Immunofluorescent Localization of the Proteins of Nuclear Ribonucleoprotein Complexes

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ABSTRACT Antibodies were raised in chickens against heterogeneous nuclear RNA (hnRNA)-binding proteins from 30S ribonucleoprotein (RNP) complexes of mouse Taper hepatoma ascites cell nuclei. The antibody preparations were characterized for immunological specificity and purity by double-diffusion gels, binding to specific bands in SDS polyacrylamide gels, and crossed immunoelectrophoresis. Antibodies raised against either whole 30S RNP complexes or purified RNP core proteins had a strong selective affinity for the four 34,000- to 40,000-dalton polypeptides which comprise the major structural proteins of hnRNP. The intracellular distribution of 30S RNP antigens in mouse ascites cells was determined by indirect immunofluorescence microscopy. In interphase cells immunofluorescent sites were restricted to the nucleus, and nucleoli were free of fluorescence. The chicken anti-mouse-RNP antibodies were also able to react with cells from many different vertebrate species, showing a similar nucleus-restricted localization of the reacting sites. The antibodies also bound chick 30S RNP-proteins and reacted with the nuclei of chick cells. An exception to this was the failure of the antibody to bind to adult chick erythrocytes, suggesting that these major hnRNA binding proteins may be found only in nuclei capable of RNA synthesis.

Fine structure analysis of transcriptionally active chromatin has suggested that newly synthesized heterogeneous nuclear RNA (hnRNA) must become rapidly associated with protein, resulting in the formation of ribonucleoprotein (RNP) fibrils (23). Because the hnRNP fibrils, rather than naked precursor RNA, are presumably the structural entities ultimately processed into cytoplasmic messenger RNA, we may anticipate that RNA-binding proteins will play a fundamental role in the mechanisms of folding, cleavage, splicing, and the subsequent transport of pre-mRNA sequences in eukaryotic cells.

Previous studies directed to the characterization of hnRNA and the proteins intimately associated with hnRNA have primarily employed biochemical approaches. Generally, extraction of hnRNP from purified nuclei has resulted in the isolation of RNP substructures which sediment at ~30–40S on sucrose density gradients (2, 21, 30). While larger RNP complexes may be obtained under appropriate conditions, these structures are rapidly cleaved to 30–40S RNP subcomplexes by low levels of endogenous or exogenous ribonuclease (13, 27, 30). This finding has led to the view that the large nascent hnRNP complexes in nuclei of eukaryotic cells are composed of chains of smaller RNP subcomplexes. Although previous work had demonstrated the great similarity in overall size and amino acid composition of polypeptides from 30S RNP isolated from a variety of vertebrate species (3, 21), we thought it important to attempt to raise antibodies against proteins known to be components of hnRNP. The availability of such specific immunological probes would provide new experimental approaches for investigations of RNP structure and physiological functions, and permit experiments to be performed at a higher level of sensitivity than that attainable by biochemical procedures. Earlier attempts to prepare and characterize antisera to hnRNP subcomplexes had indicated that these may not be easy tasks, although some preliminary results were obtained, suggesting that the major proteins of nuclear RNP were not associated with cytoplasmic polyribosomal mRNP (17).

In our study, 30S RNP subcomplexes were extracted and purified from mouse Taper hepatoma cells. The intact RNP particles, or specific polypeptides isolated from the particles, were used as immunogens in chickens to raise precipitating antibodies against the major proteins of 30S RNP which form a large part of the substructure of hnRNP. The antibody preparations, characterized for immunochemical specificity and purity, have now been employed in indirect immunofluo-

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rescence experiments on Taper hepatoma cells and other cell types to define the intracellular localization of the 30S RNP proteins.

MATERIALS AND METHODS

Preparation of 30S RNP Antigens

All antigen preparations were derived from the mouse Taper ascites hepatoma cell line (34) maintained routinely in our laboratory (20). 30S RNP complexes were isolated from purified nuclei by the procedure of Samarina et al. (29, 30), employing the modifications previously described (20, 21). In the first stage of purification, isolated nuclei were washed briefly in 0.1 M NaCl-0.01 M Tris-HCl-0.001 M $MgCl_2$ buffer at pH 7 (STM-7), then at pH 9 (STM-9), followed by extraction in STM-9 buffer for 4.5 h at 0°C. Nuclei were removed by low-speed centrifugation, and the RNP-enriched nuclear extract was centrifuged for 13 h at 27,000 rpm on 36-ml 15–30% sucrose density gradients prepared in STM-8. The 30S RNP particles were collected by centrifugation of pooled fractions for 12 h at 105,000 *g*. The pellets were resuspended in STM-8 ("crude 30S RNP"). These preparations were either reduced and alkylated (4) for analysis on analytical polyacrylamide gels (Figs. 1 *C* and 3) or were directly subjected to a second round of purification by re-centrifuging on 15–30% sucrose density gradients, as described above. Peak fractions from the 30S region of the gradients were pooled and centrifuged at 105,000 *g* for 12–16 h for collection of RNP complexes. The pellets were resuspended in STM-8, or other appropriate buffers ("purified 30S RNP"). Two types of antigen preparations were used in these studies: suspensions of purified 30S RNP particles, and purified 30S RNP core polypeptides. The latter were obtained by subjecting purified 30S RNP to electrophoresis on discontinuous SDS polyacrylamide slab gels (3-mm-thick gels containing 10% acrylamide and 0.1% SDS), as described by Laemmli (15). Bands in the 34,000- to 40,000-dalton region of the gels were localized by brief staining with Coomassie Brilliant Blue, excised, soaked for 30 min in 50% methanol containing 5% glycerol, and electroeluted into dialysis bags in the presence of 0.05 M Tris-0.4 M glycine-0.1% SDS buffer, pH 8.8. The preparations of RNP core polypeptides were then dialyzed for 48 h against distilled water, lyophilized to dryness, and dissolved in a minimal volume of 0.01 M phosphate-0.15 M NaCl buffer, pH 7.5. Total protein content and purity of the antigen preparations were determined with the Folin reagent (16) and by SDS polyacrylamide gel electrophoresis (4), respectively.

