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Human Acidic Ribosomal Phosphoproteins P0, P1, and P2: Analysis of cDNA Clones, In Vitro Synthesis, and Assembly

BENJAMIN E. RICH* AND JOAN A. STEITZ

Howard Hughes Medical Institute and Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06510-8024

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cDNA clones encoding three antigenically related human ribosomal phosphoproteins (P-proteins) P0, P1, and P2 were isolated and sequenced. P1 and P2 are analogous to *Escherichia coli* ribosomal protein L7/L12, and P0 is likely to be an analog of L10. The three proteins have a nearly identical carboxy-terminal 17-amino-acid sequence (KEESEESD(D/E)DMGFGLFD-COOH) that is the basis of their immunological cross-reactivity. The identities of the P1 and P2 cDNAs were confirmed by the strong similarities of their encoded amino acid sequences to published primary structures of the homologous rat, brine shrimp, and *Saccharomyces cerevisiae* proteins. The P0 cDNA was initially identified by translation of hybrid-selected mRNA and immunoprecipitation of the products. To demonstrate that the coding sequences are full length, the P0, P1, and P2 cDNAs were transcribed in vitro by bacteriophage T7 RNA polymerase and the resulting mRNAs were translated in vitro. The synthetic P0, P1, and P2 proteins were serologically and electrophoretically identical to P-proteins extracted from HeLa cells. These synthetic P-proteins were incorporated into 60S but not 40S ribosomes and also assembled into a complex similar to that described for *E. coli* L7/L12 and L10.

Acidic ribosomal proteins from diverse species have been studied extensively by a number of different techniques. These proteins, called A-proteins (acidic) or P-proteins (the phosphorylated eucaryotic A-proteins), are generally present in multiple copies on the ribosome and have isoelectric points in the range of pH 3 to 5, in contrast to most ribosomal proteins, which are single copy and basic. A-proteins have hydrophobic amino acid compositions (notably about 20% alanine) (34). Because of their distinct chemical properties, A-proteins can be easily dissociated from the ribosome by treatment with high salt and 50% ethanol; the A-proteins remain soluble whereas the remainder of the ribosomes or subunits precipitate (17). This procedure provides a uniform, simple protocol for the identification and isolation of A-proteins from many sources.

The best characterized A-protein is the L7/L12 protein of *Escherichia coli* (L7 is the N-acetylated form of L12 [molecular weight, 12,164]), which has been localized to the stalk of the large (50S) ribosomal subunit (5, 38). L7/L12 interacts with several translation factors, some of which bind and hydrolyze GTP, including initiation factor IF2, elongation factors EF-Tu and EF-G, and release factors RF1 and RF2 (5, 59). L7/L12 is required for binding of these factors as well as aminoacyl-tRNA to the ribosome (5, 59). Hydrodynamic and circular dichroism studies have suggested that L7/L12 and other A-proteins are prolate and have high alpha-helical contents (16). However, the crystal structure of the C-terminal domain of L7/L12 has been determined (28), and it is remarkably globular in light of the elongated shape of the whole molecule. The results of intraribosomal protein-protein cross-linking studies and reconstitution experiments have depicted a complex of two L7/L12 dimers mounted on a single L10 protein (molecular weight, 17,736) which binds directly to 23S RNA (5, 38, 42, 51).

In eucaryotes two slightly different proteins are analogous to *E. coli* L7/L12, based on functional and serological, as well as physical and chemical, criteria (21, 39, 45, 47, 52, 57, 58). Like L7/L12, these two proteins interact with eucaryotic elongation factors EF1 and EF2 and are required for aminoacyl-tRNA binding and EF2-dependent GTPase activity, as well as polypeptide synthesis (31, 32, 45). In *Artemia salina* (brine shrimp), these two P-proteins, eL12 (P2; molecular weight, 11,472) and eL12′ (P1; molecular weight, 11,423), are similar to one another in size and amino acid composition but they are derived from distinct genes (1, 2, 30). They share an identical 22-amino-acid sequence at their C termini that resembles the C-terminal sequences of the other two eucaryotic P-proteins whose sequences have been published, rat P2 (molecular weight, 11,186) (29) and *Saccharomyces cerevisiae* YPA1 (molecular weight, 11,020) (23). The C-terminal sequence contains an epitope that is recognized by autoantibodies from certain lupus patients and by a mouse monoclonal antibody raised against chicken ribosomes (10, 11, 13, 15, 50).

Antibodies reactive with the C-terminal epitope of mammalian P1 and P2 also recognize a neutral phosphoprotein with an M_r of about 37,000 that is found in the large subunit of ribosomes and in the cytoplasm (10, 11, 50). This protein is called P0 because of its similarity to P1 and P2. Based on gel filtration experiments, it has been proposed that P0 is the mammalian equivalent of L10 protein. Elkon et al. (10) have shown that complexes with an M_r of approximately 150,000 containing P0, P1, and P2 proteins can be detected free of ribosomes in cytoplasmic extracts of HeLa cells. Further evidence supporting the analogy between P0 and L10 has recently been obtained from chemical cross-linking experiments with *A. salina* ribosomes, which demonstrated intimate contact between P0 (LA_{33}) and both P1 (eL12′) and P2 (eL12); P1 and P2 homodimers were also detected (56a). In rat liver ribosomes, P0 has also been cross-linked to large-subunit proteins L4, L9, L11, and L12 (not related to eL12) and small-subunit proteins S2 and SA_{30}, as well as to elongation factor EF2 (54–56).

We describe here the isolation and analysis of three cDNA molecules that encode the human P-proteins P2, P1, and P0. The human P1 and P2 cDNA nucleotide sequences and the

* Corresponding author.

deduced amino acid sequences of the proteins are very similar to sequences that have been determined for the corresponding rat, brine shrimp, and yeast proteins and cDNAs. P0 sequences have not been previously described. The proteins encoded by these three cDNAs are functional in that they were incorporated into the 60S subunits of preexisting ribosomes and assembled into complexes similar to those previously described for cell extracts (10).

