Characterization of the Non-histone Nuclear Proteins Associated with Rapidly Labeled Heterogeneous Nuclear RNA*

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Heterogeneous nuclear RNA (HnRNA) is associated with a set of specialized RNA-binding proteins. Mild ribonuclease digestion of intact HnRNA-protein complexes released 15 S ribonucleoprotein (RNP) particles containing poly(A) and its associated protein and 40 S RNP particles containing most of the HnRNA sequences. Highly purified 40 S RNP particles have been obtained from rat liver by centrifugation of nuclear extracts on sucrose density gradients and isopycnic banding on Metrizamide density gradients. These RNP preparations contain 27% of the total HnRNA sequences of rat liver, and appear homogeneous when viewed in negative contrast in the electron microscope and by centrifugation studies using velocity sedimentation in sucrose density gradients or isopycnic banding in density gradients of cesium salts.

Analysis of the proteins in the rat liver 40 S RNP particles by two-dimensional gel electrophoresis demonstrated that the 40 S RNP particle is composed of 12 major protein components with molecular weights ranging from 29,000 to 42,000 which accounted for 75% of the total protein mass and 13 minor protein components with molecular weights greater than 42,000. The proteins in the 29,000 to 42,000 group were fractionated by ion exchange chromatography. The amino acid compositions of the purified protein fractions were strikingly similar and shared several unusual features that distinguish these HnRNA-associated proteins, as a group, from the histones and the non-histone chromosomal proteins. Each of the RNP proteins have basic charge characteristics (pI greater than 8.0) high glycine (25 mol%), low cysteine, and little detectable methionine. Like the histones, the HnRNP proteins are subject to extensive postsynthetic modification. We have identified the unusual amino acid N^2,N^6-(CH_2)_4-L-arginine in acid hydrolysates of some of the RNP proteins and shown that this amino acid arises in vivo by methylation of arginine residues with [3H]methyl groups derived from [methyl-^3H]methionine. Some proteins in the 40 S RNP particle are also subject to modification by phosphorylation of serine and threonine residues, both in vivo and in vitro by protein kinases co-isolating with crude ribonucleoprotein particle fractions. Similar groups of proteins have been observed in 40 S RNP particles prepared from human (HeLa S-3) cells and duck hepatocytes.

Evidence that the proteins co-isolating with HnRNA are closely associated components of a single macromolecular complex was obtained from analyses of the protein aggregates formed following fixation of RNP particles with formaldehyde, glutaraldehyde, or the reversible cross-linking reagent 4-methylmercaptobutyrimidate. Treatment of 40 S particles with each of these reagents resulted in a progressive and coordinate loss of free proteins fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and stabilized a high molecular weight aggregate which appeared homogeneous after electrophoresis in 1% agarose gels containing sodium dodecyl sulfate. Cleavage of the cross-links introduced by reaction with 4-methylmercaptobutyrimidate by reduction with 2-mercaptoethanol released each of the proteins in the original RNP particle fraction. These data support the hypothesis that nascent chains of HnRNA associate with sets of specialized RNA-binding proteins giving rise to repeated globular structures connected by ribonuclease-sensitive strands. The heads-on-a-string organization of HnRNP has obvious analogies to the organization of nucleosomes in DNA strands in chromatin, in both cases, endonucleolytic cleavages give rise to populations of monomer particles of apparently constant protein composition, but containing diverse nucleotide sequences.

Biochemical (1-19) and cytological data (20-25) indicate the heterogeneous nuclear RNA (HnRNA) molecules are associated with non-histone proteins in the nucleus. The HnRNA-protein complexes (HnRNP) are visible in electron micrographs of transcription units as arrays of fibrils of increasing

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† The abbreviations used are: HnRNA, heterogeneous nuclear RNA; RNP, ribonucleoprotein; HnRNP, ribonucleoprotein-containing HnRNA; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; EGT A, ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid; cAMP, adenosine 3':5'-monophosphate.
length emerging from sites of attachment along the DNA strands (24, 27–33). In every tissue examined (including observations on non-nucleolar chromatin from mammalian (24), amphibian (27, 28), and Dipteran (29) cells), the HnRNP fibrils appear as beaded structures composed of regularly spaced globular subunits throughout their lengths (28, 29). Mild ribonuclease treatment strips the nascent HnRNP fibrils from their DNA attachment sites and releases ribonucleoprotein (RNP) particles of 20 nm (26, 30, 31, 33), suggesting that the nuclear ribonucleoprotein fibrils are chains of monomeric RNA protein subunits connected by ribonuclease-sensitive strands.

Further evidence that HnRNA is organized into chains of linked ribonucleoprotein subunits has been obtained from biochemical studies of isolated HnRNP fractions (1–19). If care is taken to inhibit ribonuclease activity, HnRNP complexes may be isolated as heterodisperse structures with sedimentation coefficients as high as 200 S (3, 6, 12–15, 18–20). After mild ribonuclease treatment (or isolation in the absence of ribonuclease inhibitors), most of the ribonucleoprotein sediments as particles of about 40 S (3–5, 9–13). The 3'-terminal poly(A)-containing region of HnRNA is also released by mild ribonuclease treatment of isolated HnRNP fractions, and sediments together with its associated proteins as a particle of about 15 S (8, 10, 14, 17).

Although the original reports suggested that only one or two proteins were present in the 40 S particles (2, 3, 6, 11), later studies indicate greater complexity of the RNA-associated proteins (4, 5, 9, 12–16, 18, 19, 34, 35). Many of the discrepancies in the reported protein compositions of RNP particles could have resulted from variations in the isolation procedures for RNP particles used in different laboratories, and from failures to distinguish adequately between the structural proteins associated with the 40 S monomer particle, proteins which may be transiently associated with the undergraded HnRNP fibrils and contaminating proteins associated with other nuclear structures such as chromatin fragments or pre-ribosomal particles. In this paper we present evidence that HnRNP structures are maintained by a set of specialized RNA-binding proteins with molecular weights ranging from 29,000 to 42,000. Our observations are based on new methods for purification of 40 S RNP particles from rat liver, which include the use of isopycnic centrifugation of HnRNP particles in Metrizamide density gradients, in order to minimize the possibility of contamination of the RNP particles by other structures of similar size.

The highly purified RNP monomer particles we have obtained appear homogeneous when viewed in negative contrast in the electron microscope and by centrifugation studies using velocity sedimentation in sucrose density gradients or isopycnic banding in density gradients of cesium salts, as well as by agarose gel electrophoresis following fixation with bifunctional reagents. Analyses of the proteins in the purified RNP particles by two-dimensional gel electrophoresis show 25 components, but 75% of the protein mass of the RNP monomer particle is comprised of only 12 proteins with molecular weights ranging from 90,000 to 47,000.

The proteins in the 29,000 to 42,000 molecular weight group were fractionated by ion exchange chromatography and shown to have closely related charges and amino acid compositions, which distinguish them from the histories and the non-histone chromosomal proteins. The HnRNA-associated proteins are unusually rich in modified amino acids and display high contents of Nε, Nε (CH2)6-L-arginine, phosphoserine, and phosphothreonine. Estimates of the relative abundance of the HnRNA-associated proteins permit construction of a model for the protein structure of the 40 S particle in which each protein is present in stoichiometric amounts. These observations on the molecular organization of 40 S particles and the chemistry of the RNP proteins are consistent with the hypothesis that nascent HnRNA molecules become associated with a set of specialized RNA-binding proteins, giving rise to repeated globular structures of 20 nm along the RNA chains.

**EXPERIMENTAL PROCEDURES**

*Extraction of Ribonucleoprotein Particles from Rat Liver Nuclei*

Rat liver nuclei were purified from homogenates of 100 g (wet weight) liver by centrifugation through 2.5 M sucrose density cushions (30). HnRNP particles were extracted from the isolated nuclei by a procedure similar to that described by Samarina et al. (1–3). Because the purity of the extracted RNP particles is critically dependent on the constituents of the extraction buffers and handling of the nuclei (to avoid lysis and the consequent release of chromosomal nuclear acidic proteins) the method is described in detail below. Nuclear pellets containing a total of 1014 nuclei were washed three times with 60-ml portions of Buffer A (100 mM NaCl, containing 10 mM triethanolamine/HCl, pH 7.6, 3 mM MgCl2, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) and once with 60 ml of Buffer B (100 mM NaCl, containing 10 mM triethanolamine/HCl, pH 8.0, 3 mM MgCl2, 1 mM dithiothreitol, and 0.1 mM PMSF). Nuclei were recovered by centrifugation at 50,000 × g for 5 min. Pellets containing the washed nuclei were resuspended in 4.5 ml of Buffer B, stirred gently for 30 min at 0–4℃, and centrifuged at 50,000 × g for 5 min, saving the extract. Three more 30-min extractions with Buffer B were carried out. The four pH 8.0 extracts were combined, clarified by centrifugation at 200,000 × g for 10 min, and 3-m aliquots were applied to linear 15 to 30% sucrose gradients in Buffer B (39 ml). After centrifugation at 23,000 rpm for 15 h in a SW 27 rotor (Beckman Instruments, N. Y.), the gradients were collected by displacement with 60% sucrose. Addition of the reducing agent dithiothreitol to the extraction buffer substantially reduced contamination of the 40 S RNP particles by high molecular weight nuclear acidic proteins, probably because aggregation of proteins in the formation of the fiber bridge is minimized. PMSF is included in the buffers as a protease inhibitor.