Preparation of Anti-RNP Sera and IgG

White Leghorn chickens were injected intramuscularly at several sites along each leg and thigh with 350–400 μg of purified 30S RNP particles or purified core polypeptides emulsified in Freund's complete adjuvant. Booster injection and bleeding schedules were adjusted for each animal in accord with individual differences in the time-course of appearance of precipitating anti-RNP antibodies (determined by serial dilution Ouchterlony gels). In general, four booster injections, each containing 250–300 μg of immunogen in Freund's incomplete adjuvant, were given at ~2-wk intervals. The animals were bled from the medial wing vein 10–13 d after the second booster injection, and on days 10 and 13 after the third booster injection; terminal bleedings were performed on day 11 after the fourth booster injection. All animals were fasted for 24 h before bleeding to reduce serum lipoproteins. The blood was incubated at 37°C for 30 min and overnight at 4°C. The serum was collected, and lipids were floated off by centrifugation. Each serum was sterilized by membrane filtration and sodium azide was added to 0.1%. Identical procedures were used for treatment of immune sera and for serum samples obtained from the same animals before immunization (pre-immune sera). RNAse-free gamma globulin was prepared from each serum by successive precipitations with 18, 14, and 12.5% sodium sulfate (32). Certain sera were further purified by chromatography on Sephadex G-200 or DEAE-cellulose columns (26). Purity of gamma globulins was verified by crossed immunoelectrophoresis with rabbit antibody to chick serum proteins (35, 36).

Assays for Antibody Specificity

Ouchterlony double diffusion analyses were performed essentially as described by Muñoz (24), using 1.5-mm-thick gel layers containing 1% agarose (Bio-Rad Laboratories, Richmond, Calif.) in appropriate buffer. The buffer found to be most advantageous for routine screening of reactions between antibodies and 30S RNP was Svendsen's Tris-glycine-barbital buffer, pH 8.6 (35, 36), with 1.5 M NaCl. Reactions were also obtained using either Svendsen's buffer with 0.15 M NaCl or 0.01 M phosphate-0.15 M NaCl buffer, pH 7.2 (PBS). Reactants in high- and low-salt gels were allowed to diffuse for 48 h at 4°C in a humidified chamber. To monitor reactions between the chicken antibodies and RNP polypeptides derived from treatment of RNP with SDS, 30S RNP particles were first solubilized with 2% SDS in a boiling water bath for 2 min. The mixture was then allowed to cool to room temperature, and Triton X-100 was added to bring the final concentrations of SDS and Triton X-100 to 0.4 and 2%, respectively. Precipitin reactions between test sera and detergent-solubilized RNP were obtained at room temperature with gels containing 1% TritonX-100. The gel plates were then washed in several changes of 2% NaCl, pressed, dried to a thin film, and stained with Coomassie Brilliant Blue.

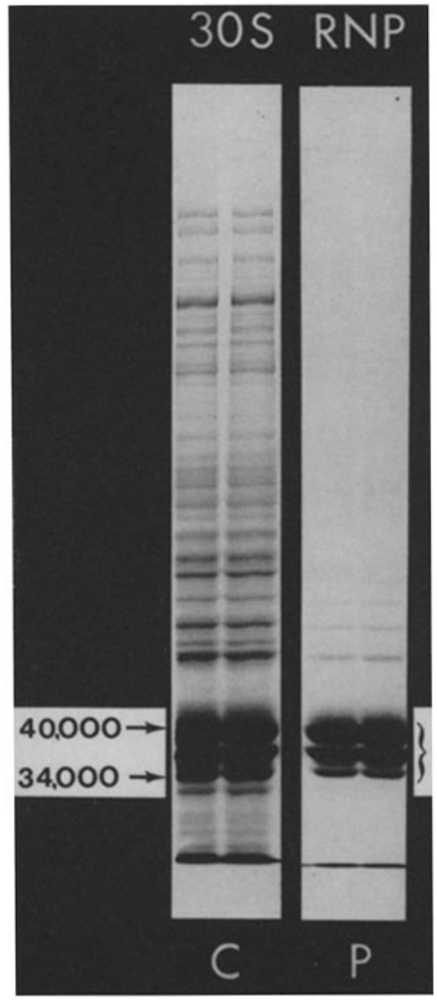

FIGURE 1 SDS polyacrylamide gel electrophoresis of proteins from crude and purified 30S RNP particles. Crude (*C*) and purified (*P*) 30S RNP particles from Taper hepatoma cells were subjected to SDS polyacrylamide slab gel electrophoresis at the end of the first and second stages of purification, respectively. The resolving gel (7.5% acrylamide) and the stacking gel (5% acrylamide) were prepared in SDS and buffers as described by Maizel (18). Bracket indicates the 34,000- to 40,000-dalton RNP-specific core polypeptides.

Procedures for detection of reactions between anti-RNP antibodies and specific RNP polypeptides in SDS polyacrylamide gels were developed through modifications of techniques described by Stumph et al. (33), Burridge (5, 6), Olden and Yamada (25), and Silver et al. (31). Fractions to be tested for reactivity with anti-RNP antibodies were electrophoresed in 0.75-mm-thick polyacrylamide slab gels (7.5% acrylamide resolving gel and 5% acrylamide stacking gel). Replicate strips were fixed for 4 h by shaking at room temperature in methanol:acetic acid:water (5:1:5). The gels were then washed for 3 d in buffer A (0.05 M Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.1% NaN_3), three changes per day. The strips were then transferred to individual parafilm-covered glass bocks, placed in a humidified chamber, and overlaid with immune or pre-immune chicken IgG (2 mg/ml, 1 ml/strip) in buffer G (buffer A with 0.25% gelatin), or buffer G alone. After incubation for 20–24 h at room temperature, the gel strips were washed for 4 d in buffer A, four changes per day. The strips were then incubated for 20–24 h with a 1-ml overlay of ^{125}I-rabbit anti-chicken IgG (2×10^5 cpm/ml, prepared as described below). The iodinated antibody solution was removed, and the strips were washed with buffer A for another 4 d, dried, and subjected to autoradiography of Kodak X-Omat R film. Replicate gel strips stained with Coomassie Brilliant Blue were used for comparison.