MATERIALS AND METHODS

Materials. Antisera were the generous gifts of J. Hardin and K. Elkon. Bacteriophage lambda gt11 and associated bacterial strains were provided by R. Young. pGEM3, phage T7 RNA polymerase, and rabbit reticulocyte lysate were obtained from Promega-Biotec. ^{125}I-labeled staphylococcal A protein, [^{35}S]methionine, [^{32}P]dATP, and [^{35}S]dATP were obtained from Amersham Corp. or New England Nuclear Corp. Protein A-Sepharose was purchased from Repligen. Other enzymes and synthetic oligodeoxynucleotides were obtained from New England BioLabs, Inc., Bethesda Research Laboratories, Inc., IBI, Promega-Biotec, Pharmacia, and Boehringer Mannheim Biochemicals. Sephacryl S-300 and protein molecular weight markers were obtained from Pharmacia. Zetabind nylon membrane was purchased from Bio-Rad Laboratories. Reagents were from Sigma Chemical Co., Pharmacia-PL, or Calbiochem-Behring. Rabbit anti-human immunoglobulin G conjugated to alkaline phosphatase was from Sigma.

Isolation of cDNA expression clones. A large human liver cDNA plasmid library (~2.3 × 10^5 recombinants, generously provided by D. Woods) (60) was digested with *Pst*I and S1 nuclease and then inserted into lambda gt11 with *Eco*RI linkers. Approximately 2 × 10^6 plaques were screened with antiserum at 10^5 plaques per 15-cm-diameter plate with duplicate filters by the method of Young and Davis (62). Regions of plates that were positive on both filters were excised, and the phage was eluted for rescreening. This process was repeated until single positive plaques were isolated, and DNA was prepared.

RNA analysis. Cytoplasmic RNA was extracted from HeLa cells by the Nonidet P-40 lysis technique (33), and poly(A)$^+$ RNA was prepared by chromatography on oligo(dT)-cellulose (33). RNA samples were fractionated by electrophoresis in formaldehyde-agarose gels and then capillary blotted onto nitrocellulose filters (33). For dot blot assays, the extracted RNA was applied directly to the nitrocellulose (33). The filters were hybridized with ^{32}P-labeled cDNA fragments and visualized by autoradiography. Alternatively, nitrocellulose filters with DNA adsorbed were hybridized with HeLa cell poly(A)$^+$ RNA, and the selected mRNAs were melted off in water for translation (33).

Isolation of full-length cDNA clones. Restriction fragments derived from the lambda expression clones were labeled to high specific activity by the random hexamer primer method (12) and used to isolate cDNA clones from a full-length fibroblast cDNA library (41) by colony hybridization on filters (33). Full-length clones were selected by agarose gel electrophoresis of excised cDNA inserts.

DNA sequence analysis. DNA sequences were determined by insertion into M13 vectors (36) and analysis by the dideoxy chain termination method of Sanger et al. (48). Staggered subclones for sequencing were generated by sonication (8) or enzymatic deletion (7) (IBI Cyclone System). Sequences were aligned and manipulated by using computer programs of R. Staden (49) and the University of Wisconsin Genetics Computer Group (9) on a DEC VAX 11/750 computer (Yale University Biomedical Computing Unit).

Construction of transcription templates. Restriction fragments derived from full-length cDNAs were inserted into pGEM3 in a configuration such that the T7 promoter directs transcription of an RNA that mimics an mRNA. pT7P0, pT7P1, and pT7P2 are the resulting plasmids containing sequences derived from P0, P1, and P2 cDNAs, respectively. pT7P0 contains a *Dra*I restriction fragment carrying all of the coding sequence of P0 inserted into the *Sma*I site of pGEM3. To construct pT7P1, P1 cDNA from the *Fok*I site (filled with Klenow enzyme to give a blunt end suitable for ligation) to the *Bam*HI site was inserted into pGEM3 between the *Eco*RI site (filled) and the *Bam*HI site. pT7P2 contains P2 cDNA from the *Mlu*I site (filled) to the *Bam*HI site inserted into pGEM3 in the same fashion. Plasmid DNA from each of the constructs was digested with *Bam*HI to create a linear template for runoff transcription. pSP65-CAT (18) (generously provided by J. Izant) was also linearized with *Bam*HI.

In vitro transcription of cDNAs. A 5-μg portion of linearized pT7P0, pT7P1, or pT7P2 was transcribed in vitro by T7 RNA polymerase by the protocol provided by the supplier to yield 10 to 20 μg of RNA per reaction. Linearized pSP65-CAT was transcribed with SP6 RNA polymerase in a similar fashion to generate chloramphenicol acetyltransferase mRNA. The reaction mixtures were extracted with phenol-chloroform, precipitated with ethanol, dried, and dissolved in water.

Translation of RNA transcripts. A 1-μg portion of RNA was denatured in 9 μl of water at 70°C for 5 min. Reticulocyte lysate, amino acids, and [^{35}S]methionine were added to 50 μl, and the reaction mixture was incubated for 1 h at 30°C by the standard protocol provided by the supplier.

Immunoprecipitations. Samples were diluted to 250 μl with TBS (50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl) containing 0.1% Nonidet P-40 and incubated for 2 h at ambient temperature with 5 μl of antiserum preadsorbed to 4 mg of protein A-Sepharose beads. ^{35}S-labeled HeLa cell extract was prepared by sonication of cells grown overnight in the presence of [^{35}S]methionine (37).

SDS gel electrophoresis. Discontinuous 15% polyacrylamide (19:1, mono-bis)-sodium dodecyl sulfate (SDS) gels (0.075 by 15 by 15 cm) (33) were run at 100 V until the dye front passed into the separating gel and then at 10 W until the front left the gel (33). The gel was then either used for blot analysis or dried on filter paper and exposed to film directly.

