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Isolation and Characterization of a 125-Kilodalton Rapidly Labeled Nucleolar Phosphoprotein

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ABSTRACT: A 125-kilodalton (kDa) phosphoprotein was isolated from nucleoli of Novikoff hepatoma cells in the presence of various inhibitors of proteases, alkaline phosphatase, and RNase. This protein was the most highly phosphorylated protein found thus far in the nucleolus. The half-life of [^{32}P]phosphate in the 125-kDa phosphoprotein was approximately 60 min. Amino acid analysis of the protein showed it had a high serine content (15.5 mol %), a high glutamine plus glutamic acid content (15.5 mol %), and a high lysine content (10.3 mol %). Phosphoserine was the only phosphorylated amino acid identified. After alkaline hydrolysis of the ^{32}P-labeled protein, ribonucleotides were found which accounted for approximately 8.5% of the [^{32}P]phosphate. After cytidine 3′,5′-[^{32}P]diphosphate ([^{32}P]pCp) labeling by RNA ligase, several oligoribonucleotide sequences were purified including $GGGC_{OH}$ and $GGGGC_{OH}$. The binding of oligonucleotides to peptides was stable under denaturing fractionation conditions including 6 M urea treatment and incubation at 100 °C for 10 min in sodium dodecyl sulfate and β-mercaptoethanol. Furthermore, when nucleotide–peptide complex was treated with ribonuclease T_2 followed by snake venom phosphodiesterase, the junctional nucleotide pCp was released. These results suggest that one or more ribonucleotides are covalently bound to the 125-kDa phosphoprotein.

Analyses of nuclear substructures of Novikoff hepatoma in this laboratory have shown a number of macromolecules contain postsynthetic modifications. Among these are the 5′ caps of U-snRNAs (Ro-Choi et al., 1975) which were also found in mRNAs and viral RNA (Rottman et al., 1974; Busch et al., 1982). Another novel posttranslational modification observed is the isopeptide conjugate linkage of protein A-24 in which histone 2A and ubiquitin are covalently linked (Goldknopf & Busch, 1977); this modification has been implicated recently in modulation of gene expression.

The nucleolus is the site of synthesis of pre-rRNA (Perry, 1962; Darnell, 1968) as well as the location of the assembly and processing of preribosomal particles (Warner & Soeiro, 1967; Liau & Perry, 1969), which are transported into the cytoplasm where they become mature ribosomes. The phosphorlation of nucleolar non-histone proteins has been implicated in the regulation of the synthesis, modification, packaging, and transport of ribosomal RNA and other nucleolar functions (Olson et al., 1978; Rose & Jacob, 1983; Busch, 1984). Studies from this laboratory have previously described the isolation and characterization of three major nucleolar phosphoproteins: protein C23 [110 kilodaltons (kDa),[1] pI = 5.2], protein B23 (37 kDa, pI = 5.2) (Busch, 1984; Mamrack et al., 1979; Lischwe et al., 1979, 1981, 1982), and more recently the 19-kDa, pI = 4.5 phosphoprotein (McRorie et al., 1984).

In this study, using inhibitors of proteases, alkaline phosphatase, and RNase (Spohn et al., 1984), it was found that the 125-kDa protein is the most highly phosphorylated nucleolar protein found thus far. This high molecular weight phosphoprotein from Novikoff heaptoma cells is in relatively low abundance in the nucleolus and has a rapid turnover ($T_{1/2}$ = 60 min). A novel feature of this molecule was the presence of RNA oligonucleotide(s) apparently covalently linked to the protein.

Materials and Methods

Nucleoli from Novikoff Hepatoma. Novikoff hepatoma ascites cells were transplanted in Holtzman rats; 6 days after transplantation, the ascites cells were harvested. Nucleoli were prepared by sonic oscillation described previously (Spohn et al., 1984). To obtain ^{32}P-labeled nucleoli, the harvested cells were incubated in vitro with [^{32}P]orthophosphate for 6 h (Mauritzen et al., 1971). Alternatively, Novikoff hepatoma tissue culture cells (Ochs et al., 1985) were labeled for 16 h with [^{32}P]orthophosphate.

Nucleolar Extraction. The isolated nucleoli were extracted with 10 mM Tris or 4 M urea/3 M LiCl. For Tris extraction, the isolated nucleoli were suspended in 3 volumes of 10 mM Tris-HCl, pH 7.6, and incubated at 0 °C for 20 min. The suspension was centrifuged at 27000g for 10 min, and the pellet was reextracted twice. The supernatants were pooled and treated with 50 μg/mL RNase A at room temperature for 1 h. Some of the isolated nucleoli of Novikoff heptoma cells were extracted with 4 M urea/3 M LiCl (Lischwe et al., 1981); the nucleoli were homogenized in 5 volumes of 8 M urea/2 mM phenylmethanesulfonyl fluoride (PMSF)/10 μg/mL leupeptin/2 mM DTT/2 mM EDTA to which an equal volume of 6 M LiCl was added. The suspension was homogenized and allowed to stand for 3–16 h at 4 °C. The proteins were separated from the residue by centrifugation at 27000g for 20 min.