In some experiments, the rat liver RNA was labeled in vivo with [5-3H]orotic acid (17.8 Ci/mmol) or [32P]orthophosphate (carrier-free). Proteins were labeled in vivo with L-[3H]leucine (50 Ci/mmol), or [14C]methionine (2.4 Ci/mmol), or [32P]orthophosphate (carrier-free). All radioactive precursors were from New England Nuclear, Inc., Boston, Mass.

*Isopycnic Banding of Nuclear RNP Particles in Metrizamide Gradients*

Fractions containing the 40 S particles from sucrose gradients were dialyzed overnight against Buffer C (50 mM sodium phosphate, pH 7.0, containing 10 mM NaCl, 1 mM MgCl2). Aliquots (8 ml) of the dialyzed sample were applied to discontinuous gradients of Metrizamide (2-3-acetamido-2,4,6-triodobenzamide-5'-amido-2 deoxy d-glucose (Nygard and Sons, Oslo) composed of 5 ml of 60% (w/v) Metrizamide in Buffer C (refractive index (R.I. = 1.421) under 10 ml of 4% Metrizamide in Buffer C (R.I. = 1.398) centrifuged at 40,000 rpm in a Ti 60 rotor (Beckman Instruments) for 60 h. In parallel experiments testing the effects of high salt concentrations on the stability and composition of the particles, 0.3 M NaCl was included in all buffers. The Metrizamide gradients were monitored for trichloroacetic acid-precipitable radioactivity and the densities of every third fraction was determined from the refractive index (37) using the formula μ = 3.560 (R.I. - 1.333).

*Preparation of Nuclear RNP Particles from Other Tissues and Species*

**Duck Liver** — 40 S RNP particles were prepared from the livers of 3-month-old Pekin ducks by successive extractions (or sonication)
of nuclei in Buffer B, following centrifugation of the nuclear extracts in linear 15 to 30% sucrose gradients, as described above.

HeLa Cells - Ribonucleoprotein particles were prepared from 2 x 10^7 HeLa S-31 cells maintained in suspension culture and labeled for 2 h by incubation with 20 mCi of [^3H]orthophosphate in the presence of actinomycin D (added to suspend ribosomal RNA synthesis) (38). The cells were lysed by Dounce homogenization in 30 ml of Buffer D (10 mM Tris/HC1, pH 7.2, 10 mM NaCl, 1.5 mM MgCl₂ and the nuclei purified by centrifugation through 2.2 M sucrose cushions (in 10 mM Tris/HC1, pH 7.2, 0.5 mM EDTA) at 25,000 rpm for 60 min in a SW 27 rotor. The purified nuclei were fractionated into chromatin, nucleoli, and ribonucleoprotein fractions by the method of Kiah and Pederson (14). The RNP fraction was dialyzed against Buffer E (100 mM NaCl, 10 mM Tris/HC1, pH 7.2, 1 mM MgCl₂) to remove sucrose and aliquots were treated for 30 min at 90°C with 0.1 ml of pancreatic ribonuclease ( Worthington) to generate 40 S RNP particles which were purified on linear 15 to 30% sucrose gradients in Buffer E at 23,000 rpm in a SW 27 rotor for 15 h. HeLa polyomaviruses were prepared as previously described (39).

Cross-linking Experiments on Nuclear RNP Particles Purified 40 S RNP particles from sucrose gradients were dialyzed against 100 mM NaCl containing 10 mM trithionamide/HC1 (pH 8.0), 1 mM MgCl₂, and 10% glycerol, and adjusted to a concentration of 2.0 A₂₆₀ units/ml. Aliquots were treated for 45 min at 25°C with either glutaraldehyde or formaldehyde at the concentrations listed in the legend to Fig. 7A. At the end of the reaction period, the samples were made 1% in SDS, 0.01% in bromophenol blue, and brought to 100°C for 2 min. The samples were analyzed by electrophoresis in 8.75 to 14% polyacrylamide gels described as below, or in 1/10% agarose gels (10 mM sodium phosphate buffer, pH 7.1, 0.1% SDS, electrophoresed for 4 h at 70 V).

For reversible cross-linking of RNP proteins, aliquots of 40 S particles were treated for 30 min at 25°C with increasing concentrations of 4-methylmercaptobutyrimidate (40, 41) (Polysciographic fractionations (42). The reaction mixtures were made 1.0 M in KCl. The column was eluted with a 400-ml linear gradient of 0-1 M (4.5 x 10⁶ cpm/pmol at the time of the reaction shown in Fig. 13). After 5- or 15-min incubations at 90°C, the reactions were terminated by addition of 0.2% (w/v) SDS. Reactions were allowed to proceed in the presence or absence of 10⁻¹⁵ M cAMP and in the presence or absence of 2.0 µg of partially purified cAMP-dependent protein kinase from calf thymus (gift of Dr. E. M. Johnson, The Rockefeller University). The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 13).

Detection of N⁶,N¹⁰(CH₃)₂ Arginine The proteins from 40 S RNP particles labeled in vitro with [^3H]orthophosphate for 60 min, were eluted from column (1.7 x 25 cm) of DEAE-Sephadex A-25 equilibrated with Buffer F. The protein was then obtained from the DEAE-Sephadex columns was eluted, dialyzed exhaustively against H₂O, lyophilized, and hydrolyzed under vacuum for 6 h at 110°C in 6 N HC1. Radioinactive inactive phosphate, phosphoserine, and phosphothreonine were separated by chromatography on an AA-15 column (0.9 x 20 cm) equilibrated and eluted with 0.05 M HCl. The column was eluted under pressure at a flow rate of 40 ml/h and fractions of 1 ml were collected and monitored for radioactivity.

Electrophoretic Analyses Proteins associated with RNP particles and other nuclear fractions were characterized first by size separation in linear 8.75 to 14% SDS-polyacrylamide gradient slab gels (1.5 x 150 x 170 mm) using the discontinuous buffer system of Laemmli (43). The protein bands were visualized after staining in 0.1% Coomassie brilliant blue R-250 in 50% ethanol, 7.5% acetic acid, and subsequent destaining in 2.5% acetic acid.

Two-dimensional gel electrophoretic analyses of nuclear proteins were based on mobility differences in acid-urea gels (46) combined with size separations in SDS-polyacrylamide gradient gels in the second dimension. The first-dimension separating gels (0.75 x 150 x 170 mm) contained 7.5% acrylamide in 6 μm urea, 0.06 N NaOH adjusted to pH 4.3 with acetic acid, and the overlying stacking gels contained 3% acrylamide in 6 μm urea, 0.06 N NaOH adjusted to pH 6.8 with acetic acid. The running buffer was 0.07 M β-alanine adjusted to pH 4.5 with acetic acid. Samples were applied in 8 μm urea, 1% T-mercaptoethanol, 0.06 N NaOH (adjusted to pH 8.8 with 6% acetic acid) after reductive alkylation with N-ethylmaleimide. After electrophoresis at 7.5 mA for 24 h, the gels were cut into vertical strips, some of which were stained while others were transferred to size-separation gels, as follows: the unstained strips were immersed in 0.125 M Tris/HC1 (pH 6.7), 0.1% SDS for 2 h, then sealed to SDS-polyacrylamide gradient gels (described above), using 1% agarose in sample buffer. After electrophoresis at 12.6 mA for 17 h, the protein spots in the two-dimensional gels were stained with Coomassie brilliant blue R-250.