Western blot (immunoblot) analysis. Gels were electroblotted onto a Zetabind charged nylon membrane as recommended by the manufacturer except that the gels were not preequilibrated with transfer buffer. Antigenic proteins were detected by incubation with antisera and then alkaline phosphatase conjugated to rabbit anti-human immunoglobulin G antibodies followed by reaction with bromochloroindole phosphate and Nitro Blue Tetrazolium (S. Edelstein, Yale University, unpublished data). Radioactive proteins blotted onto the filters were detected by autoradiography.

Gel filtration column chromatography. A mixture of mRNAs adjusted to give equivalent incorporation of [^{35}S]methionine into translation products was incubated in reticulocyte lysate as described above. The mixture was subjected directly to chromatography on a Sephacryl S-300 column (diameter, 7mm; height, 48 cm) in 10 mM $NaPO_4$–150 mM NaCl at 4°C. The flow rate was approximately 5 ml/h, and 15-drop (~300-μl) fractions were col-

lected. Proteins were precipitated with trichloroacetic acid (TCA) and analyzed by SDS gel electrophoresis and autoradiography.

Separation of ribosomal subunits. Translation reaction mixtures as described above were diluted into 10 volumes of 0.5 M KCl–10 mM $MgCl_2$–25 mM Tris hydrochloride (pH 7.5) (TMK-500). Ribosomes were pelleted through cushions of 0.5 and 2.0 M sucrose in TMK-500 in a Beckman 70 Ti rotor at 65,000 rpm (311,000 × *g* at the average radium) at 4°C for 90 min. The ribosomal pellet was resuspended in 0.5 ml of TMK-500 by gentle rocking at 4°C for 1 h. Ribosomes were dissociated into subunits by the addition of puromycin to 0.2 mM and incubation at 37°C for 15 min. Subunits were then separated by velocity sedimentation centrifugation on 10-ml 15 to 30% continuous sucrose gradients in TMK-500. The gradients were centrifuged in a Beckman SW41 rotor at 29,000 rpm (104,000 × *g* at r_{av}) for 12 h at 4°C, and 0.5-ml fractions were collected with a Gilson fraction collector. Ribosomal subunits were located by UV spectroscopy and RNA dot blot analysis with 18S- and 28S-specific hybridization probes derived from plasmid pLvB10 containing rat 18S and 28S rDNA sequences (I. Stroke, Yale University, unpublished data). Portions of each fraction were precipitated with TCA and analyzed by scintillation counting and by SDS gel electrophoresis with autoradiography to localize radiolabeled proteins.

RESULTS

Isolation and analysis of cDNA molecules that encode human P-proteins. Lambda phage clones containing fragments of cDNA were isolated from a human liver cDNA library in lambda gt11 by using serum from a lupus patient which contained anti-P-protein antibodies. Immunologically reactive clones were sorted into three groups by hybridization studies. Determination of the nucleotide sequences of these cDNAs revealed that although the three types of cDNA molecules have different sequences, they could be translated into almost identical carboxy-terminal amino acid sequences (KEESEESD(D/E)DMGFGLFD-COOH). This sequence is quite similar to carboxy-terminal regions of published sequences of P-proteins of rat (P2) (KEESEEKKDEM GFGLFD-COOH) (29), brine shrimp (eL12 and eL12′) (KKEEKKEESEEEDEDMGFGLFD-COOH) (1, 2, 30), and yeast YPA1 (KEEEAKEESDDDMGFGLFD-COOH) (23), all of which are recognized by anti-P-protein antibodies (10, 50). Although these cDNAs are quite small, containing as few as the last 23 codons of the coding sequence, all of the clones generated fusion proteins that were recognized by lupus-patient as well as monoclonal anti-P-protein antibodies (data not shown) (50).

Restriction fragments derived from lambda phage cDNA clones from each of the three groups were used as hybridization probes to screen a human fibroblast cDNA plasmid library that was constructed to maximize the number of full-length clones (41). Representative plasmids from each hybridization group were selected for sequence analysis on the basis of the sizes of their cDNA inserts by agarose gel electrophoresis. The sequences of each of the cDNA molecules were determined by random or deletion subcloning into M13 phage vectors (MP10 and MP11) and by the dideoxy chain termination method. The nucleotide and predicted amino acid sequences for the three potentially full-length cDNA clones are shown in Fig. 1.

By comparison with published sequences of P2 proteins from rats, brine shrimp (eL12), and *S. cerevisiae* (YPA1) (1, 2, 23, 29, 30), we could identify the smallest of the three cDNAs as encoding the human P2 protein. It has 115 codons and predicts a polypeptide with a molecular weight of 11,665. Beginning with the first start codon in the longest open reading frame, the human P2 amino acid sequence can be aligned so as to be 93% identical to that of rat P2, 65% identical to that of *Artemia* P2 (eL12), and 53% identical to that of yeast YPA1, but only 31% identical to the *Artemia* P1 (eL12′) sequence, with most of the identical residues in the C-terminal region of the sequence.

When the predicted amino acid sequence encoded by the other small cDNA clone was compared with the sequence of the *Artemia* P1 protein (el12′), it could be aligned, starting again from the first start codon of the cDNA, to give 61% identity. The human P1 cDNA sequence has 114 codons and predicts a polypeptide with a molecular weight of 11,514. The amino acid sequence exhibits significantly lower homology to the rat, *Artemia*, yeast, and human P2 sequences (29, 34, 26, and 33%, respectively), with the greatest similarity again in the carboxy-terminal region.

Translation of the prominent open reading frame of the third and largest cDNA (P0) revealed little homology to any published protein sequence except in the carboxy-terminal region, which is conserved among the P-proteins. From the first ATG in the open reading frame there are 317 codons, which predict a polypeptide with a molecular weight of 34,273.