Use of Inhibitors. To maintain the structural integrity of nucleolar phosphoproteins, various inhibitors were used including PMSF (0.5 mM), *N*-ethylmaleimide (1 mM), aprotinin (5 μg/mL), leupeptin (5 μg/mL), *p*-(hydroxymercuri)-benzoate (1 mM), β-glycerophosphate (10 mM), and vanadyl ribonucleoside complex (2 mM). Throughout the preparation of nucleoli and nucleolar proteins, 1 mM PMSF and 1 mM *N*-ethylmaleimide were used. The remaining inhibitors were used during the sonication and nucleolar protein extraction.

[1] Abbreviations: DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; pCp, cytidine 3′,5′-diphosphate; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Column Chromatography. The 4 M urea/3 M LiCl extract was dialyzed against 20 mM Tris–maleate (pH 6.0/6 M urea/1 mM EDTA/1 mM dithiothreitol/1 mM PMSF/5 μg/mL leupeptin overnight. The dialysate was centrifuged at 27000*g* for 15 min, and the supernatant was applied to a DEAE-cellulose (DE-52) column (1 × 25 cm) equilibrated with the same buffer. The proteins were eluted with a 0–0.5 M NaCl gradient at a flow rate of 0.1 mL/min, and 2-mL fractions were collected (Lischwe et al., 1981).

Polyacrylamide Gel Electrophoresis. Nucleolar proteins were fractionated by polyacrylamide gel electrophoresis. One-dimensional polyacrylamide–SDS gels were used to fractionate proteins according to the method of Laemmli (1970). Two-dimensional fractionation was done by isoelectric focusing (pH 3.5–10) followed by polyacrylamide–SDS gel electrophoresis (O'Farrell et al., 1977). A reverse gel modified from Swank & Munkres (1971) was devised to fractionate the tryptic peptides. This gel had a 15% acrylamide stacking gel on a 6% acrylamide separation gel in sodium phosphate buffer, pH 6.8. Identification of proteins was carried out by staining with Coomassie blue or by autoradiography. Elution of proteins was done by electroelution (Knecht & Busch, 1971) or by in situ tryptic digestion (Zweig, 1981). The eluted peptides were precipitated with 2 volumes of ethanol containing 2% potassium acetate at −20 °C. Tryptic digestion products were further fractionated by 6% polyacrylamide–SDS gel electrophoresis at pH 6.8 or 20% polyacrylamide–7 M urea gel electrophoresis at pH 8.3 (Donis-Keller et al., 1977). Elution of peptides was performed with 0.5% SDS/0.14 M NaCl/0.05 M sodium acetate–acetic acid (pH 5.1)/1 mM EDTA at 55 °C for 2 h.

Characterization of Peptides. Tryptic peptides were iodinated by the chloramine T method of Elder et al. (1977). Peptide maps were made by electrophoresis at pH 1.9 followed by chromatography (Zweig, 1981). Amino acid analysis of isolated protein was done by hydrolysis with 5.7 N HCl at 110 °C for 22 h or as modified for gels (Goldknopf & Busch, 1980). Identification of phosphoamino acids was done by the method of Hunter & Sefton (1980).

Characterization of Oligonucleotides Bound to Tryptic Peptides. RNA ligase was used to label oligonucleotides with 5′-[^{32}P]pCp after alkaline phosphatase treatment (England & Uhlenbeck, 1978). Mobility shift analyses were carried out by two-dimensional fractionation on DEAE-cellulose thin layers (Busch et al., 1976). After alkaline hydrolysis, nucleotide composition was determined by paper electrophoresis at pH 3.5 (Sanger et al., 1965). To determine the nucleotide bound to the peptide, the complex was first treated with 0.1 unit of alkaline phoshatase in 6 M urea/0.1 M Tris-HCl (pH 7.2)/10 mM $MgCl_2$ at 55 °C for 5–16 h. After inactivation of alkaline phosphatase with an equal volume of phenol in the presence of 2 mM EDTA, the complex was precipitated with 2 volumes of ethanol at −20 °C. Then the sample was digested completely with ribonuclease T_2 followed by snake venom phosphodiesterase in the presence of 25 μg of carrier RNA (Barrell, 1971).

The digest was analyzed on two-dimensional thin-layer chromatography; the first dimension was isobutyric acid/0.5 M NH_4OH (5:3), and the second dimension was concentrated HCl/2-propanol/H_2O (70:15:15). To determine the 3′,5′-diphosphonucleotide, chromatography was done in 95% ethanol/1 M ammonium acetate at pH 7.5 (70:30).

Results

Polyacrylamide Gel Electrophoresis of Tris Extracts of Nucleoli. When the protease inhibitors were used in the extractions, high molecular weight proteins were routinely found on one-dimensional gels of the nucleolar proteins (Figure 1). In addition to the phosphoproteins previously described as B23 (37 kDa, p*I* = 5.2) and C23 (110 kDa, p*I* = 5.2), a 125-kDa phosphoprotein[2] was identified. The amount of 125-kDa protein observed after Coomassie blue staining was much less than that of proteins B23 and C23 (Figure 1), but its ^{32}P labeling was much greater. Figure 2 shows the fractionation of nucleolar proteins on two-dimensional gels. The pointer shows the 125-kDa protein at pH 7.4 (Figure 2A). The microheterogeneity at pH 5.4–7.0 may result from differing amounts of phosphorylation or oligonucleotides (Figure 2B).