Chemical Analyses Protein was determined by the method of Lowry et al. (47), using bovine serum albumin as a standard. DNA was determined by the Burton modification of the diphenylamine reaction (48) and RNA was measured by absorbance at 260 nm, or by the orcinol reaction based on mobility differences in acid-urea gels (46) combined with size separations in SDS-polyacrylamide gradient gels in the second dimension. The first-dimension separating gels (0.75 x 150 x 170 mm) contained 7.5% acrylamide in 6 μm urea, 0.06 N NaOH adjusted to pH 4.3 with acetic acid, and the overlying stacking gels contained 3% acrylamide in 6 μm urea, 0.06 N NaOH adjusted to pH 6.8 with acetic acid. The running buffer was 0.07 M β-alanine adjusted to pH 4.5 with acetic acid. Samples were applied in 8 μm urea, 1% T-mercaptoethanol, 0.06 N NaOH (adjusted to pH 8.8 with 6% acetic acid) after reductive alkylation with N-ethylmaleimide. After electrophoresis at 7.5 mA for 24 h, the gels were cut into vertical strips, some of which were stained while others were transferred to size-separation gels, as follows: the unstained strips were immersed in 0.125 M Tris/HC1 (pH 6.7), 0.1% SDS for 2 h, then sealed to SDS-polyacrylamide gradient gels (described above), using 1% agarose in sample buffer. After electrophoresis at 12.6 mA for 17 h, the protein spots in the two-dimensional gels were stained with Coomassie brilliant blue R-250.
Nuclear Proteins Associated with HnRNA

(49). Unless otherwise specified, radioactivity measurements were made by collecting 5% trichloroacetic acid-precipitable material on Millipore (0.45 μm) filters, washing with 10-ml portions of 5% trichloroacetic acid followed by 100% ethanol. Samples labeled with \([{}^{32}P]\)orthophosphate were washed with 5% trichloroacetic acid containing 0.1 M NaCl and 0.01 M sodium pyrophosphate.

Proteins from the phosphocellulose columns were concentrated for amino acid analyses after dialysis against water by lyophilization. The samples were degassed, sealed under vacuum, and hydrolyzed for 18 h at 110°. The hydrolysates were lyophilized and analyzed on either a Beckman 120 B analyzer modified for a 10-fold increase in sensitivity with a Honeywell scale expander or on a Durrum amino acid analyzer (50).

RESULTS

Isolation of 40 S HnRNP Particles from Rat Liver Nuclei – The procedure of Samarina et al. (1-3) has been modified for large scale purification of HnRNP particles from rat liver nuclei. The nuclei, purified by centrifugation through a sucrose density barrier, were incubated at pH 8.0. This leads to cleavage of the perichromatin RNP fibrils by endogenous endoribonuclease activity and to a release of HnRNP complexes that were subsequently isolated as 40 S particles by velocity centrifugation in sucrose density gradients. Further purification of the RNP fraction by isopycnic centrifugation in Metrizamide density gradients yields milligram quantities of HnRNP particles with characteristic density, morphology, and protein composition.

The distribution of RNA, protein, and DNA at successive stages in the fractionation of hepatocyte nuclei is shown in Table I. Incubation of nuclei at pH 8.0 leads to release of about 45% of the total nuclear RNA together with 12% of the nuclear protein, but less than 4% of the original DNA content of the purified nuclei (Table I). Since more than 80% of the RNA remaining in the insoluble fraction is associated with nucleoli and probably represents ribosomal RNA sequences, we conclude that the pH 8.0 extract contains over 80% of the newly synthesized nonribosomal RNA molecules. This estimate is in close agreement with the original data of Samarina et al. (1) who reported that nearly 80% of the nuclear RNA of DNA-like base composition is extractable from rat liver nuclei in hypotonic buffers.

Ribonucleoprotein particles were isolated from the nuclear extracts by centrifugation in 15 to 30% sucrose density gradients. Fig. 1 shows the distribution of RNA and protein labeled in vivo with \([{}^{32}P]\)orthophosphate for 1 h, \([{}^{3}H]\)orotic acid for 2 h, and with \([{}^{3}H]\)leucine for 20 h, in the sucrose gradient. Over 80% of the \([{}^{3}H]\)-labeled RNA, 65% of the \([{}^{32}P]\)-labeled nucleic acid, and nearly 40% of the radioactive proteins

| Fraction analyzed | Volume | Protein | DNA (mg) | % total | RNA (mg) | % total | Protein/RNA |
|-------------------|--------|---------|----------|--------|----------|--------|------------|
| Homogenate        | 720    | 18,000.0| 224.4    | 100.0  | 36.0 × 10^6| 100.0  | 72.2       |
| Nuclei sedimented through 2.2 M sucrose | 50 | 505.0 | 130.2 | 58.0 | 23.63 | 42.4 × 10^6 | 67.2 |
| Nuclear washes    |        |         |          |        |          |        |            |
| pH 7.0            | 91     | 62.8    | 2.8      | 0.9    | 0.05    | 8.0 × 10^4 | 0.3 |
| pH 8.0            | 40     | 14.8    | 0.8      | 0.4    | 0.05    | 8.1 × 10^4 | 0.3 |
| * Nuclear extract, pH 8.0 | 24 | 53.7 | 5.2 | 2.3 | 10.65 | 11.7 × 10^6 | 32.5 | 5.95 |
| Nuclear residue   | 50     | 350.0   | 127.5    | 66.8   | 8.01    | 19.0 × 10^6 | 20.8 |
| Sucrose gradient fractions |      |         |          |        |          |        |            |
| 40 S              | 45     | 23.4    | n.d.     | 0      | 6.17    | 6.8 × 10^4 | 19.0 | 3.79 |
| 12-20 S           | 16     | 5.1     | n.d.     | 0      | 0.25    | 2.8 × 10^4 | 0.8 |
| Metrizamide gradient fraction | 21 | 14.7 | n.d. | 0 | 3.98 | 4.1 × 10^4 | 10.8 | 3.70 |

\(a\) Radioactivity incorporated into RNA after 2 h labeling in vivo with [5-\(H\)]orotic acid (2 mCi/kg body weight).

\(b\) Expressed as per cent of total DNA in the original homogenate.

\(c\) n.d. = not detectable.

![Figure 1](http://www.jbc.org/)

Fig. 1. Isolation of rat liver 40 S ribonucleoprotein particles from nuclear extracts by sucrose gradient centrifugation. RNA was labeled by intraperitoneal injections of 200 μCi of [5-\(H\)]orotic acid, 2 h before killing, or 1 mCi of [\({}^{32}P\)]orthophosphate 1 h before killing. Protein was labeled over a 20-h period in rats receiving three successive intraperitoneal injections of [4,5-\(H\)]leucine at 6-h intervals. Aliquots of nuclear extracts in Buffer B were applied to 39-ml linear 15 to 30% sucrose gradients and centrifuged for 15 h at 23,000 rpm using a SW 27 rotor. The gradients were fractionated by displacement with 60% sucrose and the absorbance at 240 nm and trichloroacetic acid-precipitable radioactivity monitored. Bars indicate fractions of the sucrose gradients containing material sedimenting at 12 to 20 S and at approximately 40 S. --- \(1\) --- \(2\), radioactivity, [5-\(H\)]orotic acid; --- \(3\), radioactivity, [\({}^{32}P\)]orthophosphate; --- \(4\), radioactivity, [4,5-\(H\)]leucine; --- \(5\), [\({}^{32}P\)]orthophosphate, --- A, A, A.
Nuclear Proteins Associated with HnRNA are recovered in a peak of UV absorbing material with a sedimentation coefficient of approximately 40 S. The particulate nature of this material is evident in electron micrographs which reveal a relatively uniform population of discrete particles approximately 20 nm in diameter (Fig. 2). No contamination of these particles by chromatin, membraneous material, or ribosomal subunits has been observed and no DNA was detectable in the 40 S peak region by the diphenylamine reaction. Direct chemical analysis showed that nearly 25% of the total nuclear RNA can be recovered in the form of 40 S particles containing about 5% of the total nuclear protein (Table I).

In addition to the 40 S RNP particle peak, a smaller peak of \(^{32}P\) activity and UV absorption occurs in the 12 to 20 S region of the gradient (Fig. 1, lower panel). Particles of this size have been shown to contain the 3′-terminal poly(A)-rich sequences of newly synthesized HnRNA chains (10, 14). The relative increase in the labeling of the 12 to 20 S peak with \(^{32}P\)orthophosphate compared to the labeling of the 12 to 20 S peak with \(^{3}H\)orotic acid (Fig. 1, upper panel) is consistent with the presence of \(^{32}P\)phosphate in pyrimidine-deficient poly(A)-containing RNA fragments.

Further purification of the 40 S HnRNP particles was achieved by centrifugation in gradients of Metrizamide. Fig. 3A shows the distribution of radioactivity in 40 S particles doubly labeled in vivo with \(^{32}P\)orthophosphate and \(^{3}H\)leucine and centrifuged to equilibrium in Metrizamide gradients of low ionic strength. Most of the \(^{32}P\) and \(^{3}H\) activity bands as a sharp peak of buoyant density at 1.29 g/cm\(^3\). Purification of the HnRNP particles in this way is accompanied by a loss of 10 to 20% of the \(^{3}H\)leucine-labeled protein present in the 40 S peaks from sucrose gradients. These proteins are recovered as a small peak of \(^{3}H\)leucine-labeled material banding at 1.38 g/cm\(^3\). After Metrizamide gradient centrifugation the RNP fraction contains protein and RNA in a ratio of 3.70 to 1, and more than 10% of the total hepatocyte RNA rapidly labeled in vivo with \(^{3}H\)orotic acid (or 27% of the total HnRNA (Table I)).