^{125}I-rabbit anti-chicken IgG was prepared by lactoperoxidase-catalyzed iodination (1, 6–9, 19). CNBr-activated Sepharose 4B (2.4 g, Pharmacia Inc., Piscataway, N. J.) was swelled in 1 mM HCl, collected on a glass filter, and washed with 400 ml of 1 mM HCl. The gel was then washed extensively and equilibrated with 0.1 M borate-0.5 M NaCl buffer, pH 8.0 (coupling buffer, CB). Purified chicken IgG (66 mg) in 3 ml of CB was added and the mixture was rotated (end-over-end) for 4 h at room temperature. The gel was collected on a glass filter, washed with excess CB, then rinsed and resuspended in 1 M Tris·HCl, pH 8.0, to block any remaining active groups. The mixture was rotated for 2 h at room temperature, collected on a glass filter, and washed alternately with 50-ml aliquots of CB and 0.1 M acetate-0.5 M NaCl buffer, pH 4.0 (four cycles). The gel was then washed with 0.2 M glycine·HCl, pH 2.8, equilibrated with PBS, and adjusted with PBS to a 50% (vol/vol) slurry. A 3- to 4-ml aliquot of rabbit anti-chicken IgG (N. L. Cappel Laboratories Inc., Cochranville, Pa.) containing 100 mg of total protein and 33 mg of specific antibody was then added to the slurry, and the mixture was rotated for 2–4 h at room temperature. The conjugate was centrifuged, resuspended in PBS, poured into a chromatography column, and washed extensively with PBS until the effluent had negligible absorbance at 280 nm. The entire immuno-adsorbent mixture was transferred to a plastic tube and adjusted to a 50% slurry (16 ml) with PBS. Reagents were added in the following order: 555 μg of lactoperoxidase (Sigma B grade, Sigma Chemical Co., St. Louis, Mo., in 500 μl of PBS), 8 mCi of ^{125}I-Na (New England Nuclear, Boston, Mass., NEZ-033; 1.3 ml of isotope neutralized with 1.3 ml of 0.1 N HCl), 0.68 ml of PBS (isotope vial wash), and 220 μl of freshly prepared 8.8 mM H_2O_2 (100 μM at final concentration). The mixture was rotated for 30 min at room temperature, repacked into the chromatography column, and washed overnight with PBS at 0°–4°C. ^{125}I-rabbit anti-chicken IgG was eluted with 0.2 M glycine·HCl, pH 2.8. 5-ml fractions were collected and quickly neutralized with 1 M Tris·HCl, pH 8.0. Peak fractions were pooled and dialyzed for 24 h at 0°–4°C against buffer A. Gelatin was added to the final preparation (2.5 mg/ml) and aliquots were stored at −70°C with a sp act of 2.5×10^6 cpm/mg.

Indirect Immunofluorescence

Cells either were grown directly on sterile glass coverslips or were attached to coverslips which were pre-coated with a solution of 5 μg/ml poly-L-lysine (Sigma Chemical Co.). The coverslip preparations were rinsed in Earle's balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.) and fixed for 30 min at 0°–4°C with a mixture of one part formalin and nine parts 95% ethanol. The cells were then treated with absolute acetone at 0°–4°C for 30 min, rinsed in PBS, incubated in 3% Tween-80 (Atlas Chemical Industries, Inc., Wilmington, Del.) for 1 min at room temperature, and rinsed again in PBS. All coverslips were covered with normal rabbit serum diluted 1:32 with PBS and allowed to incubate at 37°C for 45–60 min in a humidified chamber. The coverslips were rinsed with PBS and overlaid with 0.2 ml (20 μg) of immune or pre-immune chicken IgG; controls were treated with 0.2 ml of PBS. The preparations were then incubated at 37°C for 1 h, rinsed extensively with PBS, returned to the 37°C chamber, and incubated for 1 h with a 1:32 dilution of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-chicken IgG (Miles Laboratories, Inc., Elkhart, Ind.); all FITC conjugates were absorbed with an acetone extract of mouse liver (N. L. Cappel Laboratories Inc.) before use. The coverslips were again washed thoroughly in PBS and mounted on slides with FA Mounting Fluid (Difco Laboratories, Detroit, Mich.). Slides were viewed with a Zeiss Universal microscope equipped with phase-contrast and epi-fluorescence condensers, and excitation and barrier filters optimized for maximal FITC fluorescence. Photomicrographs were taken with Tri-X Pan film (Kodak). The film was developed with Microdol-X (Kodak), and all negatives were printed under equivalent darkroom conditions.

RESULTS

Purification of the RNP Antigens

Purified 30S RNP particles were isolated from mouse Taper hepatoma ascites cells using the two-stage purification procedure of Martin et al. (21), developed by modification of the method originally described by Samarina et al. (30). The effective purification of the 30S RNP by a second round of sucrose gradient centrifugation is shown by the comparison of the polypeptide composition of crude (*C*) and purified (*P*) RNP (Fig. 1). The purified 30S RNP particles contain hnRNA sequences, and have a very limited number of polypeptides (21). Analysis of the purified particles by SDS polyacrylamide gel electrophoresis demonstrates that >90% of the total protein of the particles is contained in four polypeptides with apparent mol wt in the range of 34,000–40,000 (bracketed in Fig. 1 *P*). The four major RNP polypeptides are components of a basic protein complex which is integral to the maintenance of RNP particle structure (22). They are prevalent only in the 30S region of sucrose density gradients of the nuclear extracts, and are neither histones nor ribosomal proteins (3).