In addition to similarities in their predicted amino acid sequences, the nucleotide sequences of the human P1 and P2 cDNAs show similarities to the comparable *Artemia* cDNAs. In their coding regions, the human P2 cDNA sequence is 66% homologous to the eL12 sequence and the human P1 cDNA sequence is 65% identical to the eL12′ sequence. For approximately 100 nucleotides at the 3′ ends of the coding regions all three of the human P-protein cDNAs exhibit 60 to 75% homology with each of the *Artemia* cDNAs, but the 5′ regions are much more specific to P1, P2, or P0.

To estimate the sizes of the human mRNAs encoding P0, P1, and P2, Northern blots (RNA blots) were probed with the three cDNAs (Fig. 2). The right lane of each blot was loaded with 10 μg of HeLa cell poly(A)$^+$ RNA. Poly(A)-containing RNAs of approximately 1,200, 650, and 600 nucleotides were detected by P0, P1, and P2 cDNA probes, respectively, whereas no signal was detected with poly(A)$^-$ RNA (data not shown). The strength of the hybridization signals suggests that the P1 and P2 mRNAs represent roughly 0.01% of the mass of poly(A)$^+$ RNA of HeLa cells, whereas the P0 mRNA appears severalfold more abundant. Translation of poly(A)$^+$ selected by hybridization to P0 cDNA yielded a polypeptide with an M_r of approximately 37,000 that was immunoprecipitable with anti-P-protein serum. Similar experiments using the P1 and P2 cDNAs gave appropriate-size bands corresponding to M_rs of approximately 15,000 and 14,000, respectively (data not shown).

Synthesis of P-proteins from cDNAs in vitro. To confirm that our cDNAs encode the full-length human P0, P1, and P2 proteins, we transcribed the cDNAs and translated the resulting mRNAs in vitro. The first ATG codons of the P1 and P2 cDNA open reading frames were identified as the initiator codons based on phylogenetic homologies. The first ATG codon in the P0 cDNA open reading frame also seemed likely to be the initiator, because neighboring sequences resemble those surrounding the initiators of the two other cDNAs (Fig. 1). We therefore generated plasmid constructs that placed the cDNAs immediately 3′ to a T7 phage RNA

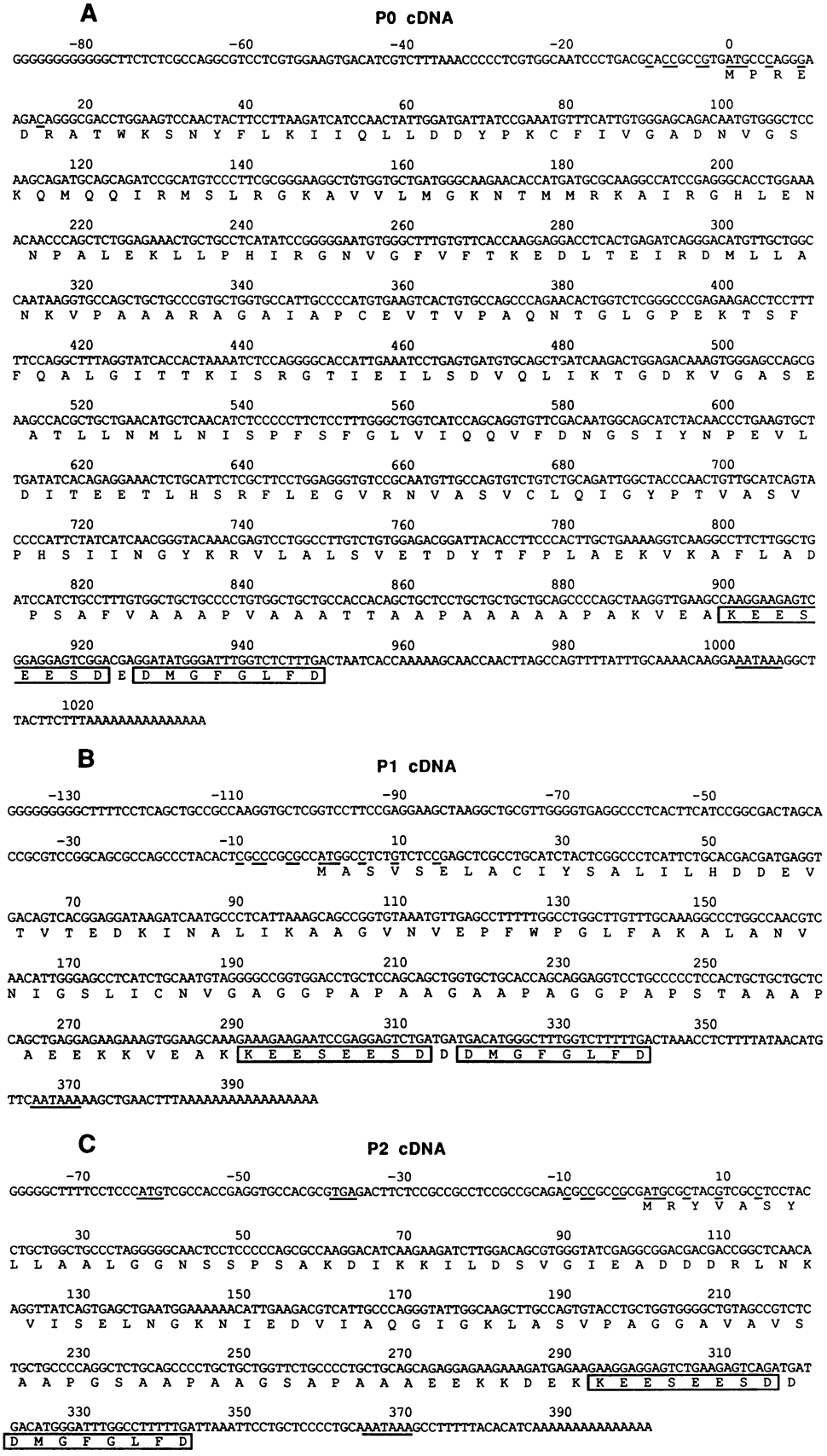

FIG. 1. Nucleotide sequences of P0 (A), P1 (B), and P2 (C) cDNA molecules, with encoded amino acid sequences. The C-terminal amino acids common to each of the three P-proteins are boxed. The polyadenylation signal (AATAAA) of each cDNA and nucleotides common to all three cDNAs in the initiator regions are underlined, as are the upstream ATG and TGA codons of the P2 cDNA.