Bandng RNP particles in Metrizamide gradients of low ionic strength does not substantially alter either the sedimentation coefficient of the 40 S particles (data not shown) or the equilibrium buoyant density of the particles in gradients of cesium salts (see below), indicating that the 40 S RNP particle structure remains intact throughout the isolation procedure.

Characterization of Proteins Co-purifying with HnRNA by Polyacrylamide Gel Electrophoresis - The proteins appearing in nuclear subfractions at successive stages in the preparation of HnRNP particles have been characterized by electrophoresis in SDS-polyacrylamide gradient gels. Complex mixtures of proteins ranging in molecular weight from less than 20,000 to more than 150,000 are released during the washing of the isolated nuclei (data not shown). The subsequent extractions at pH 8.0 remove the bulk of the nuclear HnRNP particles, together with other nuclear proteins which were separated by sucrose density gradient centrifugation (Fig. 4a). About 35 protein species ranging in molecular weight from 25,000 to glutaraldehyde was removed by dialysis against the original buffer, and samples were applied to carbon-coated Formvar grids, stained with 1% neutralized phosphomolybdate, and examined in a Siemens Elmskop model 102. Bar indicates 0.1 \(\mu m\). Micrograph courtesy of Dr. H. P. Hoffmann, The Rockefeller University.

![Fig. 2. Visualization of rat liver nuclear ribonucleoprotein particles by negative staining. Aliquots of rat liver 40 S RNP particles, purified by sucrose gradient centrifugation, were dialyzed against 50 mM PO\(_4\) buffer, pH 7.6, 1 mM MgCl\(_2\), and fixed by dialysis against phosphate buffer containing 1% glutaraldehyde. Excess glutaraldehyde was removed by dialysis against the original buffer, and samples were applied to carbon-coated Formvar grids, stained with 1% neutralized phosphomolybdate, and examined in a Siemens Elmskop model 102. Bar indicates 0.1 \(\mu m\). Micrograph courtesy of Dr. H. P. Hoffmann, The Rockefeller University.](http://www.jbc.org/Downloaded from http://www.jbc.org/).
over 150,000 co-sediment with RNA in the 40 S RNP particle fraction. Of these proteins more than 70% are represented by a group of proteins of molecular weight from 29,000 to 42,000 (Fig. 4b). The proteins of this group are in constant relative proportions in replicate preparations and the same proportions are observed whether the relative amount of each band is determined by densitometry of gels stained with Fast Green, or Coomassie brilliant blue R-250 or of autoradiographs of ³²P-labeled protein bands. By each of these methods, the major proteins that co-purify with HnRNA as 40 S particles appear as a unique and constant subset of the total proteins present in the pH 8.0 extract (compare Fig. 4, a and b). Other proteins mainly of molecular weight greater than 42,000 distribute characteristically elsewhere on the sucrose gradients, some of them appearing in the 12 to 20 S particle region (e.g. Fig. 106).

All the proteins in the 29,000 to 42,000 molecular weight group remain tightly associated with RNA during centrifugation of the RNP particles in Metrizamide density gradients; however, many of the proteins of higher molecular weight are separated from the RNP particles (compare Fig. 4, b and c) and observed to band at a density corresponding to that of free protein (Fig. 3). It seems likely that the major proteins co-purifying with RNA through the Metrizamide gradients are structural proteins required for the assembly of the 40 S complex. Treatment of the complex with pancreatic ribonuclease (0.01 µg/ml) for 30 min at 4° or exposure to nonionic detergents such as Triton X-100 or Brij 35 (0.1% (v/v)), and centrifugation on gradients of sucrose or Metrizamide does...
not alter either the proportions or numbers of the proteins in the complex. In contrast, exposure of the HnRNP particles to high ionic strength (see below) or to protein-denaturing conditions (such as concentrated urea or ionic detergents) permits selective solubilization of some of the proteins (2, 3, 13, 18) but results in disaggregation of the 40 S particles.

The association of a characteristic subset of nuclear proteins with HnRNA molecules does not appear to be an artifact of a particular method of nuclear fractionation. We have prepared 40 S particles by three other methods; involving sonication, hypotonic lysis, and hypertonic lysis of isolated nuclei, followed by centrifugation of the released particles in sucrose gradients. Although recoveries of 40 S particles varied somewhat with the method used, the electrophoretic banding patterns of the proteins in the 40 S particles prepared by all four methods are virtually indistinguishable (data not shown).

Two-dimensional Gel Electrophoresis of the Protein Components of Purified HnRNP Particles – A better estimate of the complexity of the protein complement of the 40 S HnRNP particles isolated by Metrizamide gradient centrifugation was obtained by two-dimensional gel electrophoresis. The HnRNP proteins (solubilized in urea) are separated according to charge by electrophoresis in denaturing gels at pH 4.3, and further fractionated by electrophoresis using polyacrylamide gels containing 0.1% SDS, as shown in Fig. 5. Twenty-five proteins are reproducibly resolved; they have been numbered consecutively in order of increasing molecular weight. It is significant that protein bands which appear to contain only one or two components in charge separating gels show higher orders of complexity in the two-dimensional gel system. Thus, the major subset of proteins in the 29,000 to 42,000 molecular weight class (75% of the protein mass of the Metrizamide gradient-purified HnRNP particles) all have very similar mobilities in denaturing gels at pH 4.3. (This accounts for earlier reports on the apparent simplicity of the HnRNA-associated proteins (e.g., 3, 6).) The two-dimensional gel separations show this group of proteins to include five major species (Numbers 4, 5, 6, 8 and 9) and seven minor species (Numbers 1, 2, 3, 7, 10, 11, and 12) (Fig. 5).

HnRNP Particles Are Macromolecular Complexes Stabilized by Electrostatic Interactions Between RNA and Proteins – The close association between 32P-labeled HnRNA and a characteristic subset of [3H]leucine-labeled proteins persists throughout centrifugation of the unfixed 40 S particles in neutral sucrose or Metrizamide gradients provided the ionic strength of the medium is kept low (Fig. 3A, Fig. 4c), but raising the salt concentration to 600 mM NaCl leads to a separation of the particles and release of the RNA-associated proteins. Analysis of the doubly labeled 40 S particles in Metrizamide gradients containing 600 mM NaCl shows a separation of the 32P-labeled nucleic acids, which band at 1.25 g/cm³, a buoyant density which corresponds to that of free RNA in Metrizamide (37), from the [3H]leucine-labeled proteins, which band at a density of 1.33 g/cm³ (Fig. 2B).

The stability of the HnRNP particles at low ionic strength and their dissociation at high salt concentrations was confirmed by isopycnic banding of the peaks from Metrizamide gradients in density gradients of Cs₂SO₄ or CsCl (Fig. 5). HnRNP particles, labeled in vivo with nucleotide precursors were purified in sucrose gradients and fixed with glutaraldehyde before centrifugation in gradients of cesium salts. The 40 S particles, unable to dissociate because of fixation, banded as sharp peaks with a density of 1.43 g/cm³ in Cs₂SO₄ and 1.385 g/cm³ in CsCl (Fig. 6, A and D). After further purification of the 40 S particles by centrifugation in Metrizamide gradients of low ionic strength, the glutaraldehyde-fixed RNP particles banded with a density of 1.44 g/cm³ in Cs₂SO₄ and 1.395 g/cm³ in CsCl (Fig. 6, B and E). However, 40 S particles from sucrose gradients were exposed to 600 mM NaCl during Metrizamide gradient centrifugation, the particles dissociated and most of the resulting RNA banded in Cs₂SO₄ at a buoyant density of 1.66 g/cm³, very close to the density of free RNA (Fig. 6C). Similarly, when the 40 S particles were exposed to high ionic strength during Metrizamide gradient centrifugation, most of the radioactivity applied to the CsCl gradients was recovered in the pellet, while less than 10% of the applied radioactivity was observed in a small peak at 1.44 g/cm³ (Fig. 6F).