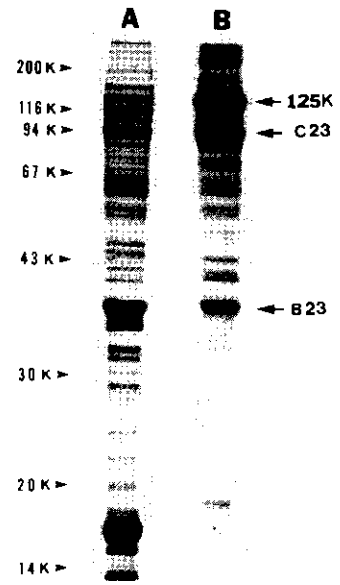

FIGURE 1: One-dimensional electrophoresis of the Tris extract (10 mM, pH 7.4) of nucleoli from Novikoff hepatoma cells labeled with [^{32}P]orthophosphate overnight. (A) Coomassie blue staining of 12% SDS–PAGE. (B) Autoradiography of ^{32}P-labeled proteins. The arrows show the positions of the 125-kDa, C23, and B23 proteins.

Turnover of the 125-kDa Phosphoprotein. Figure 3 shows a time course study of the ^{32}P-labeling patterns for nucleolar phosphoproteins. Novikoff hepatoma cells were labeled with [^{32}P]phosphate for 16 h and subsequently chased with McCoy's 5A medium containing 4.2 mM sodium phosphate (pH 7.4).[3] The 125-kDa protein was highly labeled, and the decrease of [^{32}P]phosphate in this protein was rapid compared to the other proteins. The half-life for [^{32}P]phoshate in the 125-kDa protein calculated from densitometric scans was approximately 60 min.

DEAE-cellulose Chromatography. Figure 4 shows an elution profile of ^{32}P-labeled proteins from DEAE-cellulose column chromatography. Five ^{32}P-labeled peaks were eluted with a linear 0–0.5 M NaCl gradient in 20 mM Tris–maleate buffer (pH 6.0). SDS–PAGE and autoradiography indicated that the 125-kDa protein was enriched in peak D (fractions 56–64 Figures 4 and 5A,B).

Analysis of Amino Acid Composition and Tryptic Peptides. DEAE-cellulose fractions tube numbers 56–64 which enriched the 125-kDa protein were pooled and fractionated on a preparative SDS–PAGE. The 125-kDa protein was identified by Coomassie blue staining or autoradiography and cut out carefully from the preparative gel. The 125-kDa protein was

[2] The molecular weight of this protein was determined on 6% SDS–polyacrylamide gels.

[3] The relatively highest labeling of the 125-kDa protein with respect to protein C23 was found in the mid-log phase.