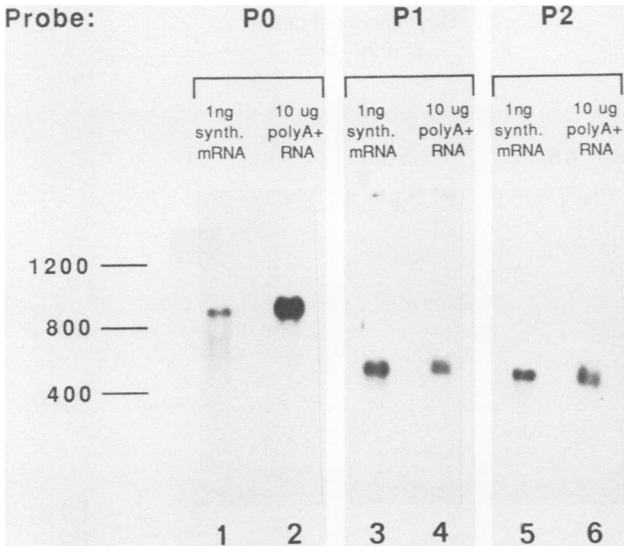

FIG. 2. Northern blot analysis of polyadenylated HeLa cell RNA (lanes 2, 4, and 6) and synthetic (synth.) mRNAs (lanes 1, 3, and 5) hybridized with radiolabeled P0 (lanes 1 and 2), P1 (lanes 3 and 4), or P2 (lanes 5 and 6) cDNA. Mobilities and sizes in nucleotides of single-stranded DNA markers are indicated at the left.

polymerase promoter. After linearization by restriction enzyme digestion at the end of the 3′ untranslated region (P0) or at the end of the short poly(A) tail (P1 and P2), the plasmids were transcribed in vitro with T7 RNA polymerase. The left lane of each blot in Fig. 2 was loaded with 1 ng of the resulting transcript from the corresponding plasmid. These RNA transcripts were comparable in size to the poly(A)$^+$ RNAs extracted from HeLa cells.

Upon incubation in rabbit reticulocyte lysate containing [^{35}S]methionine, each of the synthetic P-protein mRNAs programmed the synthesis of a single prominent polypeptide which was immunoprecipitable with anti-P-protein serum (Fig. 3A). In addition to the major translation product, P0 translation reactions consistently generated a second polypeptide with an M_r of about 25,000 that was not immunoprecipitable with anti-P-protein antibodies and was probably a premature termination product (Fig. 3A, lane 1, and Fig. 3B, lane 2). P1 and P2 synthesized in vitro comigrated with P1 and P2 immunoprecipitated with anti-P-protein serum from HeLa cell extracts labeled with [^{35}S]methionine in vivo; however, the identification of P0 in our anti-P-protein immunoprecipitates of HeLa cell extracts was difficult (compare with references 10 and 13). Moreover, our synthetic P0 was significantly precipitated by the normal control serum and to some extent by protein A-Sepharose alone (data not shown). We attribute these results to limited solubility of P0.

Each of the three in vitro translation products comigrated with a P-protein detected by immunoblotting HeLa 80S ribosomal proteins with anti-P-protein serum (Fig. 3B). These proteins have previously been identified as P0, which migrates at an M_r of about 37,000, and a doublet of P1 and P2 that migrates in the 15,000- to 19,000-M_r range. We conclude that our cDNAs contain the full-length coding sequences for these human ribosomal proteins.

In vitro-translated P-proteins assemble into complexes and specifically associate with 60S ribosomal subunits. Rabbit reticulocyte lysates were programmed with synthetic mRNAs encoding P0, P1, P2, or chloramphenicol acetyltransferase, and the ^{35}S-labeled products were analyzed by gel filtration on Sephacryl S-300 (Fig. 4). Instead of eluting in the fully included fractions of the column, as predicted by their monomeric or dimeric molecular weights, the bulk of the synthetic P-proteins were detected at an intermediate position corresponding to a molecular weight of approximately 150,000. In addition, a significant fraction of the P-proteins were found in the void volume of the column. These positions correlate well with the expected behaviors of a P-protein complex previously described (10) and of whole ribosomes. The fully included fractions contained chloramphenicol acetyltransferase (molecular weight, 25,663) and a small amount of the synthetic P2 protein. This may indicate that there is an excess of P2 in the reaction or that some of the synthetic P2 is somehow defective.

To determine whether the large structures with which the synthetic P-proteins associated were in fact ribosomes, we performed the following experiment. Ribosomes from the translation reaction described above were pelleted through sucrose cushions, suspended in buffer containing 0.5 M KCl, and dissociated with puromycin. The subunits were then separated by centrifugation on a 15 to 30% sucrose gradient. The profiles of A_{260} and TCA-precipitable counts per minute are shown in Fig. 5. Dot blot analyses of 18S and 28S rRNAs

