protease inhibitor (PI) cocktail (~640 μL total of a 200-mL solution containing 0.78 g of benzamidine, 13.1 g of ε-amino-*n*-caproic acid, 1.25 g of *N*-methylmaleimide, 666 μL of phenylmethanesulfonyl fluoride, and 0.1 M EDTA) was layered on the eluate before defrosting. Prior to DEAE-cellulose chromatography, each sample was dialyzed for 2 days in the cold against three large volume changes of 0.05 M Tris, pH 8.2, containing PI, which gave about an 8-mL sample for application to the column. After addition of sample the column was run at room temperature, first with Tris buffer and then with a gradient in Tris between 0 and 0.5 M NaCl (150 mL of each). The flow rate was 0.7 mL/min and 3-mL volumes were collected. The eluate was monitored for protein at 230 nm and for radioactivity by using 200 μL per tube in 10 mL of 3a70B cocktail. Conductivity readings were taken from spaced elution tubes to monitor the salt gradient. The contents of eluate tubes were combined on the basis of radioactive peaks.

Amino Acid Analysis. Amino acid analysis of fractions within the salt gradient of the DEAE-cellulose eluate was carried out by using an HPLC amino acid ion-exchange analyzer sensitive to the picomole level. Samples were hydrolyzed for 22 h at 105 °C in constant boiling HCl. Following evaporation the amino acids were dissolved in 0.01 N HCl. A postcolumn *o*-phthalaldehyde derivatization and fluorescence detection system was used.

Results

Translation Efficiency. Figure 1 shows the incorporation of serine and proline into protein in the translation system with the rat incisor total RNA at 0.9 mg/mL. In 120 min 4000 cpm above background was incorporated for [^{3}H]serine and 11 700 cpm for [^{3}H]proline. It is important to note that the stock proline had more than 5 times the specific activity of the [^{3}H]serine. In the same period [^{35}S]methionine was incorporated only to the level of 6000 cpm. These data indicate that the mRNA was directing the synthesis of proteins relatively rich in serine and proline.

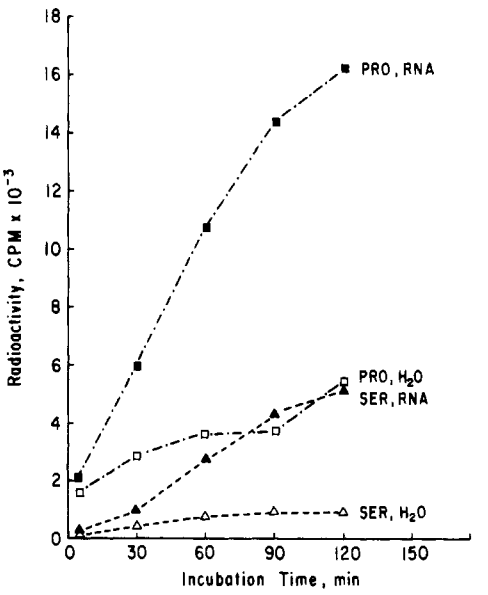

FIGURE 1: Time course of incorporation of [^{3}H]serine and [^{3}H]proline into peptide-bound form by translation of rat incisor mRNA. Note that the [^{3}H]proline used has a 6.3 times higher specific activity than the serine. Thus, serine incorporation was relatively very efficient. (■) [^{3}H]Pro, system + mRNA. (□) [^{3}H]Pro, system − mRNA. (▲) [^{3}H]Ser, system + mRNA. (△) [^{3}H]Ser, system − mRNA.

Gel Fluorograms. Densitometer scans of a 5-week fluorogram of gel electrophoretograms of the rat incisor mRNA translated proteins and the proteins produced by the lysate in the absence of added mRNA are shown in Figure 2. The two most prominent bands are common to both systems, but several unique bands or regions are evident in the system plus mRNA. The set of bands near the top of the gel are probably related to procollagen chains. The most prominent of the unique bands has a molecular weight of 153 000, calibrated by globular protein standards. Numerous bands are in the range M_r 80 000–143 000. Sliced and counted gels provided essentially the same data.

Identification of α-Phosphophoryn. Control experiments with the electrophoretic transfer of phosphophoryns to nitrocellulose and subsequent staining with anti-phosphophoryn antibody were not successful. We therefore went directly to the use of an affinity column in which anti-α-phosphophoryn was coupled to the Sepharose. In a preparation in which poly(A+) rat incisor mRNA was used, the translated proteins were passed over the column. As in control experiments, phosphophoryn was avidly bound and could only be eluted with 4.0 M guanidine hydrochloride. It did not elute in a high ionic strength NaCl wash (Tsay & Veis, 1985). Since the most prominent distinctive peaks shown in Figure 2 were in the high molecular weight range, the column eluate was applied to 5–8% gradient gels for electrophoresis. As seen in Figure 3, the protein retained by the column is primarily in one band, with a relative molecular weight, again as calibrated from globular protein standards, of 155 000. At high loading, lower molecular weight bands are also seen, with prominent bands in the distinctive ranges already noted in Figure 2.