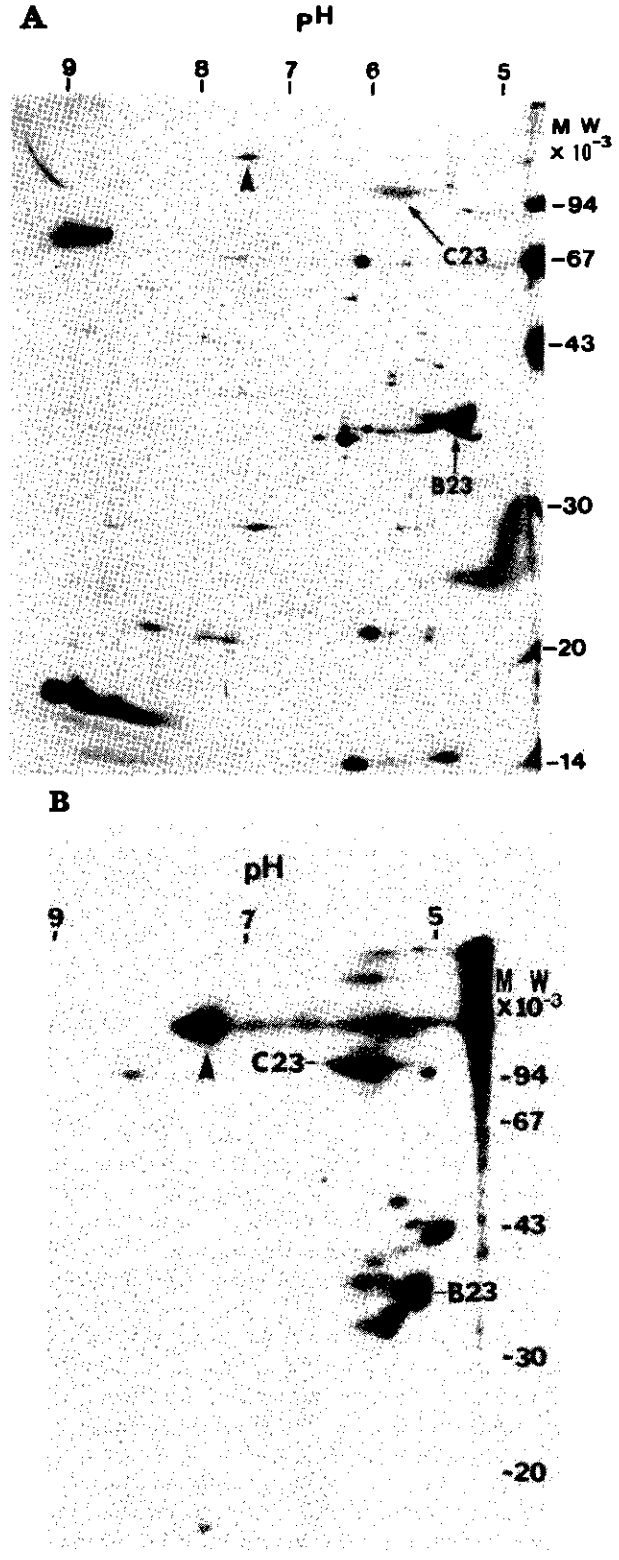

FIGURE 2: Two-dimensional isoelectric focusing (pH 3.5–10)/SDS–PAGE (12%) separation of proteins of the Tris extract of nucleoli from Novikoff hepatoma cells. Arrowheads show the position of the 125-kDa, p*I* = 7.4 protein. (A) Silver-stained gel. (B) Autoradiograph of the gel.

then electroeluted from the gel pieces. Figure 6 shows the two-dimensional gel of the purified 125-kDa protein. The purified 125-kDa protein was either hydrolyzed in the gel matrix (Goldknopf & Busch, 1980) or electroeluted from the gel for amino acid analysis (Knecht & Busch, 1971). As shown in Table I, the 125-kDa protein had a high serine content (15.5 mol %) as well as a high glutamine plus glutamic acid content (15.5 mol %) and a high lysine content (10.3 mol %). Phosphoamino acid analysis by acid hydrolysis followed by electrophoresis on thin-layer cellulose plates showed that only phosphoserine was present (Figure 7). As expected (Table I) from the tyrosine content (2.3 mol %), the two-dimensional map of the ^{125}I-labeled tryptic peptides had 22 major spots (Figure 8).

Table I: Amino Acid Composition[a] of the 125-kDa Nucleolar Phosphoprotein

amino acid	mol %[b]	amino acid	mol %[b]
Asx	9.0	Met	1.1
Thr	3.7	Ile	3.0
Ser	15.5	Leu	6.8
Glx	15.5	Tyr	2.3
Pro	8.0	Phe	1.4
Gly	6.1	Lys	10.3
Ala	6.4	His	3.0
Val	4.5	Arg	3.3

[a] The 125-kDa protein eluted from the gel or in the gel matrix was hydrolyzed with 5.7 N HCl at 110 °C for 22 h (Goldknopf & Busch, 1980) and analyzed on a Beckman 121 amino acid analyzer. [b] The values are the averages of three analyses.

Table II: Distribution of [^{32}P]Phosphate in Tryptic Peptides of 125-kDa Protein

peptide	% distribution[a]	peptide	% distribution[a]
1	20.5	5	4.9
2	18.2	6	12.0
3	18.2	7	14.7
4	11.5	total	100.0

[a] The tryptic digestion products were cut out from the reverse gel after autoradiography, and the radioactivity was measured in Cerenkov units.

Table III: Distribution of [^{32}P]Phosphate in Peptide 3 after Alkaline Hydrolysis

component in peptide 3	% distribution	nucleotide in peptide 3	% distribution
P_i	53.1	C	42.9
nucleotide	46.9	A	9.0
		G	33.5
		U	14.6

Electrophoresis of the ^{32}P-labeled tryptic digestion products separated seven bands (Figure 9A). After alkaline hydrolysis, the majority of ^{32}P was released as [^{32}P]orthophosphate. Figure 10 shows that band 1 migrated as an alkaline-resistant streak and most of the bands did not migrate. Interestingly, band 3 contained all four ribonucleotides. Tables II and III show the distributions of [^{32}P]phosphate in each peptide and the nucleotides in peptide 3. The ^{32}P in the nucleotides accounted for 8.5% of the total in the 125-kDa protein. On 20% polyacrylamide–7 M urea gel electrophoresis, all the ^{32}P-labeled tryptic digestion products migrated between bromophenol blue and xylene cyanol (Figure 3B), which suggests that the oligonucleotide length was less than 10.

Partial Characterization of the Oligonucleotides. The ^{32}P-labeled and unlabeled tryptic peptides were further digested with proteinase K and fractionated on reverse polyacrylamide gels (Figure 11A). After excision of the bands, the digestion products were extracted with 0.5% SDS/0.14 M NaCl/50 mM sodium acetate–acetic acid (pH 5.1)/1 mM EDTA at 55 °C and precipitated with 2 volumes of ethanol containing 2% potassium acetate. After alkaline phosphatase treatment, bands 1 and 2 were labeled with [^{32}P]pCp by RNA ligase. The labeled products were fractionated on a 20% polyacrylamide–7 M urea gel (Figure 11B). The major bands were purified by two-dimensional homochromatography and then subjected to