Production and Characterization of the Anti-RNP Antibodies

Antibodies against hnRNA-binding proteins were produced by immunizing White Leghorn chickens with either whole purified 30S RNP particles from mouse Taper hepatoma cells, or with the four 34,000- to 40,000-dalton polypeptides obtained by elution from slices of SDS polyacrylamide gels after electrophoresis of purified 30S RNP proteins. Both the particulate and the detergent-solubilized antigens were effective in raising moderate to high titers of precipitating antibodies in chickens.

Fig 2 *B* demonstrates that dilutions of immune sera from chickens immunized with purified 30S RNP particles (outer wells) reacted strongly with 30S RNP (center well) in high-salt Ouchterlony gels. The inclusion of 1.5 M NaCl in the gels promoted optimal immunoprecipitation (10, 12), while also facilitating the dissociation of protein from RNA during diffusion (22). Immunological reactions were also apparent when the assay was performed with low salt (0.15 M NaCl) Ouchterlony gels. In this case, however, reactions were less reproducible and more difficult to assess because of the relative insolubility of the antigens. With the use of either high- or low-salt gels, antibodies raised against the major RNP polypeptides (eluted from SDS polyacrylamide gels) also effectively precipitated 30S RNP complexes which had not been exposed to detergent. Reactions between antibodies and SDS-solubilized antigens were also monitored, using a mixed detergent-micelle system to keep the 30S RNP polypeptides soluble, while preventing antibody inactivation or artifactual precipitations mediated by

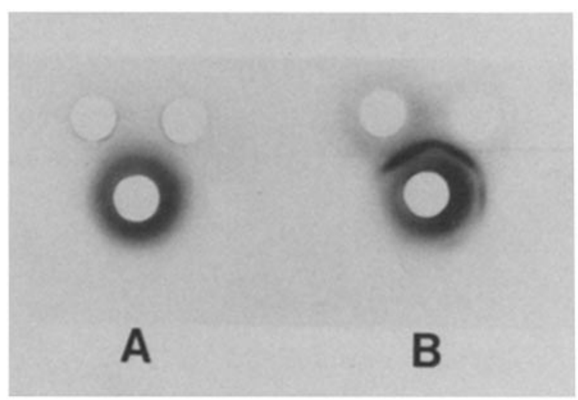

FIGURE 2 Ouchterlony double-diffusion analysis of anti-RNP reactions. Crude 30S RNP particles (10 μg) were placed in the center well of each set, and serial dilutions (10 μl) of either pre-immune serum (*A*) or antiserum against whole purified 30S particles (*B*) were placed in the outer wells. Serum dilutions shown are 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32 (clockwise from eleven o'clock). Gels were allowed to diffuse for 48 h at 0°–4°C in a humidified chamber and were stained with Coomassie Brilliant Blue R-250. Similar Ouchterlony patterns have been obtained with low-salt gels and with mixed-detergent micelle gels (see text) using antibodies against either the 34,000- to 40,000-dalton RNP-specific polypeptides or purified 30S RNP particles.

SDS. These experiments demonstrated that antibodies raised against either the isolated major RNP polypeptides, or the whole purified 30S RNP particles, reacted strongly with RNP polypeptides derived from treatment of 30S RNP with SDS. Regardless of the Ouchterlony system used for analysis, sera obtained from animals before immunization produced no precipitin lines (Fig. 2*A*). None of the immune sera reacted with RNA extracted from 30S RNP or with ribosomal RNA.

RNase-free gamma-globulins, isolated from each immune and pre-immune serum were used in subsequent tests of antibody specificity and in all immunofluorescence experiments. Direct and indirect immunoprecipitation of 30S RNP complexes was always found to be severalfold greater with gamma-globulin from immune sera than from pre-immune sera (results not shown). However, varying degrees of nonimmune precipitation (aggregation) of the antigen sometimes occurred during the incubation periods required for immunoprecipitation. Accordingly, this limitation precluded the use of conventional immunoprecipitation assays for estimation of antibody titers.

Specificity of the anti-RNP antibodies for hnRNA-binding proteins was established by monitoring antibody binding to specific bands in SDS polyacrylamide slab gels, using modifications of the procedure described by Burridge (5, 6). For these tests, crude 30S RNP and total nuclear STM-9 extract were selected to maximize the number of potentially reactive nuclear proteins available for binding of the antibodies. Parallel gel strips were fixed and then either stained with Coomassie Brilliant Blue or incubated with the preparation of gamma-globulin being tested. Binding of the primary antibodies was detected by applying ^{125}I-rabbit anti-chicken IgG to the gel strips, followed by autoradiography.

All of the immune gamma-globulins had a strong selective affinity for the major RNP polypeptides in the 34,000- to 40,000-dalton region of the gels (Fig. 3). Gamma-globulin from animals immunized with whole 30S RNP complexes also showed slight antibody activity against some of the minor proteins found in crude preparations of 30S RNP (Fig. 3*E*). Antibodies raised against just the major 34,000- to 40,000-dalton RNP-specific polypeptides were found to react slightly with one to three higher molecular weight species (Fig. 3 *C* and *H*), suggesting that these may share antigenic determinants with the major polypeptides, or are more likely to be oligomers of one or more of them; cross-links can be formed between members of the 34,000- to 40,000-dalton group to yield oligomers of this size (22). There was no antibody binding apparent on parallel gel strips treated with the corresponding preparations of pre-immune gamma globulin (Fig. 3 *B* and *F*). Similarly, there were no reactions in controls not treated with the primary antibody (Fig. 3*A*). The higher level of background binding apparent in Fig. 3 *C* seems to result from the presence in these immunoglobulins of antibodies directed against components of the acrylamide gel; this is not surprising because the RNP proteins were isolated from gels, and we have subsequently found that preincubation of the gamma globulins with blank gel pieces reduces this background (Fig. 3 *H*).