**Fig. 5.** Two-dimensional gel electrophoresis of the proteins associated with 40 S RNP purified by centrifugation in Metrizamide (Fig. 3). Reduced and alkylated proteins were subjected to electrophoresis at pH 4.3 in 7.5% acrylamide gels containing 8 M urea. Strips of the first dimension gel were applied to SDS-polyacrylamide gradient gel slabs and electrophoresed for 18 h. The fractionated proteins were visualized by staining with Coomassie Brilliant Blue R-250. The composite photograph at right shows the distribution of the 25 major RNP protein spots after two-dimensional electrophoresis. Strip at the top of the two-dimensional gel shows the protein-banding pattern after electrophoresis in the first dimension of pH 4.3, while the strip at the right of the two-dimensional gel shows protein banding pattern after one-dimensional SDS-gradient gel electrophoresis. The schematic diagram at left plots the distribution of protein spots resolved by two-dimensional electrophoresis and assigns numbers to the spots in order of increasing molecular weight. A molecular weight scale plotting the mobilities of standard proteins of known molecular weight (Fig. 4) is shown at the far left.
40 S RNP particles are exposed to increasing concentrations of NaCl. Salt concentrations greater than 600 mM NaCl results in velocity sedimentation and circular dichroism measurements.3

Panels D and E, centrifugation in Metrizamide gradients containing 600 mM NaCl (F). The gradients were then analyzed by collecting 12-drop fractions which were monitored for trichloroacetic acid-precipitable radioactivity. The density at every fifth fraction was determined from the refractive index. Panels A to C, CsCl density gradients of 40 S RNP particles after sucrose density gradient centrifugation (A), centrifugation in Metrizamide gradients containing 10 mM NaCl (B) and centrifugation in other gradients (C). Panels D to F, CsCl density centrifugation of 40 S RNP particles after sucrose density gradient centrifugation (D), centrifugation in Metrizamide gradients containing 10 mM NaCl (E) and centrifugation in Metrizamide gradients containing 600 mM NaCl (F).

We have confirmed that exposure of 40 S RNP particles to salt concentrations greater than 600 mM NaCl results in complete disaggregation of the RNA and protein moieties by velocity sedimentation and circular dichroism measurements.

By each of these techniques no evidence for salt-resistant RNP particle subfractions was obtained, suggesting as do the electron microscopic and centrifugation experiments cited above that the HnRNP particle fraction isolated by Metrizamide gradient centrifugation is homogeneous.

Cross-linking of Proteins in HnRNP Particles—Direct evidence that the proteins co-isolating with HnRNA are closely associated components of a single macromolecular complex and not a heterogeneous mixture of particles of 40 S that differ in their protein compositions was obtained by electrophoretic analysis of the aggregates formed after fixation with formaldehyde, glutaraldehyde, or the reversible cross-linking reagent, 4-methylmercaptobutyrimidate (40, 41). Treatment of 40 S particles with increasing concentrations of formaldehyde (above 5 mM) or with glutaraldehyde (above 0.5 mM) results in a progressive and coordinate integration of free protein bands seen in SDS-polyacrylamide gels (Fig. 6), into high molecular weight aggregates containing RNA and protein. These complexes cannot enter the 3% polyacrylamide-stacking gels (Fig. 6A and B), but migrate as a single band in 1% agarose gels containing SDS (Fig. 6C). Extensive treatment of the cross-linked aggregates with ribonuclease does not release protein from the HnRNP complex, supporting the idea that protein-protein cross-links rather than RNA-protein cross-links, are primarily responsible for maintaining these complexes. No products of greater size than the 40 S particle are generated, even at high glutaraldehyde concentrations.

Fixation of the 40 S particles with high concentrations of the cleavable cross-linking reagent 4-methylmercaptobutyrimidate (greater than 8 mM) also results in the formation of a stable macromolecular complex which migrates as a single sharp band in 1% agarose gels (Fig. 6B). Cleavage of this complex, by reduction with 2-mercaptoethanol, releases each of the proteins of the original 40 S particle (Fig. 8). The individual proteins are not well resolved by electrophoresis in 1% agarose gels, but produce an electrophoretic pattern on SDS-polyacrylamide gradient gels, equivalent to that shown by proteins of unfixed HnRNP particles (compare Fig. 6A and Fig. 10). All the cross-links introduced by 4-methylmercaptobutyrimidate are protein-protein linkages, since this reagent does not react appreciably with free RNA or RNA in ribosomes (41).

Thus, three types of fixation indicate a close apposition of the proteins tightly associated with HnRNA in 40 S RNP particles, a structure with characteristic buoyant density, sedimentation coefficient, and EM morphology.

Chromatographic Fractionation of 40 S HnRNP Proteins—The preceding experiments show that nearly all the HnRNA molecules of rat liver may be isolated together with specific nuclear proteins. We have fractionated the major RNP proteins by ion exchange chromatography and compared the amino acid compositions of the separated protein species in order to determine whether the RNP proteins (which displayed very similar mobilities in both change and size separations using polyacrylamide gel electrophoresis) are chemically related to one another.

Chromatography on DEAE-Sephadex A-25 was employed to fractionate the proteins of the 40 S RNP particle into

\[4 \text{ M guanidine hydrochloride and 0.25% SDS are also completely effective in dissociating the RNA and protein as measured by circular dichroism. In contrast, the RNP particle structure is resistant to 50 mM EDTA. J. Karn, G. Vidali, and G. Fasman, unpublished observations.}\]
Typical purified protein fractions are shown in Fig. 10 which depicts a SDS-polyacrylamide gel analysis of the homogeneity of the proteins. Proteins 1, 3, 4, 5, and 8 had electrophoretic mobilities in the presence or absence of 2-mercaptoethanol that were indistinguishable from each other. Proteins 6 and 7 were not separated by these chromatographic procedures.

**Fig. 7.** Generation of a stable 40 S macromolecular RNA-protein complex by fixation with formaldehyde or glutaraldehyde. 40 S ribonucleoprotein particles in 100 mM NaCl, 10 mM triethanolamine/ HCl, pH 8.0, 1 mM MgCl₂, 10% (v/v) glycerol were incubated for 45 min at 25°C with formaldehyde or glutaraldehyde concentrations as indicated, and the cross-linked proteins subjected to electrophoresis on SDS-polyacrylamide gradient gels or on neutral SDS-1% agarose gels. A, SDS-polyacrylamide gel electrophoresis of 40 S proteins incubated with formaldehyde concentrations ranging from 0 to 100 mM. B, SDS-polyacrylamide gel electrophoresis of 40 S particle proteins incubated with glutaraldehyde concentrations ranging from 0 to 50 mM. Note progressive and coordinate loss of proteins at glutaraldehyde concentrations greater than 0.5 mM. C, analysis of glutaraldehyde-stabilized 40 S particles in SDS-1% agarose gels. The 40 S particles were stabilized by incubation with glutaraldehyde concentrations ranging from 0 to 10 mM. Note the appearance of an apparently homogeneous macromolecular aggregate at glutaraldehyde concentrations greater than 2.5 mM.

The major HnRNA-associated proteins were separated by a series of chromatographic steps using the cation exchange resins phosphocellulose and carboxymethylcellulose (Fig. 9). Typical purified protein fractions are shown in Fig. 10 which depicts a SDS-polyacrylamide gel analysis of the homogeneity of the isolated proteins. Using this fractionation scheme it was possible to obtain samples of proteins 3, 4, 5, and 8 with greater than 80% electrophoretic homogeneity. Proteins 6 and 7 were only partially resolved by the chromatographic procedures, and unresolved by SDS-gel electrophoresis.

The 40 S RNP proteins have characteristic and specific amino acid compositions – amino acid compositions for the major RNP proteins fractionated by ion exchange chromatography are presented in Table II.

**Fig. 8.** Reversible fixation of 40 S RNP particles by cross-linking with 4-methylmercaptobutyrimidate. A, 40 S particles in 100 mM NaCl, 10 mM triethanolamine/HCl, pH 8.0, 1 mM MgCl₂, 10% (v/v) glycerol were incubated for 30 min at 25°C with concentrations of 4-methylmercaptobutyrimidate ranging from 0 to 10 mM and the protein products separated by SDS-polyacrylamide gel electrophoresis in the presence or absence of 1% (v/v) 2-mercaptoethanol. Note the coordinate loss of free proteins at 4-methylmercaptobutyrimidate concentrations greater than 4 mM, and complete restoration of the free protein-banding pattern by addition of 2-mercaptoethanol. B, analysis by SDS-1% agarose gel electrophoresis of 40 S RNA-protein complexes stabilized with 4-methylmercaptobutyrimidate-stabilized 40 S RNP complexes. RNP particles were incubated for 30 min at 25°C with 10 or 15 mM mercaptobutyrimidate, and applied to neutral 1% agarose gels in the presence or absence of 1% (v/v) 2-mercaptoethanol. Note release of free proteins with high electrophoretic mobility in the presence of 2-mercaptoethanol from the electrophoretically homogeneous, stabilized 40 S RNP particle.