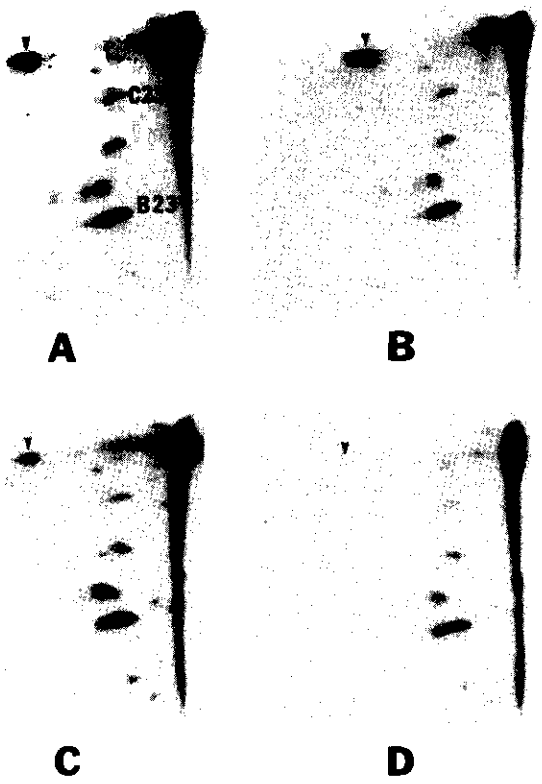

FIGURE 3: Turnover experiment of ^{32}P-labeled nucleolar phosphoproteins. Novikoff hepatoma tissue culture cells were labeled with [^{32}P]orthophosphate overnight and then chased with cold P_i for 0 (A), 30 (B), 60 (C), and 120 (D) min. Arrowheads indicate the 125-kDa protein.

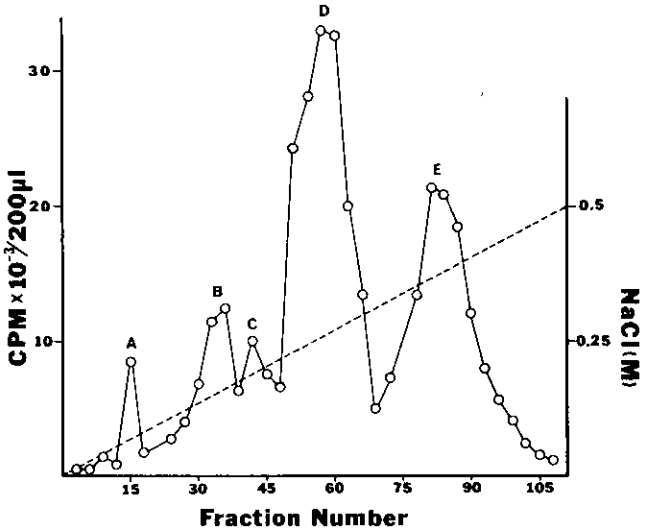

FIGURE 4: DEAE-cellulose column chromatography of 3 M LiCl/4 M urea extract of nucleoli from Novikoff hepatoma cells. The column was eluted with a 0–0.5 M NaCl gradient containing 20 mM Tris–maleate (pH 6.0), 6 M urea, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, and 5 µg/mL leupeptin. Radioactivity was measured in Cerenkov units.

alkaline hydrolysis followed by electrophoresis to determine the 3′ termini. Homochromatographically purified oligonucleotides labeled with [^{32}P]pCp were subjected to mobility shift analysis after partial alkaline hydrolysis. Figure 12 shows the sequence $GGGC_{OH}$ which was identified in band a on Figure 11B, lane 1. The satellite spot "S" was not identified. Band d on Figure 11B, lane 2, contained a $GGGGC_{OH}$ sequence.

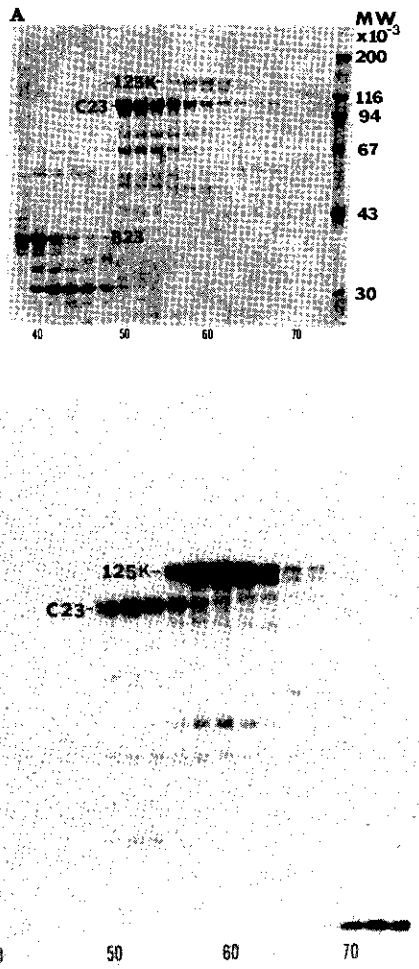

FIGURE 5: One-dimensional separation of ^{32}P-labeled nucleolar proteins obtained by DEAE-cellulose chromatography. (A) Coomassie blue stained gel (7.5% SDS–PAGE). (B) Autoradiograph. Bottom numbers are the fraction numbers shown on Figure 4.