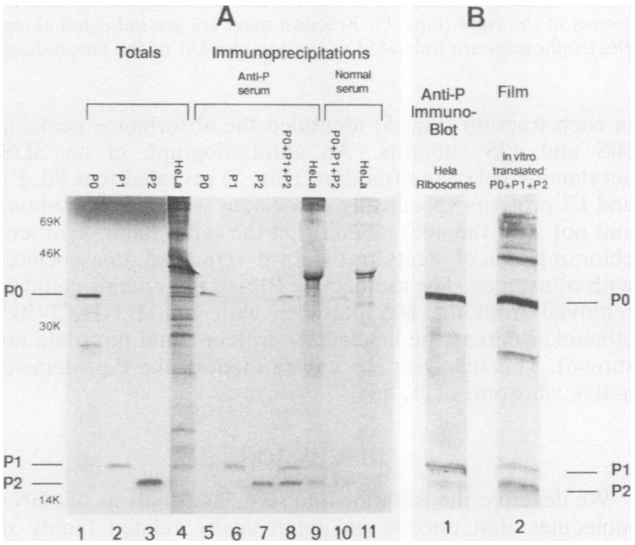

FIG. 3. Analysis of translation products of synthetic mRNAs derived from cloned cDNAs. (A) Translation reaction mixtures containing [^{35}S]methionine, programmed by P0, P1, or P2 cDNA transcripts, and [^{35}S]methionine-labeled HeLa cell extract were resolved by electrophoresis on a 15% acrylamide-SDS gel and visualized by autoradiography (lanes 1 through 4). Portions of the same translation reaction mixtures, a combination of all three reaction mixtures, and HeLa cell extract were immunoprecipitated with anti-P-protein serum (lanes 5 through 9). As a control, the pooled translation reaction mixtures and the HeLa cell extract were immunoprecipitated with serum from a normal patient (lanes 10 and 11). (B) Photograph of anti-P-protein immunoblot of HeLa 80S ribosomal proteins (lane 1) and autoradiograph of the same mixture of P0, P1, and P2 translation products as in panel A in an adjacent lane of the filter (lane 2). An artifact band visible in lane 1 at an M_r of about 30,000 (30K) was also seen when anti-P-protein antibodies were omitted (data not shown). The prominent band between P1 and P0 in lane 2 (and also pane A, lane 1) is probably a premature P0 translation termination product (see Results).

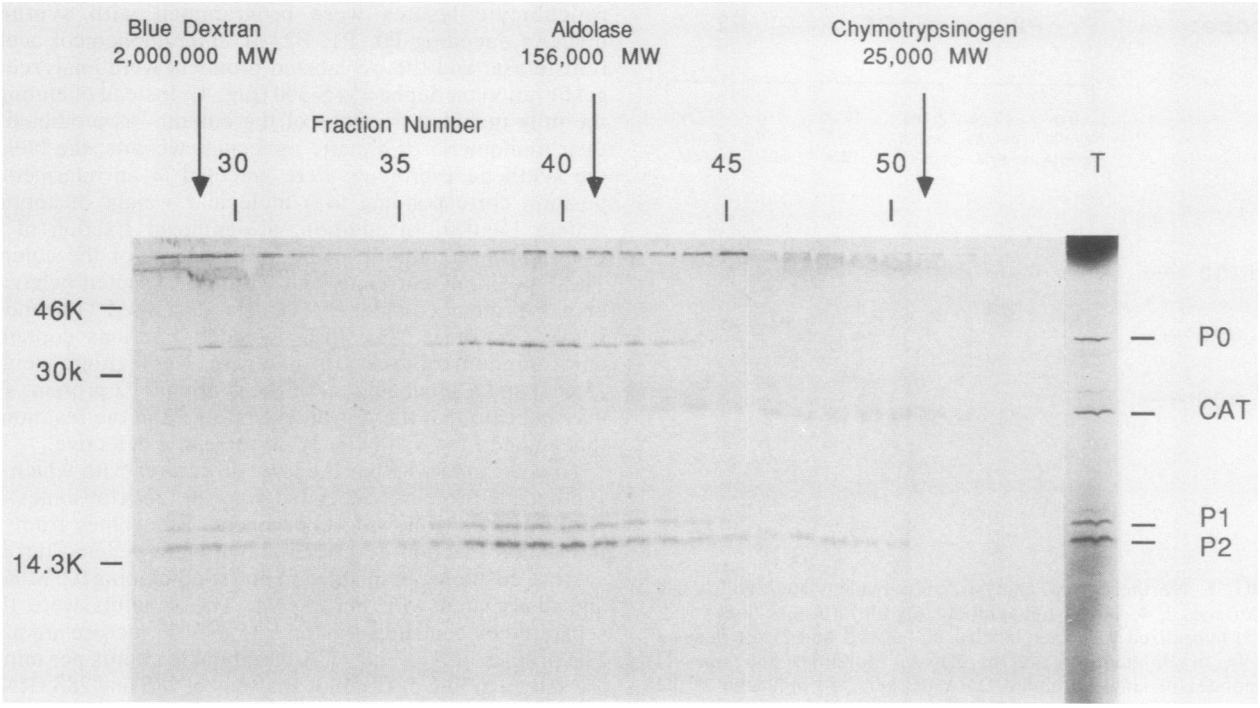

FIG. 4. Autoradiograph of SDS-acrylamide gel of Sephacryl S-300 elution profile of mixture of translation products from reactions programmed by synthetic P0, P1, P2, and chloramphenicol acetyltransferase (CAT) mRNAs. The initial mixture loaded onto the column is shown at the right (lane T). Fraction numbers are indicated along the top of the gel. Molecular weight (MW) markers for gel filtration and electrophoresis are indicated at the top and left of the autoradiograph, respectively.

in each fraction (Fig. 5) identified the absorbance peaks as 60S and 40S subunits. An autoradiograph of an SDS-acrylamide gel of the fractions (Fig. 5) revealed that P0, P1, and P2 proteins specifically associated with the 60S subunit and not with the 40S subunit. On the other hand, synthetic chloramphenicol acetyltransferase remained unassociated with ribosomes. The radioactive P1 and P2 proteins could be removed from the 60S particles with 0.5 M NH_4Cl–50% ethanol, whereas the labeled P0 protein could not (data not shown). This behavior is similar to that of the P-proteins of native ribosomes (11, 56a).