The above results indicate that *in situ* binding of antibodies to specific bands in polyacrylamide gels appears to be of particular value in the study of nuclear proteins and membrane components because detection of antigen-antibody reactions does not rely on the immunoprecipitation or antigen solubility. It should be particularly useful where iodinated staphylococcal protein A cannot be used directly because of the source of the primary antibody. Using this approach, we have been able to

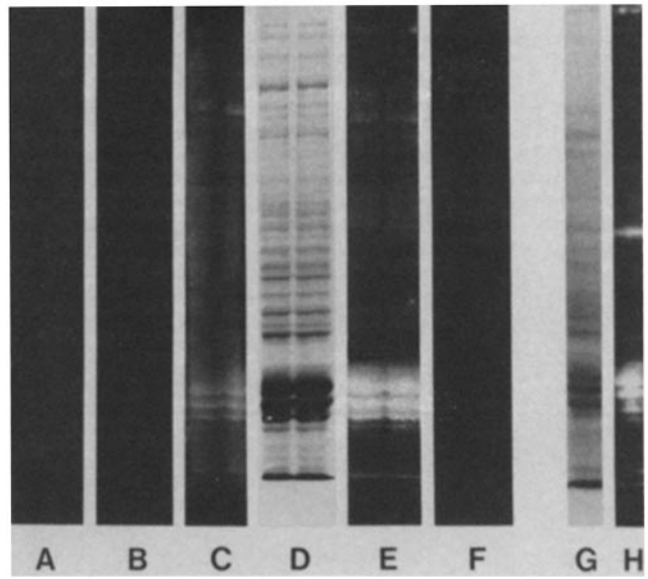

FIGURE 3 Selective binding of anti-RNP antibodies to RNP polypeptides in SDS polyacrylamide gels. Proteins from crude 30S RNP were separated in SDS polyacrylamide gels, fixed, and reacted with gamma-globulins isolated from pre-immune and anti-RNP sera. Binding of antibodies to protein bands was detected by autoradiography, after treatment of all gels with ^{125}I-rabbit anti-chicken IgG. Antibody-binding specificities were compared for (*A*) control (buffer G), (*B*) IgG from animal *C* before immunization, (*C*) IgG from animal immunized with just the 34,000- to 40,000-dalton RNP polypeptides, (*D*) parallel gel stained with Coomassie Brilliant Blue, (*E*) IgG from animal immunized with whole purified 30S RNP particles, and (*F*) IgG from animal *E* before immunization. Proteins from total nuclear STM-9 extracts were reacted with IgG from animal *C* (*H*). A duplicate strip stained with Coomassie Brilliant Blue is shown in *G*.

detect reactions between the antibodies and 30S RNP proteins in total nuclear extracts of Taper hepatoma cells (Fig. 3 *G* and *H*), and also show that the anti-RNP antibodies do not react with ribosomal proteins or histones. This demonstration of the specificity of our antibodies for proteins known to be components of hnRNP has allowed us to investigate the intracellular distribution of these antigens in Taper hepatoma cells by indirect immunofluorescence microscopy.

Immunofluorescent Localization of 30S RNP Antigens in Taper Hepatoma Cells

Intact hepatoma cells attached to poly-L-lysine-coated coverslips were fixed and treated with 20 μg of the chicken gamma-globulin under investigation. The cell layer was subsequently treated with FITC-conjugated rabbit anti-chicken IgG for visualization of the binding of the primary antibodies to the cells by fluorescence microscopy.

Results of a typical experiment are shown in Fig. 4. All cells treated with the immune gamma-globulins were consistently found to be brightly fluorescent (Fig. 4, center). Strong fluorescence was apparent using antibodies against either whole 30S RNP particles or against just the major RNP-specific polypeptides. There was no detectable fluorescence in cells treated with gamma-globulin taken from the same animals before immunization (Fig. 4, top), or in controls, where treatment with the primary antibody was deleted (Fig. 4, bottom).

Examination of the cells under higher magnification (Fig. 5) showed more clearly that the fluorescence was restricted to the nucleus, with some areas more intensely fluorescent than others. Moreover, the nucleolar areas were free of fluorescence. Absence of fluorescence from nucleoli and cytoplasm was obtained under a variety of different conditions of fixation, and was not caused by general loss of antigens or lack of