Determination of Nucleotide That Binds to Peptide. When ^{32}P-labeled nucleotide–peptide complex was treated with alkaline phosphatase, 23.9% of total phosphate still remained in the complex. After alkaline phosphatase treatment, the sample was digested consecutively with ribonuclease T2 and snake venom phosphodiesterase. The digest was analyzed by two-dimensional chromatography (Figure 13). Four spots (Ap, Cp, Gp, and Up) were identified as 3′-nucleotides by comparison with 5′- or 3′-nucleotide standard. The mobility of another spot (pCp) was consistent with that of cytidine 3′,5′-diphosphate (Seno et al., 1968). For confirmation of the mononucleoside diphosphate, the sample was rechromatographed in an ethanol/1 M ammonium acetate system in which the pCp spot did not migrate (Laskowski, 1966). The dense phosphoserine spot at the origin (0) was identified by phosphoamino acid analysis (Goldknopf & Busch, 1980).

Discussion

With the aid of inhibitors of proteases, alkaline phosphatase, and RNase, a novel 125-kDa phosphoprotein was found in the nucleoli of Novikoff hepatoma cells. This phosphoprotein was in a relatively high concentration in the nucleolus compared to the nucleoplasm where the 125-kDa protein was present in a negligible amount (data not shown). amino acid analysis showed that it has a high serine content (15.5 mol %), a high glutamine plus glutamic acid content (15.5 mol %), and a high lysine content (10.3 mol %). Phosphoamino acid analysis

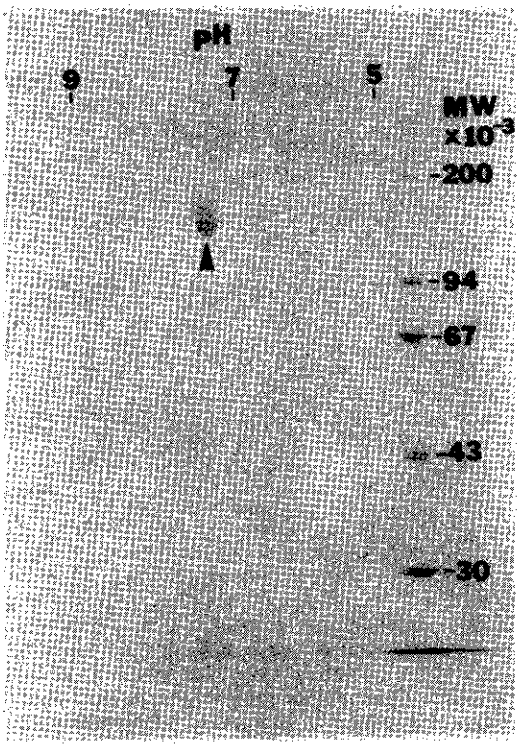

FIGURE 6: Two-dimensional isoelectric focusing (pH 3.5–10)/SDS–PAGE (7.5%) of the purified 125-kDa protein from preparative SDS–PAGE following DEAE-cellulose chromatography.

FIGURE 7: Phosphoamino acid analysis of the 125-kDa protein by high-voltage electrophoresis on a TLC plate: 0, sample application point; P_i, inorganic phosphate; P-ser, phosphoserine; P-thr, phosphothreonine; P-tyr, phosphotyrosine.

showed that only phosphoserine was present. The 125-kDa protein has a rapid phosphate turnover in comparison to other nucleolar phosphoproteins.

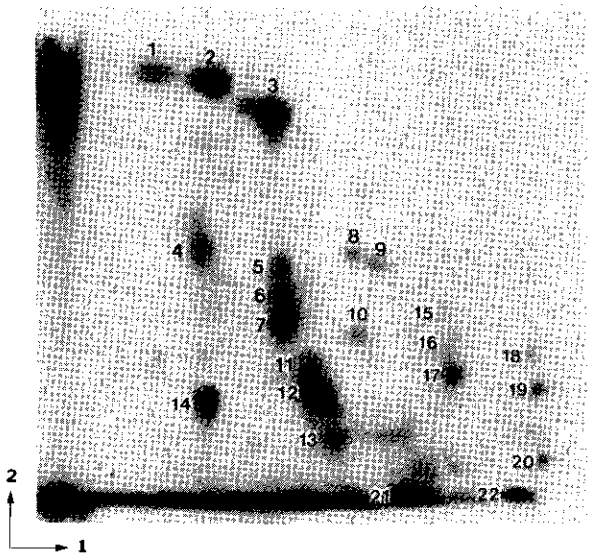

FIGURE 8: Two-dimensional fractionation of tryptic peptides of the 125-kDa protein labeled with ^{125}I by chloramine-T. High-voltage electrophoresis was run at 1 kV for 40 min (1) followed by ascending chromatography (2).

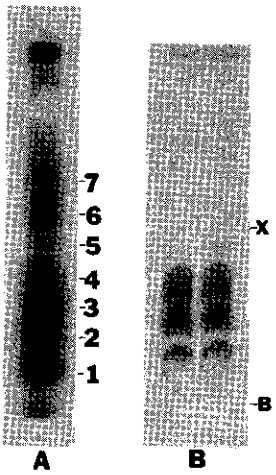

FIGURE 9: Autoradiographs of polyacrylamide gel electrophoresis of tryptic digestion products of ^{32}P-labeled 125-kDa protein. (A) 6% reverse SDS–PAGE shows seven ^{32}P-labeled bands which were analyzed for nucleotides as shown in Figure 10. (B) 20% polyacrylamide–7 M urea gel separation: X, xylene cyanol; B, bromophenol blue.