and 11 were not separated by these chromatographic or electrophoretic procedures. The major 40 S RNP proteins have characteristic and specific amino acid compositions – amino acid compositions for the major RNP proteins fractionated by ion exchange chromatography are presented in Table II. The amino acid compositions of the purified proteins are strikingly similar, and share several unusual features that distinguish the RNP proteins from the histones (Table II). Each of the proteins in this group have extremely high glycine contents (typically 25 mol %), low detectable methionine, and low cysteine contents. The proteins have between 1.55 and 2.26 mol % histidine, and between 4.93 and 8.04 mol % lysine. Protein fractions 2, 6 + 7, 8, and 10 + 12 contain the unusual amino acid N⁶,N⁶(CH₃)₂-arginine. The identification and in vivo synthesis of this amino acid is described below. The arginine contents of the proteins vary from 4.98 to 7.62 mol % (including the contribution of N⁶,N⁶(CH₃)₂-arginine), while the dimethylarginine content of the protein fractions varies from undetectable (protein 4) to 32% of the arginine residues (proteins 6 + 7). In contrast, amino acid analyses of total rat liver histone fractions show the presence of considerably more methionine, lysine, and arginine (1.80, 12.00, and 11.10 mol %, respectively) in the histones than in the HnRNA-associated proteins. The histones have significantly less glycine than do the RNP proteins (8.7 mol % compared to 25.0 mol %) and no detectable N⁶,N⁶(CH₃)₂-arginine (Table II). Differences in amino acid composition between the chromosomal non-histone proteins and the HnRNA-associated proteins are also evident (Table II).
**L-Arginine** — An unusual amino acid eluting between ammonium and arginine was detected in acid hydrolysates of 40 S RNP proteins analyzed by standard chromatographic procedures (50). We have identified this amino acid as N^6,N'^6(CH_3)_2-L-arginine by chromatography with synthetic standards using an amino acid analyzer modified to permit complete separation of all the methylated derivatives of the basic amino acids (Fig. 11, Refs. 43 and 44). The evidence may be summarized as follows: (a) the amino acid elutes from the amino acid analyzer in the same position as N^6,N'^6(CH_3)_2-L-arginine but is clearly separated from its symmetrically methylated analogue N^6,N'^6(CH_3)_2-L-arginine; (b) in mixing experiments in which known quantities of N^6,N'^6(CH_3)_2-arginine are added to acid hydrolysates of 40 S RNP proteins a single homogeneous peak is quantitatively eluted in the position where dimethylarginine elutes; (c) the amino acid is labeled in vivo with arginine; (d) the amino acid is labeled in vivo with [methyl-^3]methionine (Fig. 11).

It is significant that the N^6,N'^6(CH_3)_2-L-arginine is rapidly labeled in vivo with [^3]H]methyl groups derived from [methyl-
Nuclear Proteins Associated with HnRNA

FIG. 10. SDS-polyacrylamide gradient gel analysis of protein fractions prepared by cation exchange chromatography (Fig. 9). a, nuclear extracts (pH 8.0) containing 40 S particles; b, proteins in 12 to 20 S region of sucrose gradients (Fig. 1); c, 40 S nuclear RNP particles prepared by sucrose gradient centrifugation (Fig. 1); d, proteins excluded from DEAE-Sephadex A-25 (Fig. 4d). e, protein Fraction 3; f, protein Fraction 6 + 7; g, protein Fraction 4; h, protein Fraction 5; i, protein Fraction 8. Scale at left indicates mobility of protein molecular weight standards (Fig. 4).

3Hmethylmethionine, suggesting that this unusual amino acid arises from postsynthetic modification of arginine residues in vivo (43, 51, 52). After labeling in vivo with [methyl-3H]methylmethionine (Fig. 11) more than 70% of the radioactivity found in the 40 S particle proteins could be recovered as N\textsuperscript{\textsubscript{6}},N\textsuperscript{\textsubscript{\textsterisk}}(CH\textsubscript{3})\textsubscript{2}-l-arginine. Almost 10% of the radioactivity was found in the region where N\textsuperscript{\textsubscript{6}},N\textsuperscript{\textsubscript{\textsterisk}}(CH\textsubscript{3})\textsubscript{2}-l-arginine elutes (although no ninyhdrin-positive material could be detected). This amino acid may represent an intermediate in the formation of the dimethylarginine. A small unidentified peak of radioactivity was also observed to elute between ammonium and dimethylarginine. No modification of lysine or histidine residues was detectable, either by ninyhdrin reaction or uptake of label in vivo, although methylation reactions of this kind are known to occur in histones (44).

Quantitative analysis of the N\textsuperscript{\textsubscript{6}},N\textsuperscript{\textsubscript{\textsterisk}}(CH\textsubscript{3})\textsubscript{2}-l-arginine content of various nuclear fractions demonstrates that more than 67% of the total nuclear N\textsuperscript{\textsubscript{6}},N\textsuperscript{\textsubscript{\textsterisk}}(CH\textsubscript{3})\textsubscript{2}-l-arginine may be recovered in 40 S particles isolated on sucrose gradients (data not shown). Since the ribonucleoprotein particles are not quantitatively recovered during the nuclear fractionation procedures (Table I), these data suggest strongly that the methylation of arginine residues found in nuclear protein fractions almost exclusively involves the proteins associated with HnRNP complexes.

Phosphorylation of RNP Proteins—Evidence that the 40 S proteins contain phosphorylated amino acids was obtained from analyses of the 3\textsuperscript{\textsubscript{2}}P-labeled amino acid content of 40 S RNP proteins subjected to partial acid hydrolysis. In a typical experiment, 53% of the total protein 3\textsuperscript{\textsubscript{2}}P activity was recovered in 40 S proteins isolated on sucrose gradients (data not shown). Since the ribonucleoprotein particles are not quantitatively recovered during the nuclear fractionation procedures (Table I), these data suggest strongly that the methylation of arginine residues found in nuclear protein fractions almost exclusively involves the proteins associated with HnRNP complexes.

Amino acid compositions of protein components of 40 S ribonucleoprotein particles and other rat liver nuclear subfractions

Rat liver 40 S HnRNP particles were purified by sucrose gradient centrifugation as described under "Experimental Procedures." The 40 S nuclear RNP particle proteins were solubilized in 6 M urea and fractionated by ion exchange chromatography on DEAE-Sephadex A-25, phosphocellulose, and carboxymethylcellulose. Aliquots of the purified proteins (numbered as indicated in Figs. 5 and 9) were hydrolyzed in 6 M HCl, 18 h, 110°, and analyzed with a Beckman model 120 B amino acid analyzer or a Durrum amino acid analyzer.

| Amino acid | Total 40 S HnRNP protein\textsuperscript{a} | HnRNP protein fractions | Total non-histone chromosomal protein\textsuperscript{a} | Total histone\textsuperscript{a} |
|------------|---------------------------------|-------------------------|---------------------------------|------------------------|
|            | moles per 100 moles total amino acid\textsuperscript{b} | moles per 100 moles total amino acid\textsuperscript{b} | moles per 100 moles total amino acid\textsuperscript{b} | moles per 100 moles total amino acid\textsuperscript{b} |
| Aspartic acid | 10.83 | 10.36 | 11.55 | 10.74 | 10.83 | 10.58 | 10.34 | 12.66 | 7.90 | 6.34 |
| Threonine | 3.90 | 3.09 | 3.02 | 3.23 | 3.89 | 3.22 | 2.90 | 3.49 | 4.12 | 4.36 |
| Serine | 4.97 | 12.36 | 6.79 | 7.15 | 9.16 | 7.91 | 6.83 | 7.69 | 5.60 | 5.22 |
| Glutamic acid | 10.97 | 9.52 | 10.52 | 10.63 | 12.83 | 10.65 | 11.87 | 13.82 | 11.36 | 11.27 |
| Proline | 6.81 | 3.51 | 4.24 | 3.65 | 3.29 | 2.93 | 3.98 | 4.59 | 4.65 |
| Glycine | 17.88 | 25.98 | 24.67 | 25.54 | 25.95 | 25.70 | 25.05 | 19.95 | 14.75 | 8.75 |
| Alanine | 5.55 | 3.76 | 4.32 | 3.63 | 4.04 | 3.60 | 5.00 | 4.97 | 6.17 | 11.23 |
| Half-cystine | Trace | 1.26 | 0.65 | 0.63 | 0.57 | 1.67 | 1.85 | 1.94 | Trace | Trace |
| Valine | 5.68 | 2.92 | 3.98 | 4.16 | 1.33 | 3.96 | 4.38 | 4.19 | 7.04 | 6.77 |
| Methionine | 0.71 | 0.42 | 0.32 | n.d.\textsuperscript{c} | n.d. | n.d. | n.d. | n.d. | 0.31 | 3.69 | 1.80 |
| Isoleucine | 2.70 | 2.75 | 2.91 | 2.41 | 2.37 | 2.53 | 2.32 | 2.80 | 5.73 | 3.99 |
| Leucine | 4.68 | 3.93 | 2.68 | 2.58 | 3.32 | 3.92 | 4.55 | 4.11 | 7.85 | 7.73 |
| Tyrosine | 3.33 | 3.26 | 4.27 | 5.37 | 4.38 | 4.28 | 2.84 | 3.11 | 3.41 | 1.62 |
| Phenylalanine | 3.26 | 3.76 | 3.13 | 5.68 | 5.57 | 5.74 | 4.23 | 4.43 | 3.16 | 1.41 |
| Lysine | 7.71 | 4.93 | 5.13 | 5.94 | 8.16 | 5.46 | 5.81 | 5.90 | 5.59 | 12.00 |
| Histidine | 2.51 | 2.26 | 2.07 | 2.21 | 2.07 | 1.73 | 2.04 | 1.55 | 1.87 | 2.07 |
| N\textsuperscript{\textsubscript{6}},N\textsuperscript{\textsubscript{\textsterisk}}(CH\textsubscript{3})\textsubscript{2}-l-Arginine | 1.07 | 0.82 | n.d. | n.d. | n.d. | 1.59 | 1.17 | 0.62 | 0.23 | n.d. |
| Arginine | 7.97 | 5.10 | 6.70 | 7.29 | 7.62 | 3.39 | 7.53 | 4.97 | 7.07 | 11.10 |

\textsuperscript{a} Single determination.