HPLC and DEAE-cellulose Fractionation of Reticulocyte Lysate Mixture. The [^{3}H]serine-labeled proteins of a rat incisor total RNA translation were passed over a gel filtration HPLC column. A parallel blank sample was treated similarly. Under these nondenaturing conditions the hemoglobin in the mixture, M_r 68 000, served as a convenient internal molecular

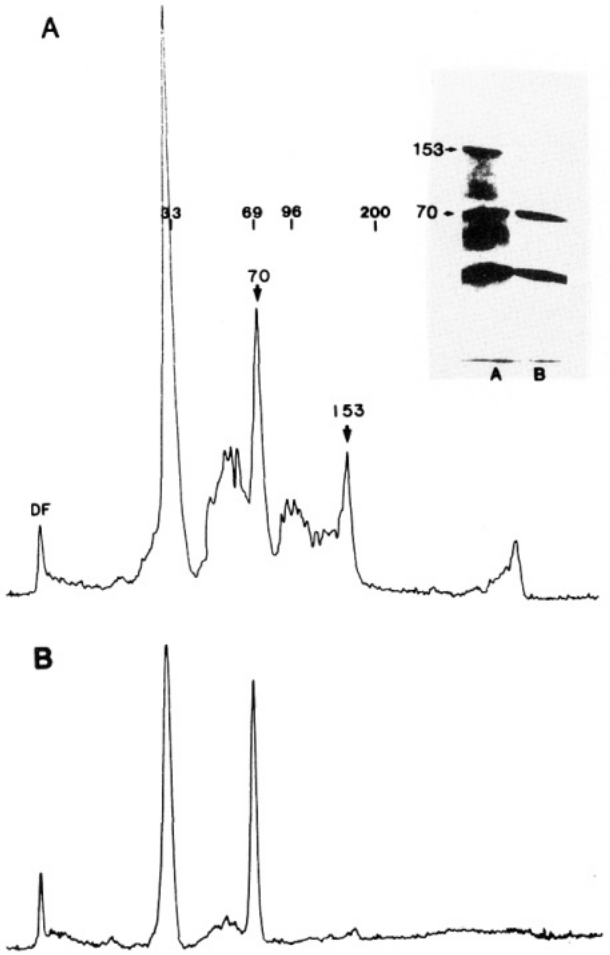

FIGURE 2: Densitometer tracings of rat incisor mRNA and blank translated proteins, [^{3}H]serine labeled, 120 min, after sDS–polyacrylamide gel electrophoresis. The molecular weights indicated for the two major peaks were determined by comparison with the migration of Coomassie-stained protein molecular weight standards. (A) Translation with tooth mRNA; (B) blank. Migration was from right to left. DF indicates dye front.

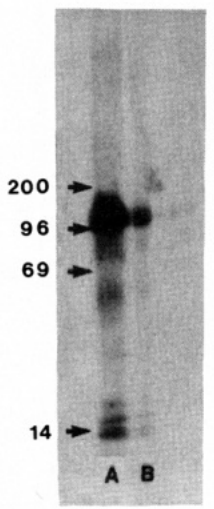

FIGURE 3: Fluorogram of gel electrophoretogram of the [^{3}H]-serine-labeled rat incisor poly(A+) mRNA translated protein eluted from the α-phosphophoryn antibody affinity column. The major band corresponds to M_r 150 000 as determined from ^{14}C-labeled globular protein standards, whose positions are indicated by the arrows. Lane B is one-tenth the concentration of lane A.

weight reference point. Figure 4 compares a portion of the radioactivity elution patterns for equal volumes of translated proteins and blank. The high radioactivity at >11 mL of

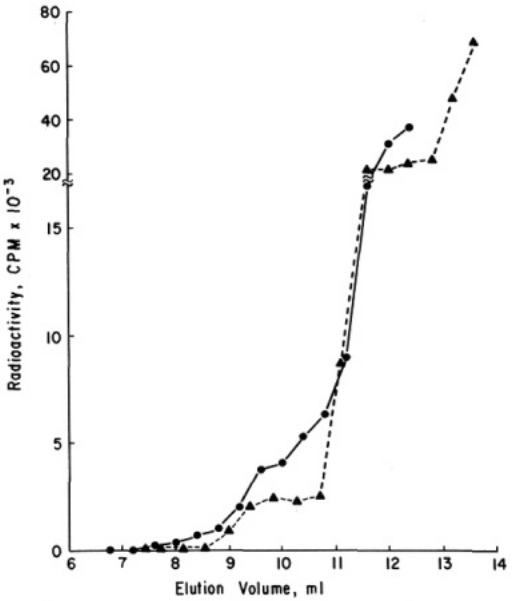

FIGURE 4: Gel exclusion chromatography of [^{3}H]serine-labeled proteins from the translation of rat incisor mRNA and reticulocyte lysate blank. Only the high molecular weight region is shown. Chromatography was under nondenaturing conditions. (●) Translated proteins; (▲) blank. Fractions were collected from 6 to 11 mL.