Markinson & McSwigan (1978) and Markinson et al. (1978) described a phosphoprotein with a molecular weight of 135 000 which is similar to the 125-kDa protein reported here in terms of its high phosphorylation. A 135-kDa phosphoprotein in mouse and hamster and a 128-kDa phosphoprotein in human tissue (Pfeifle & Anderer, 1984) had a similar nucleolar localization and a high phosphorylation level. There is insufficient data for a direct comparison. The 145-kDa protein of *Xenopus laevis* nucleoli (Franke et al., 1981; Krohne et al., 1982; Benavente et al., 1984) is not the same as the 125-kDa phosphoproteins. First, its extractability by salt was quite different. After salt extraction, the *Xenopus* 145-kDa protein remained in the insoluble nucleolar skeletons (Franke et al., 1981; Benavente et al., 1984). The 125-kDa phosphoprotein is easily extracted at low salt concentration (10 mM Tris). Second, the p*I*'s of the two proteins are different (Franke, 1981). In addition, Figure 8 shows the two-dimensional map of the tryptic peptide is different (Krohne, 1982).

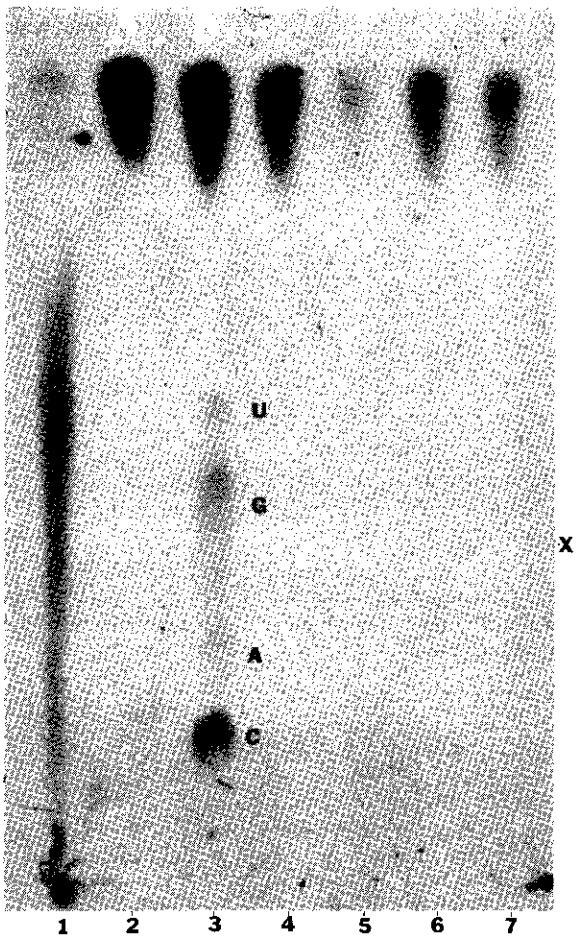

FIGURE 10: Identification of ^{32}P-labeled ribonucleotides associated with band 3 (Figure 9A). Each peptide (Figure 9A) eluted from the gel was hydrolyzed with 0.4 N NaOH at 37 °C overnight, and electrophoresis was done on Whatman 3MM paper at pH 3.5. X, xylene cyanol. Only band 3 contained ribonucleotides, and their relative amounts are shown in Tables II and III.

RNA oligonucleotides in the tryptic peptide 3 contained approximately 8.5% of the [^{32}P]phosphate in this protein. The binding of oligonucleotide to peptide 3 was stable in 4 M urea/3 M LiCl and at high temperature (100 °C for 10 min) in the presence of sodium dodecyl sulfate and β-mercaptoethanol, and 7 M urea. When the nucleotide–peptide complex was treated with alkaline phosphatase, ribonuclease T2, and snake venom phosphodiesterase consecutively, pCp was released along with 3′-nucleotides, which are not found in free RNA. Accordingly, the oligonucleotide is covalently bound to the 125-kDa protein via pCp. Initial structural characterization of the oligonucleotides showed the presence of $GGGC_{OH}$ and $GGGGC_{OH}$ in this structure. The amino acid that bound to pCp was not identified at this moment as the alkaline phosphatase did not cleave all the phosphate in the peptide. A P–O linkage was found in polio virus where the 5′ end of the RNA is linked to viral protein VPg through a phosphotyrosine (Ambros & Baltimore, 1978). The oligonucleotides linked to tryptic peptide 3 may be a breakdown product of a polynucleotide, a point which is currently under further study.