\textsuperscript{b} Average of two determinations.

\textsuperscript{c} Values not corrected for hydrolytic losses.

\textsuperscript{d} n.d. = not detectable.
The phosphoproteins appearing in the HnRNP particle fraction have been characterized by SDS-polyacrylamide gel electrophoresis of RNP proteins labeled in vivo for 45 min with \[^{32}P\]orthophosphate (Fig. 12). Most of the \[^{32}P\] activity in the 40 S particle fraction is incorporated by proteins 6, 7, 8, and 9 (Fig. 12c). In contrast, protein 4, which is the protein present in greatest molar quantities in the 40 S particle fraction was not detectably phosphorylated. Since the serine and threonine contents of the RNP proteins do not differ significantly (Table II), these differences in the content of phosphorylated amino acids probably result from sequence-specific recognition of phosphorylation sites by nuclear protein kinases. Other nuclear phosphoproteins are recovered in pH 8.0 nuclear extracts (Fig. 12a) and in the 12 to 20 S region of sucrose gradients (Fig. 12b).

The proteins in the 40 S RNP complex may also be phosphorylated in vitro by protein kinases (Fig. 13). We have confirmed a previous report (53) that 40 S RNP particles contain an associated protein kinase activity, simply by incubating 40 S particles in the presence of \(\gamma\)-\[^{32}P\]ATP and an appropriate buffer. Consistent with the results of the in vivo phosphorylation experiments, proteins 6, 7, 8, and 9 rapidly incorporate \[^{32}P\] radioactivity while protein 4 does not appear to incorporate \[^{32}P\] radioactivity. It is of interest that proteins 6, 7, and 8 are also subject to modification by methylation of arginine residues while protein 4 is not methylated (Table II). The protein kinase also labeled to high specific activities a number of proteins with molecular weights less than 27,000 and greater than 67,000 that were not phosphorylated in vivo. Addition of cAMP did not modify the rate or the specificity of the 40 S RNP-associated protein kinase. Addition of partially purified protein kinase from calf thymus (gift of Dr. E. M. Johnson, The Rockefeller University) increased the rate of phosphorylation of the 40 S RNP proteins (Fig. 13), but did not significantly alter the substrate specificity of the reaction. The rate of phosphorylation of protein 9 was particularly increased by the exogenous kinase. Even in the presence of protein kinase from another tissue, protein 4 remained unphosphorylated.

Estimates of Protein Stoichiometry in Rat Liver 40 S HnRNP Particle—Table III summarizes the available chemical data concerning the major proteins found in rat liver 40 S ribonucleoprotein particles. The proteins in the 29,000 to 42,000 molecular weight group appear to be the structural proteins of the HnRNP particles by the following criteria. These proteins represent more than 75% of the protein mass of RNP particle fractions and are closely related by charge (Table III) and amino acid compositions (Table II). Each of
the proteins in this fraction are present in apparently constant proportions throughout a rigorous isolation procedure (Fig. 4). The proteins of higher molecular weight which are sometimes present in HnRNP particle preparations (4, 9, 12, 13, 18) are not likely to be structural proteins. These proteins are present in limited quantities of 40 S particles isolated by sucrose gradient centrifugation, and many are lost when 40 S particles are further purified by centrifugation on Metrizamide gradients (Fig. 4). Proteins in this fraction contain enzymatic activities, including a protein kinase activity (54), endoribonuclease (55), and a homopolymer synthetase activity (56).

Making the assumptions that only the proteins in the 29,000 to 42,000 molecular weight group are required for the maintenance of the 40 S particle, and that the RNP fractions represent a homogeneous population of structures, can a model for the 40 S HnRNP be derived? The relative abundance of the proteins in this group was estimated by measurement of the total radioactivity in protein fractions labeled in vitro and separated by phosphocellulose chromatography, and confirmed by integration of densitometry tracings of the stained proteins separated by SDS-polyacrylamide gel electrophoresis (Table III). Assuming that the proteins present in lowest yields are present in one copy per 40 S particle, the copy number per particle of the more abundant proteins may be estimated. By multiplying the estimated copy number per particle by the molecular mass of the proteins estimated by SDS-polyacrylamide gel electrophoresis, and adding the calculated protein masses, the total protein mass of the 40 S particle may be estimated to be 1.1 to 1.2 \times 10^6 daltons.

Assuming a protein to RNA ratio of 3.7:1 (Table I, Refs. 3, 11, 13, and 52), the total mass of the 40 S particle may then be estimated to be 1.3 to 1.5 \times 10^6 daltons. This molecular mass estimate is in close agreement with the estimates for the molecular mass of the 40 S particle (1.4 to 1.6 \times 10^6 daltons) based on comparison to the molecular masses of ribosomal subunits (57, 58).

Although these estimates should not be regarded as accurate until confirmed by alternative procedures, they provide a useful illustration that a unique protein structure composed of major basic RNP proteins can be reasonably proposed. Whether other proteins are required to maintain this core structure, and whether the high molecular weight proteins have organizational roles in the RNP structure awaits further investigation.

**Similarity of Protein Components of 40 S HnRNP Particles from Different Species**—To what extent are the organizational principles of rat liver ribonucleoprotein particles we have described applicable to ribonucleoprotein structures in other species? HnRNP particles have been isolated from human (HeLa S-3) cells, and from duck liver cells and their protein components compared by electrophoresis in polyacrylamide gradient gels containing 0.1% sodium dodecyl sulfate (Fig. 14). Side by side comparisons with proteins of rat liver 40 S particles (Fig. 14c) show that the proteins obtained from each

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**Fig. 13.** Phosphorylation of 40 S RNP particle proteins in vitro by protein kinase. The 40 S RNP particles were dialyzed overnight against 0.1 M sodium acetate, pH 6.3, 10^{-3} M papaverine, 1.0 mM EGTA. Protein kinase activity was assayed as uptake of radioactive phosphate from [γ-32P]ATP into 40 S RNP proteins fractionated by SDS-polyacrylamide gradient gel electrophoresis. Incubations were initiated by addition of MgCl_2 (10 mM) and ATP, and terminated after 2 or 15 min by addition of SDS. Reactions were in the presence or absence of 10^{-6} M cAMP, and in the presence or absence of 2 μg of partially purified calf thymus cAMP-dependent protein kinase (gift of Dr. E. M. Johnson) as indicated in the legend at the top of the right-hand panel. Left panel, proteins detected by Coomassie brilliant blue R-250 staining; right panel, phosphoproteins detected by autoradiography (48-h exposure). The ninth gel slot (far right) depicts purified protein kinase control. Note appearance of protein band of molecular weight 84,000 in samples containing added protein kinase (left panel). Scale at far left indicates the mobilities of protein molecular weight standards (Fig. 4).