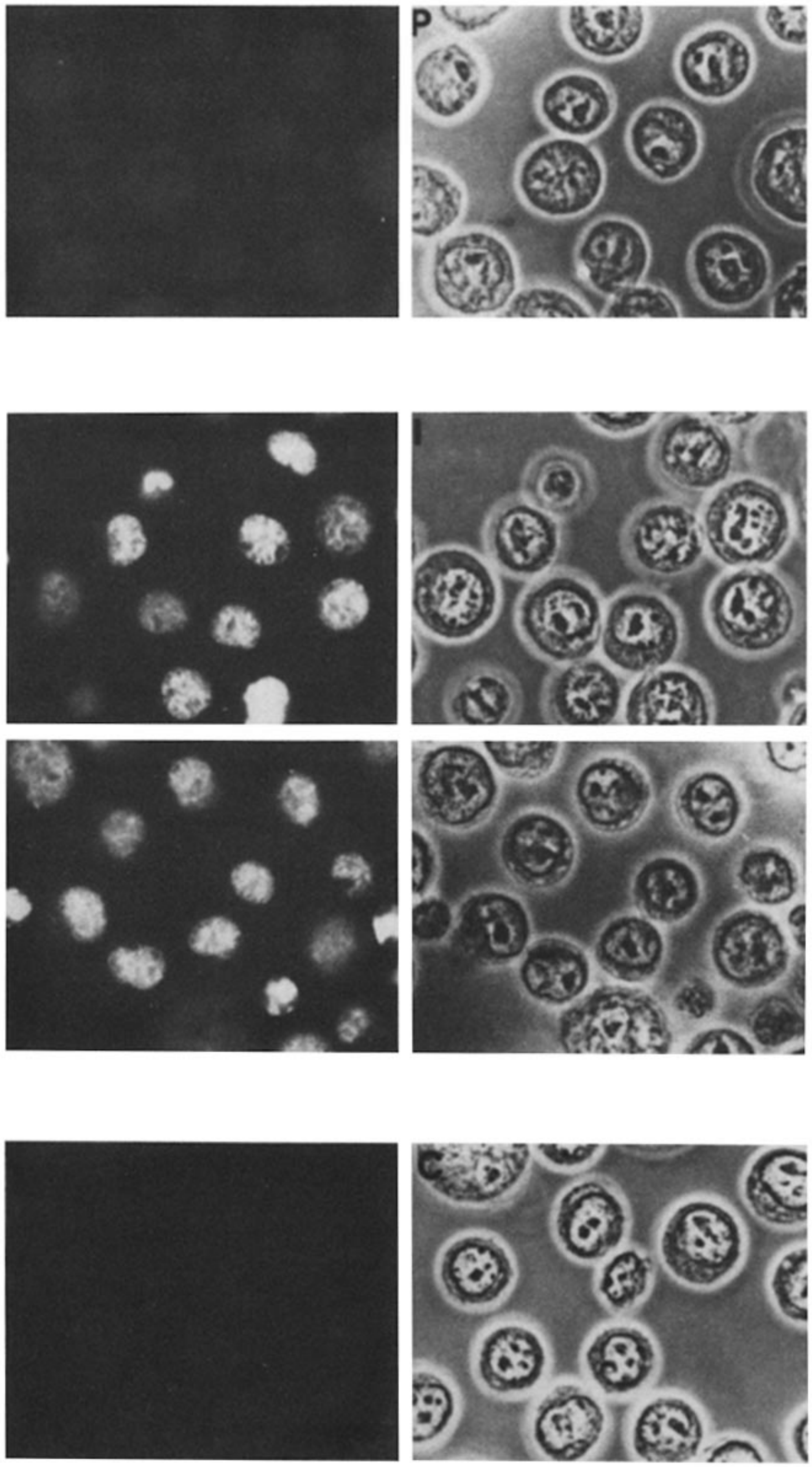

FIGURE 4 Localization of 30S RNP antigens in mouse ascites cells by indirect immunofluorescence. Coverslip preparations of Taper hepatoma cells were processed for indirect immunofluorescence microscopy and identical fields were examined using phase-contrast and epi-fluorescence optics (right and left panels, respectively). Before treatment with fluorescein-conjugated rabbit anti-chicken IgG, the cells were incubated with either (*I*) 20 μg of IgG from an animal immunized with whole purified 30S RNP particles, (*P*) 20 μg of IgG from the same animal prior to immunization, or (*C*) an equivalent volume of PBS (control). Fluorescence of cells treated with IgG from animals immunized with the gel-purified 34,000- to 40,000-dalton RNP polypeptides did not differ significantly from that shown in *I*. × 600.

antibody penetration. This conclusion is supported by the fact that, under identical experimental conditions, nucleoli were found to be brightly fluorescent when treated with selected human auto-immune sera known to contain antinucleolar antibodies (not shown). The masking of antigens may also prevent or limit their detection by the immunofluorescence technique as shown with mouse sperm protein (28) and actin (11). In an attempt to expose these antigens, we have treated cells with various reducing agents (beta-mercaptoethanol, dithiothreitol) and a protein denaturant, guanidinium chloride as described by Rodman et al. (28). In addition, we have monitored the distribution of 30S RNP antigens during their extraction in STM-9 buffer. In neither of these experiments have nucleoli or cytoplasm shown any fluorescence. Thus, the lack of fluorescence in cells treated with our anti-RNP gamma-globulin under various conditions suggests an absence of the 30S RNP antigens at these sites.

Reaction of Antibodies to Mouse 30S RNP Proteins with Chicken Cells

Because we had previously demonstrated that the proteins of 30S subcomplexes of hnRNP from various vertebrate species had similar electrophoretic properties and amino acid composition (21), we had some confidence that our antibodies to mouse antigens might also react with the analogous proteins in other species. If so, the utility of our antibodies as biological probes would be greatly increased. Initial studies using the indirect immunofluorescence technique showed that the antibodies would bind to human (HeLa) and to amphibian (Triturus) cells (results not shown). A nuclear restriction of the reacting antigens was also observed in these heterologous cell types. Perhaps more surprising was the finding that the antibodies would also bind to the nuclei of cells from chickens, i.e., the species in which the antibodies had been induced. The reaction of the chicken anti-mouse RNP immunoglobulins with chick cerebral cells is shown in Fig. 6. Fluorescence in both glial and neural cells was restricted to the nucleus, and as with mouse cells the nucleoli appeared to be less fluorescent than the surrounding nucleoplasmic (or euchromatic) regions.