DISCUSSION

We describe the isolation and sequence analysis of cDNA molecules that encode an antigenically related family of three human ribosomal phosphoproteins, P0, P1, and P2. The 5′ untranslated regions of the P0, P1, and P2 cDNAs are 77, 129, and 74 base pairs (bp) long, and the 3′ untranslated regions are 66, 38, and 38 bp long, respectively (Fig. 1). The poly(A) stretches of each of the cDNAs start 18 to 20 bp after the beginning of the hexanucleotide polyadenylation signal AATAAA (43). The initator codons are located in sequences that are somewhat similar to the optimal sequences described by Kozak (25). All three of the initiators have a purine (G) at the critical −3 site. Of note is the upstream ATG at −63 bp of the human P2 cDNA sequence (Fig. 1C). After this potential start codon are eight codons and a TGA terminator. The upstream ATG, with a pyrimidine (C) at position −3, is in a less optimal environment than the one at the beginning of the coding region, so it may not be utilized. Even if initiation occurs at the upstream ATG codon this would not be expected to be a barrier to translation of the P2 sequence because it is followed by an in-frame terminator and the context of the downstream ATG is fairly strong (see reference 25, Fig. 7 and 8). It is possible that the octapeptide MSPPRCHA is produced.

Two of the human P-protein cDNAs are readily identifiable by the extensive homologies of their encoded amino acid sequences to those of similar proteins from rat, brine shrimp, and yeasts and by their homologies to the coding regions of cDNA sequences that have been determined for the two brine shrimp proteins. The protein encoded by the third cDNA, P0, has previously been identified only by gel electrophoresis and immunoblotting. This cDNA encodes a polypeptide sequence that shares features with the other two sequences. First, our data confirm that the antigenically cross-reactive C termini of these three proteins are nearly identical (16 of 17 terminal residues; Fig. 1). Second, common to the P-proteins is an alanine-, glycine-, and proline-rich region of 20 to 30 residues adjacent to the conserved C termini. Third, large portions of the amino acid sequences of each of the P-proteins, including much of the shared sequence, are predicted to form alpha-helical structures by the algorithms of Chou and Fasman (6) or Garnier et al. (14). This is consistent with the observed high alpha-helical content and thermostability of eL12 (16). In SDS-acrylamide gels, P1 and P2 migrated more slowly than expected on the basis of their molecular weights. We found this to be true of P0 as well; although it has a predicted molecular weight of 34,273, it appeared to have a molecular weight of 37,000 to 38,000 by SDS gel electrophoresis (56). This may be due to the unusual secondary structure that these proteins possess.

We used our cDNA clones to develop an experimental system for the synthesis of the three P-proteins by in vitro transcription of the cDNA molecules and subsequent translation of the RNA transcripts. This system allows the synthesis of small amounts of highly radioactive proteins

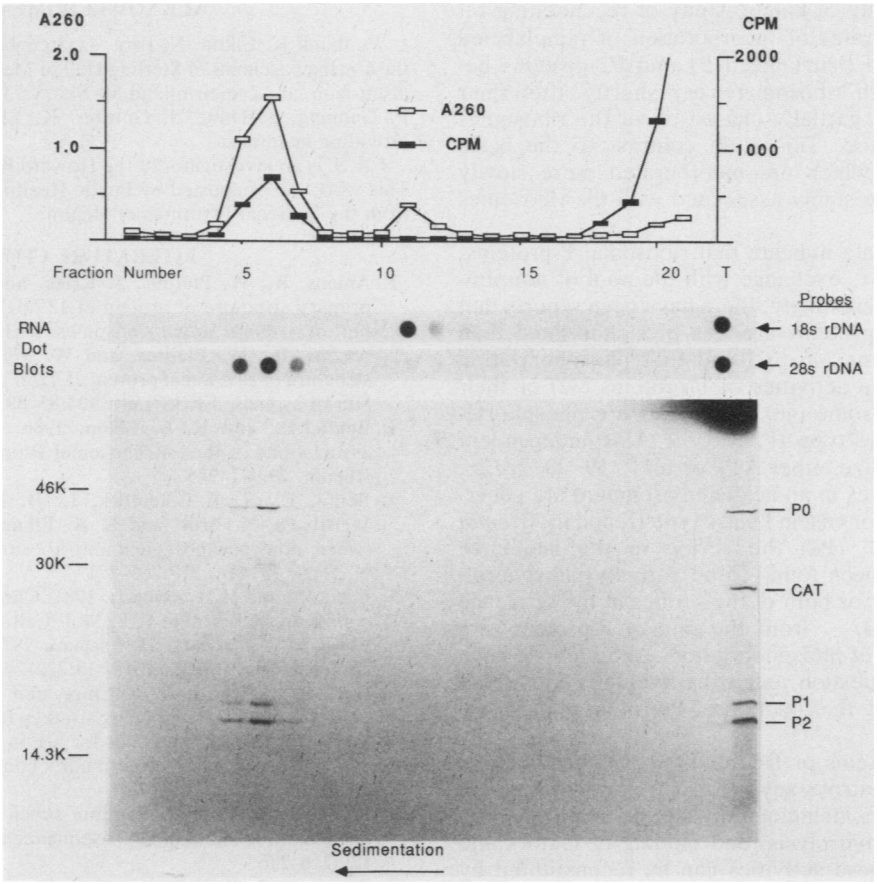

FIG. 5. Analysis of fractions of a velocity sedimentation sucrose gradient containing puromycin-dissociated 60S and 40S ribosomal subunits and radiolabeled synthetic P-proteins. The same mixture of P0, P1, P2, and chloramphenicol acetyltransferase translation products as in Fig. 4 was diluted 10-fold in buffer containing 0.5 M KCl, and the ribosomes were pelleted through sucrose cushions. The ribosomes were dissolved in the same buffer, treated with puromycin, and then sedimented into a 15 to 30% sucrose gradient. The graph at the top shows both the A_{260} (▭) and the TCA-precipitable counts per minute (▬) of portions of each fraction. Immediately below, dot blots of RNA extracted from each fraction hybridized with either 18S- or 28S-specific probes are shown. The large panel at the bottom is an autoradiograph of an SDS-acrylamide gel of the fractions. T, Total unfractionated mixture.

that can be readily detected biochemically. It should be stressed that the quantities of protein that are actually synthesized are very small; in relation to the amounts of rabbit P-proteins present in the reticulocyte lysate, the radiolabeled P-proteins are present in only trace amounts (roughly 1 part in 10^4).