**Fig. 14.** Similarity in the protein complements of HnRNP particles from different species, analyzed by SDS-polyacrylamide gel electrophoresis. a, duck liver 40 S RNP particles prepared by extraction of nuclei with Buffer B; b, duck liver 40 S RNP particles prepared by sonication of duck liver nuclei; c, rat liver 40 S RNP particles prepared by extraction of nuclei with Buffer B; d, HeLa S-3 cell 40 S HnRNP particles prepared from total HnRNP by digestion with 0.01 μg/ml of pancreatic ribonuclease (30 min, 4°C) and sedimentation in sucrose density gradients as described under "Experimental Procedures"; e, HeLa S-3 cell total HnRNP prepared by the method of Kish and Pederson (14); f, HeLa S-3 cell nuclear proteins. Note the large quantities of the two major HeLa cell 40 S RNP-associated proteins (31,000 and 32,000 daltons) in this fraction. The intensely stained proteins of apparent molecular weights 29,000 and 28,000 are HeLa cell histone H-1 proteins; g, HeLa S-3 cell polysomal proteins. Polyosomes (three to eight ribosomes per polysome) were prepared from postnuclear supernatant fractions of HeLa cell lysates as described under "Experimental Procedures."
The molecular mass of the proteins was estimated by comparison of their mobilities in SDS-polyacrylamide gradient gels to protein standards of known molecular mass (Fig. 4). The isoelectric points of the proteins (pl) were determined as follows: 40 S RNP particles were isolated from sucrose gradients, the proteins solubilized in Buffer F, and applied to columns of DEAE-Sephadex A-25 as described under "Experimental Procedures." The proteins excluded from the column were applied to a 150-ml LKB isoelectric focusing column containing 1% (v/v) Ampholines, pH 3 to 10, 4 M urea, and a gradient of 10 to 40% sucrose. The apparatus was operated at 4°C for 12 h at 200 V and for 24 h at 500 V using 0.1 M HCl as the anodic buffer, and 0.1 M NaOH as the buffer at the cathode. At the end of the electrophoresis period, when the current had stabilized, 1.5-ml samples were collected and assayed for pH. Aliquots of each fraction were analyzed for protein content by electrophoresis in SDS-polyacrylamide gels. The content of N°°N°°(CH₃)₃-l-arginine was determined by amino acid analysis of acid hydrolysates of proteins fractionated by ion exchange chromatography (Table I) and the presence of phosphorylated amino acids detected by uptake of [³²P]orthophosphate in vitro into proteins fractionated by SDS-polyacrylamide gel electrophoresis (Fig. 12). The relative abundance of the proteins was estimated by comparing the radioactivity incorporated in vitro into proteins separated by chromatography on phosphocellulose (Fig. 9A) and confirmed by analysis of densitometry tracings of the stained proteins fractionated by SDS-polyacrylamide gradient gel electrophoresis (Fig. 4).

| Protein fraction | Molecular mass | pl | N°°N°°(CH₃)₃-l-Arginine | Phosphoserine and phosphothreonine | Relative abundance | Copies per 40 S particle | Mass per 40 S particle |
|------------------|---------------|----|------------------------|---------------------------------|------------------|------------------------|----------------------|
| daltons
| 1 | 29,000 ± 1,000 | 8.2 | - | - | 0.12 | 1 | 29,000 |
| 2 | 30,000 ± 1,000 | 8.5 | + | - | 0.11 | 1 | 30,000 |
| 3 | 31,000 ± 1,000 | 8.3 | - | - | 0.36 | 3 | 91,000 |
| 4 | 32,000 ± 1,000 | 8.6 | - | - | 1.00 | 9 | 288,000 |
| 5 | 34,000 ± 1,000 | 8.0 | - | - | 0.32 | 3 | 102,000 |
| 6 | 34,000 ± 1,000 | 8.4 | +++ | +++ | 0.59 | 6 | 204,000 |
| 7 | 34,500 ± 1,000 | 8.4 | +++ | +++ | 0.15 | 1 | 34,500 |
| 8 | 35,000 ± 1,000 | 9.2 | + | + | 0.31 | 3 | 105,000 |
| 9 | 40,000 ± 1,500 | 7.1 | - | + | 0.25 | 2 | 82,000 |
| 10 | 41,500 ± 1,500 | 9.4 | + | + | 0.30 | 9 | 83,000 |
| 11 | 42,000 ± 1,500 | 9.5 | + | + | 0.10 | 1 | 42,000 |
| 12 | 42,000 ± 1,500 | 9.5 | + | + | 0.11 | 1 | 42,000 |

Species contain major components in the 29,000 to 42,000 molecular weight range, with similar electrophoretic characteristics, although the overall patterns are clearly not identical. Taken together with reports on the similarity of the total amino acid compositions of 40 S RNP particle proteins obtained from several different species including mouse Taper Hepatoma cells (11), duck liver (11), and HeLa S-3 cells (52), and immunological experiments showing that antibodies prepared against proteins of rat liver 40 S particles will cross-react with proteins from avian and amphibian ribonucleoprotein particles (59), these observations support the view that the RNP-associated proteins are similar in most eukaryotes. Whether apparent homologies in protein compositions between species would vanish with the greater resolving power of two-dimensional gel electrophoretic systems, or whether the difference in banding patterns would be reduced in comparable studies of the HnRNP particles prepared by Metrizamide gradient centrifugation, remain to be determined.

**DISCUSSION**

Our experiments on the protein complement of rat liver 40 S HnRNP particles demonstrate that these structures contain sets of specific RNA-binding proteins. Only 25 components are observed in two-dimensional gel analyses of the proteins of rat liver 40 S HnRNP particles which have been purified by centrifugation on Metrizamide density gradients. We have identified 12 of these proteins as the most likely structural proteins in the RNP particle fraction. The proteins in this group range in molecular weight from 29,000 to 42,000 and together comprise more than 75% of the protein mass of the isolated 40 S HnRNP particles. Amino acid analysis of individual proteins from this group, separated by ion exchange chromatography, reveal that each of these proteins is similar in both charge and amino acid content. The RNP proteins are rich in glycine and the unusual modified amino acid N°°N°°(CH₃)₃-l-arginine, but contain little methionine or cysteine. These unusual amino acid compositions distinguish the HnRNA-associated proteins, as a group, from the rest of the non-histone nuclear proteins and from the histones.

In considering the structural organization of HnRNP particles a key question is whether isolated HnRNP fractions represent a uniform or mixed population of structures. The 40 S particles we have isolated appear to be homogeneous when examined by electron microscopy and by centrifugation studies using both sedimentation and isopycnic banding techniques. However, it is conceivable that these techniques could fail to resolve subpopulations of RNP particles that differ in their protein composition and that even the major proteins in the isolated HnRNP fractions might occur in a random distribution in the 40 S particle populations. For this reason, the results of fixation experiments using bifunctional reagents are particularly significant, for they show that all of the proteins in the HnRNP particles are capable of forming cross-links to stabilize a macromolecular complex which appears homogeneous after electrophoresis in agarose gels. It is noteworthy that the free protein bands disappear coordinately as fixation proceeds (Figs. 7 and 8) and that ribonuclease digestion does not disrupt the resulting complexes, suggesting, as do the observations of the lability of HnRNP complexes to high salt (Refs. 2, 4, 13, and 18, and Fig. 3), that protein-
protein interactions are essential for the organization of the HnRNP particle. The fact that all protein bands are "integrated" into the fixed RNP complex when cross-linking is carried out with 4-methylmercaptopuburyrimidate, and that all bands are recovered when the disulfide cross-linkages are reductively cleaved (Fig. 8) offers further support for the view that the major structural proteins exist in closed apposition in all the HnRNP particles. This is not a proof that all HnRNP particles are identical, but it does indicate a common structural organization of a presumptive core complex. In this paper, we have proposed a possible model for the 40 S RNP particle of rat liver, based on the assumption that each of the proteins in the 29,000 to 41,000 group is present in at least one copy per 40 S particle (Table III).

These experiments, together with a wide range of previous knowledge about the structure and labeling kinetics of HnRNP complexes, support the view that nascent RNA sequences are organized into structures consisting of repeated subunits arrayed linearly along the polynucleotide chains. Such structures, appearing in electron micrographs as arrays of 20 nm diameter particles connected by ribonucleosensitive strands (3, 11, 23–32), are degraded rapidly unless precautions are taken to inhibit endonucleolytic action (3, 13). The beads-on-a-string organization of HnRNP has obvious analogies to the organization of nucleosomes on DNA strands in chromatin (60, 61), in both cases, endonucleolytic cleavages give rise to populations of monomer particles of apparently constant protein composition, but containing diverse nucleotide sequences.

While the unmodified complement of HnRNP proteins is a constant set of non-histone nuclear proteins, containing no more than 25 components, posttranslational modifications (18, 43, 51, 54, 62) may introduce additional charge heterogeneity. The HnRNP associated proteins are extensively modified by methylation of arginine residues and phosphorylation of serine residues. Some of the HnRNP proteins have as much as 30% of their arginine residues modified by methylation generating the amino acid \( N^\text{methyl} \, N^\text{O-acetyl} \, L-\text{arginine} \). The role of this modification reaction and the phosphorylation reaction in the metabolism of the HnRNP proteins is not yet understood, but it seems likely that such modifications serve to specifically alter the charge and conformation, of RNA-associated proteins to allow processing and transport of HnRNA. The report (18) that RNP proteins which are phosphorylated in the metabolism of the HnRNP proteins is not yet understood, but it seems likely that such modifications serve to specifically alter the charge and conformation, of RNA-associated proteins to allow processing and transport of HnRNA. The extent of homologies between HnRNP-associated proteins in different species will not be properly understood until sequence studies of the purified proteins have been completed.

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Characterization of the non-histone nuclear proteins associated with rapidly labeled heterogeneous nuclear RNA.

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