Absence of RNP Antigens from Adult Chick Erythrocytes

The 30S RNP proteins are associated with the rapidly synthesized hnRNA in vertebrate cells, and we may anticipate that these polypeptides would be absent from cells not actively synthesizing RNA. The ability of our anti-mouse RNP immunoglobulins to react strongly with the analogous chicken antigens enabled us to carry out a simple extreme test of this prediction. When adult chicken erythrocytes were mixed with chick embryonic cerebral cells and both exposed to the anti-RNP antibodies, the cerebral cells reacted strongly while the erythrocytes were entirely negative (Fig. 6). Although the objection regarding masked antigens again arises, the finding that the transcriptionally inactive nuclei of the erythrocytes were accessible to immunoglobulin under our conditions as demonstrated by their ability to bind antihistone antibodies, reinforces the negative result with anti-30S RNP immunoglobulins.

DISCUSSION

The utility and reliability of antibodies as biological probes are critically dependent on the titer and specificity of the immunoglobulins obtained. As previous workers had reported (17), we also found great difficulty in eliciting the production of effective antisera to rodent hnRNP core proteins (of the 30S RNP subcomplexes) in rabbits. A probable cause of this difficulty is the apparent conservation of the structure of these 34,000- to 40,000-dalton polypeptides amongst vertebrates (21). The response of chickens to the mouse antigens is perhaps the result of minor differences which are also reflected in the slightly different electrophoretic behavior of avian 30S RNP proteins compared with mammalian polypeptides (3). Fortunately, once induced, the chicken anti-mouse RNP immunoglobulins will also react with avian and lower vertebrate antigens, therefore extending the range of experiments of potential biological interest available to us.

The antibodies induced in chickens with either intact purified 30S RNP subcomplex or the 34,000- to 40,000-dalton

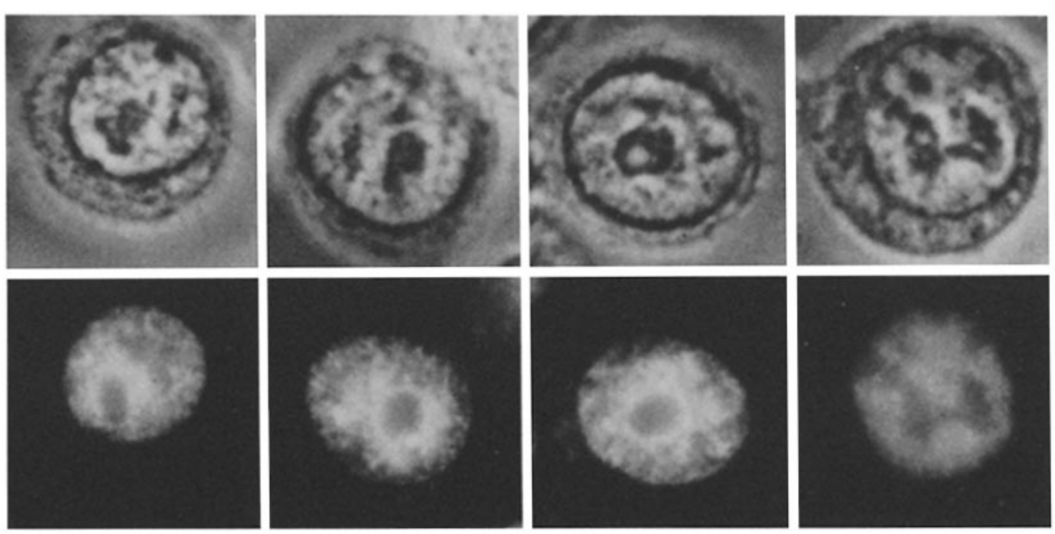

FIGURE 5 Distribution of 30S RNP antigens in interphase mouse ascites cells. Taper hepatoma cells were treated with anti-RNP antibodies and prepared for fluorescence microscopy as in Fig. 5. Upper panels: phase-contrast micrographs of interphase cells. Lower panels: same cells photographed under epi-fluorescent illumination. × 1,900.