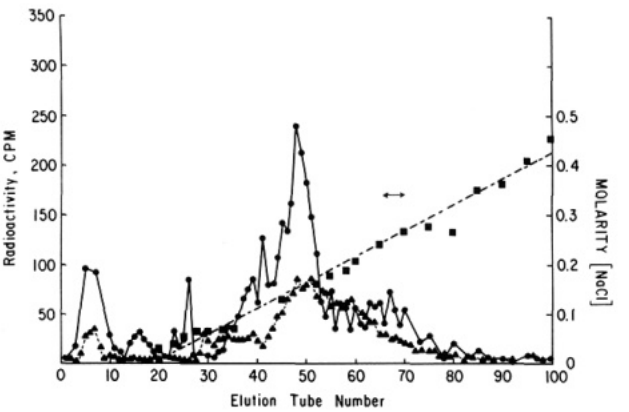

FIGURE 5: DEAE-cellulose chromatography of the high molecular weight region of the HPLC fractions of Figure 3. [^{3}H]Serine labeled. (●) Translation + mRNA; (▲) translation − mRNA; (■) NaCl gradient.

eluate corresponds to the first common peak at M_r 70 000 seen in Figure 2 and coincided with the elution of native hemoglobin, which was readily visible. The eluate of the high molecular weight fractions from 6 to 11 mL was collected and passed over a DEAE-cellulose column. There was sufficient protein from the lysate present in this high molecular weight fraction to preclude the necessity for addition of further carrier protein. The data shown in Figure 5 indicates that while many of the rat incisor specific translated proteins eluted early in the salt gradient, a significant portion eluted in the region expected for phosphophoryn and nonphosphorylated phosphophoryn, at 0.25 M NaCl, tubes 66–70. It is difficult to judge the significance of the larger peak eluting at 0.1–0.15 M since the cutoff at 11 mL of the HPLC elution did not entirely eliminate the beginning of the large peak. The relative intensity of counts in this region, however, is in general accord with numerous peaks seen in the region from M_r 70 000 to 150 000 in Figure 2 and the higher number of counts in the eluate between 10 and 11 mL in Figure 5.

Dot-Blot Immunodetection. Analyses were carried out on aliquots from tubes 49 and pooled tubes 66–70 from the DEAE chromatography, along with unfractionated translate and blank

and purified rat phosphophoryn. Tubes 66–70, the unfractionated total translated proteins, and purified rat phosphophoryn were positive. Tube 49 and the unfractionated blank translation were negative for rat α-phosphophoryn.

Optical density measurement along the DEAE chromatogram indicated that very little protein was present in the tube 66–70 region, or immediately adjacent regions. The entire remaining eluates from these regions after counting and immunoblot assays were concentrated and hydrolyzed in 6 N HCl, and an HPLC amino acid analysis was carried out in a qualitative fashion. The distinctive phosphophoryn fingerprint of high and nearly equivalent aspartic acid and serine peaks, with very little background of other components, was evident in translated protein fraction 66–70 but not in equivalent aliquots of the adjacent eluates.

Discussion

The gel of Figure 2 shows that a large number of serine-labeled proteins are produced by the combined total incisor pulp and odontoblast mRNA. The major band at M_r 153 000 (±5000) is the component that is taken up preferentially by the α-phosphophoryn affinity column (Figure 3). This component, as identified by the dot-blot immunoassay, also chromatographs as a very acidic component on DEAE-cellulose, eluting in the position expected for a phosphophoryn. It has, on a qualitative basis, nearly equivalent contents of aspartic acid and serine, and these two amino acids are present at a greater concentration than all others. Thus, this protein with M_r 153 000 can be concluded to contain the α-phosphophoryn moiety. The presence of relatively faint bands at M_r <150 000 in the electrophoretogram of the eluate from the anti-α-phosphophoryn column indicates that some of the bands in the range from M_r 70 000 to 150 000 and M_r <70 000 may be related to α-phosphophoryn and could represent incompletely finished or degraded nascent chains.

The cell-free translation system should produce a pre form for phosphophoryn. In most cases the signal sequence is on the order of 20–25 amino acid residues (Blobel & Dobberstein, 1975), contributing 2000–3000 to the molecular weight. Thus, the high molecular weight of the primary gene product compared with the tissue-isolated phosphorylated rat phosphophoryn at M_r ~90 000 (Butler et al., 1981; Stetler-Stevenson & Veis, 1983) is strong evidence that the protein is produced in a pro or precursor form and, as with so many other extracellular matrix proteins, must be subject to posttranslational, secretory, or postsecretory proteolytic processing.

Our studies at present provide no data on the other potential phosphophoryns or other odontoblast-specific proteins. The very specific anti-α-phosphophoryn used reacts only weakly with a few of the other serine-labeled products (Figure 3, lane A).

We are in position to prepare a cDNA to the prepro-α-phosphophoryn mRNA. That work is now in progress. Identification of other pulp and odontoblast-specific proteins will require the preparation of suitable antibodies, work already initiated.

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