The unique features of the 125-kDa phosphoprotein include (a) exceptionally high labeling and rapid turnover of ^{32}P, (b) nucleolar localization, (c) the presence of RNA oligonucleotides, and (d) low abundance in the nucleolus. These findings raise several questions. Is the 125-kDa protein a product of a posttranscriptional modification in the nucleoplasm or the nucleolus? Is the oligonucleotide an integral part of the 125-kDa protein? Is the 125-kDa protein a transcription factor in the initiation of nucleolar transcription, a factor for processing of 45S pre-rRNA, or a primase involved in DNA replication? It has been reported that a high molecular weight 9S DNA polymerase α from calf thymus and HeLa cells contained a large subunit (Gronostajski et al., 1984; Grosse & Krauss, 1985). The subunit had a similar molecular weight to the 125-kDa protein and had a primase activity which synthesized 8–15 nucleotide long RNA primer. Further at-

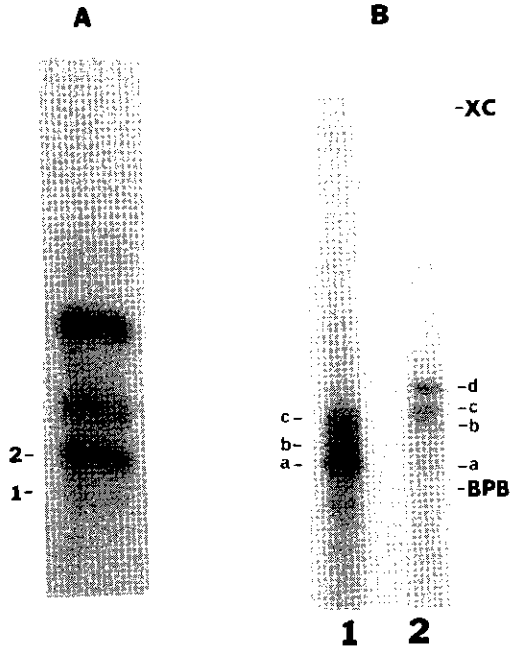

FIGURE 11: [^{32}P]pCp labeling experiment by RNA ligase. (A) 6% reverse SDS–PAGE separation of ^{32}P-labeled peptides digested with trypsin followed by proteinase K. Cold peptides were run along with ^{32}P-labeled peptides, and each band was eluted for the ligase experiment. (B) 20% acrylamide–7 M urea gel electrophoresis of bands 1 and 2 (panel A) after [^{32}P]pCp labeling by RNA ligase: XC, xylene cyanol; BPB, bromophenol blue.

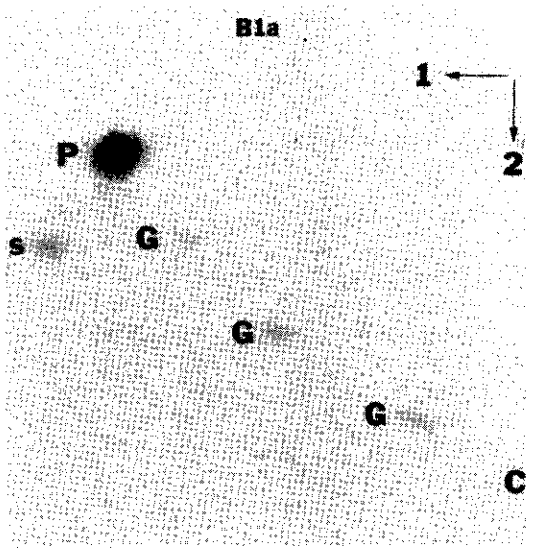

FIGURE 12: Mobility shift analysis of RNA associated with the 125-kDa protein after [^{32}P]pCp labeling. [^{32}P]pCp-labeled bands from Figure 11 were partially hydrolyzed with alkali (pH 10), and electrophoresis was done on cellulose acetate at pH 3.5 (1) followed by homochromatography (2) on a DEAE-cellulose TLC plate. B1a, band a on Figure 11B, lane 1. Band 2d contained $GGGGC_{OH}$. The other bands had more complex patterns.

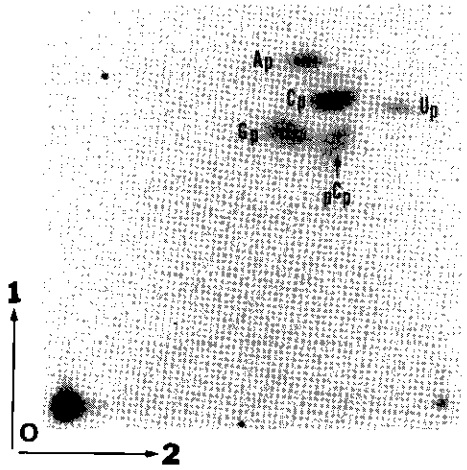

FIGURE 13: Two-dimensional thin-layer chromatography of ribonuclease T2 digest followed by snake venom phosphodiesterase digestion of the nucleotide–peptide complex. First dimension (1), isobutyric acid/0.5 M ammonium hydroxide (50:30); second dimension (2), concentrated HCl/2-propanol/water (15:70:15). Migration of all the separated nucleotides was consistent with that reported by Seno et al. (1968). Ap, adenosine 3′-monophosphate; Cp, cytidine 3′-monophosphate; Gp, guanosine 3′-monophosphate; Up, uridine 3′-monophosphate; pCp, cytidine 3′,5′-diphosphate; O, sample application point.

tempts to evaluate the role of the 125-kDa protein in nucleolar function are in progress.

Registry No. P-Ser, 407-41-0.

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