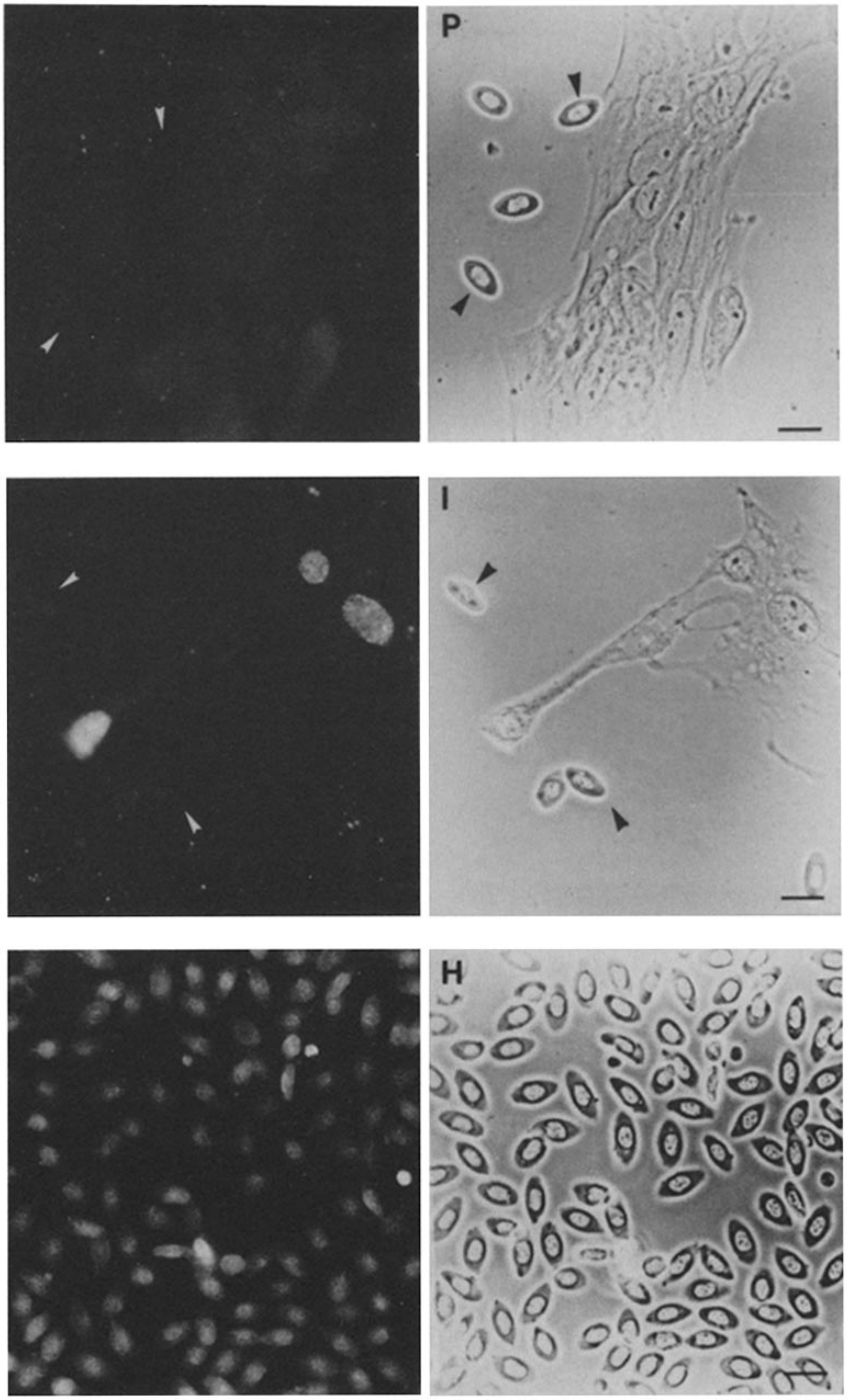

FIGURE 6 Comparison of the amounts of 30S RNP antigens in chicken embryonic cerebral cells and mature erythrocytes. Cerebral cells from a 10-d chick embryo were cultured in vitro for 7 d, fixed, and washed with Hank's balanced salt solution. The same coverslips were rinsed briefly in poly-L-lysine (5 μg/ml) and adult chicken erythrocytes were allowed to attach. Preparations were then fixed and prepared for fluorescence microscopy as in Fig. 5 with either pre-immune (*P*) or immune (*I*) IgG. Erythrocytes (arrows) show no fluorescence, while the cerebral cells have a typical nuclear-restricted distribution. Similarly fixed adult erythrocytes were treated with rabbit-anti-histone H3 (*H*) serum (1:20) obtained from Dr. B. D. Stollar; the nuclear fluorescence indicates that erythrocyte nuclei were penetrable by immunoglobulins under our conditions. Bar, 10 μm.

polypeptide group appear to be quite specific for the proteins of the complex as judged by the reaction of the gamma-globulins of immune sera with nuclear proteins electrophoretically resolved on acrylamide gels (Fig. 3). Both types of antibodies detect all of the major 30S RNP core polypeptides. Because the antibodies raised against RNP detect the free protein components, and the antibodies to electrophoretically purified polypeptides react with intact RNP, they cannot readily be used to distinguish between the location in the cell of hnRNP as such, or any "free" pool of the proteins. This reservation must be borne in mind when considering our immunofluorescent localization experiments. On the other hand, the broad ability of our antibodies to detect the protein antigens in both bound and free form gives us some confidence that we will not overlook some cryptic state of these proteins in the various differentiated cell types.

In light of the above it must be considered significant that we cannot detect the RNP proteins in the cytoplasm of interphase cells. The biochemical approaches of Lukanidin et al. (17) using similarly induced antisera had failed to detect nuclear 30S RNP antigens on polysomal mRNP, and polypeptide analyses of preparations of cytoplasmic free mRNP and derived polysomal mRNP have failed to show significant levels of the 34,000- to 40,000-dalton group. Taken together with the findings reported here, we must assume that these polypeptides have an entirely intranuclear function, or that if they do indeed move with mRNA to the cytoplasm they are very rapidly displaced so that the steady-state level in the cytoplasm is extremely low.

The apparent absence of the 30S RNP antigens from the nucleoli of mammalian and avian cells (Figs. 5 and 6), raises different questions. The biochemical evidence suggests that the 30S RNP core proteins bind to hnRNA sequences very soon after they are synthesized (21, 30), and that they therefore may comprise the protein associated with nascent hnRNA chains visualized by electron microscopy (23). It is possible that the same proteins might bind to nascent pre-rRNA transcripts, even though analyses of nucleolar preribosomal particles have not shown a particular enrichment in polypeptides of the relevant size range (14), and despite the failure to detect significant amounts of ribosomal RNA in purified 30S RNP. Our immunofluorescent studies clearly suggest a minimal involvement of the major hnRNP proteins in the processing of pre-rRNA. Such a negative result with immunological techniques used on whole cells clearly demands evidence that the negative site is indeed accessible to immunoglobulins. The positive reaction of sera containing anti-nucleolar antibodies with similarly treated cells lends some weight to the negative result using the anti-RNP antibodies.

Because we presume that the 30S RNP polypeptides are primarily involved in the early processing of hnRNA, we may expect that transcriptionally inactive nuclei would lack these proteins. An extreme case of such a nucleus is that of the mature avian erythrocyte. The failure to detect any 30S RNP antigens by immunofluorescence in nuclei of these cells, while other chicken cells are strongly positive, certainly supports this view of the role of these proteins in the nucleus (Fig. 6). Again, the negative result requires a positive control to demonstrate antibody accessibility, in this case provided by the strong binding of antihistone antibodies to the erythrocyte nucleus. While the results therefore strongly suggest that hnRNP core proteins are absent from transcriptionally inactive nuclei, it must be considered that the erythrocyte is a terminal cell, and it will be of some interest to examine transcriptionally dormant cell types for the presence of proteins presumably required immediately upon reactivation of the transcriptional process.

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