Using this system, we demonstrated that each of the three synthetic P-proteins can assemble onto preexisting 60S subunits of rabbit ribosomes and remain associated with them under stringent salt conditions (0.5 M KCl). The observation that the in vitro-synthesized P1 and P2 were removed from the subunit by extraction with 0.5 M NH_4Cl–50% ethanol, while P0 remained associated with the particle indicates that the interactions between newly synthesized P-proteins and ribosomes mimic those of the in vivo-synthesized components. We also found that the synthetic P-proteins can assemble with rabbit proteins to form a smaller multimeric complex similar to that initially observed by Elkon et al. (10). This complex has been postulated to be a pentamer comprised of one P0 molecule and two molecules each of P1 and P2, similar to the L10-L7/L12 complex that has been described for *E. coli* (42). Such pentamers may be incorporated with little structural alteration into the large subunits of ribosomes.

Although most ribosomal proteins are found exclusively in ribosomes or in the nucleolus, cytoplasmic pools of A-proteins (or P-proteins) free of ribosomes have been observed in *E. coli*, *S. cerevisiae*, *A. salina*, and HeLa cells (10, 40, 46, 57, 63). This suggests that, in contrast to most ribosomal proteins, the acidic ribosomal proteins may be able to exchange on and off of the ribosome in vivo. Zinker and Warner (64) described the exchange of newly synthesized ribosomal proteins onto preexisting ribosomes in a strain of *S. cerevisiae* with a temperature-sensitive defect in rRNA synthesis. Three large-subunit ribosomal proteins were identified as exchangeable because they became selectively labeled by radioactive amino acids in the absence of ribosome synthesis. Two of these, E1 and E3 (also called P1 and P5; M_rs, ~40,000 and ~10,000 to 14,000, respectively) are acidic phosphoproteins that probably correspond to P0 and an unresolved doublet of P1 and P2. In *E. coli*, the presence of L10 is required for incorporation of L7/L12 into ribosomes or large subunits. L7/L12 forms complexes with L10 in vitro, and these may be able to assemble as such onto the 50S subunit (5, 38). Similarly, microinjection of tritiated *Artemia* eL12 protein (probably a mixture of eL12 and eL12′) into stage IV/V *Xenopus* oocytes results in high-salt-resistant incorporation of labeled eL12 into *Xenopus*

ribosomes (24). Finally, a kinetic study of regenerating rat liver that measured rates of incorporation of radiolabeled leucine demonstrated that labeled P1 and P2 proteins become associated with ribosomes very shortly after their synthesis but can be partially chased off of the ribosomes with unlabeled leucine. This is in contrast to the basic ribosomal proteins, which are incorporated more slowly but then remain more stably associated with the ribosomes (53).

All of the above data indicate that ribosomal P-proteins, possibly as a complex, exchange with the pool of nonribosomal P-proteins. Interestingly, there have been reports that the nonribosomal P-proteins are less phosphorylated than those on the ribosome (46, 63) and that phosphorylation seems to increase the activities of P-proteins (32) or their affinities for the ribosome (46). P-proteins are phosphorylated by casein kinase type II, a cyclic AMP-independent kinase which can utilize either ATP or GTP (19, 20, 22, 26, 27, 35). Serine residues in an acidic environment are generally good substrates for casein kinase type II, and in *Artemia* eL12 (P2) or eL12′ (P1) the serine in the sequence . . .EESEE. . . has been found to be partially phosphorylated (1, 2, 30). Either or both of the serines in the sequence . . .EESEESD(D/E)D. . . from the human P-proteins are likely to be the sites of phosphorylation. Although we have not addressed this question, our experimental system may be useful for studying the function of P-protein phosphorylation.

Removal of A-proteins or P-proteins from ribosomes by ethanol extraction destroys several in vitro activities of the ribosomes or subunits including polypeptide synthesis, factor-dependent GTP hydrolysis, and binding of factors and aminoacyl-tRNA. These activities can be reconstituted by adding the extracted proteins back to the stripped ribosomes or subunits (17, 31, 32, 47). Mixing experiments have demonstrated that factor-dependent GTP hydrolysis and aminoacyl-tRNA-binding activities can be recovered in hybrid ribosomes or large subunits composed of either combination of procaryotic or eucaryotic ribosomes and extracted proteins (39, 44, 47). In the microinjection experiments described above, it was found that *E. coli* L7/L12 protein, when coinjected with L10, only weakly interacts with the *Xenopus* ribosomes, being dissociated by a high-salt wash. However, the L7/L12-L10 complex was able, to some extent, to inhibit the incorporation of eL12 into the *Xenopus* ribosomes (24). Furthermore, although it is not obvious by inspection of the primary structures, reports of serological cross-reaction between bacterial L7/L12 and eucaryotic P-proteins (21, 61) indicate that these proteins have remained highly conserved throughout evolution, structurally as well as biochemically. The presence of identical sequences at the C termini of the three eucaryotic P-proteins that have been highly conserved throughout evolution is intriguing. It seems likely that these structures interact with other components of the translation machinery that are also very highly conserved, perhaps by virtue of their central role in the process.

Of interest is the observation of an association between the presence of antibodies which recognize the conserved C termini of the P-proteins and the incidence of psychotic complications in systemic lupus erythematosus patients. Moreover, there are indications that the titer of these anti-P-protein antibodies may rise by as much as 30-fold during critical phases of lupus psychosis (3, 4). In addition to being useful for understanding the structure and function of the ribosome, this work may help to elucidate the disease process of lupus psychosis.

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