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THE SMALL NUCLEAR RIBONUCLEOPROTEINS THAT REACT WITH ANTI-Sm AND ANTI-RNP ANTIBODIES

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SUMMARY: We have examined the polypeptide pattern of small nuclear ribonucleoprotein particles that react with monoclonal anti-Sm antibodies or polyclonal anti-(U1)RNP antibodies. The fresh nuclear extracts analyzed were prepared from human cells that had been pulse-chased with a mixture of 15 ^{3}H-labeled amino acids. In contrast to previous reports in the literature, the apparent molecular weights of the major polypeptides that remained in the 1 M NaCl-washed ribonucleoprotein-antibody complexes were approximately 80000, 55000, 28000, 25000, 14000 and 9000, when probed with monoclonal anti-Sm antibodies, and about 69000, 58000 and 35000, when polyclonal anti-(U1)RNP antibodies were used.

U1, U2, U4, U5 and U6 snRNAs* are present in eukaryotic cells in snRNPs, and antibodies termed anti-Sm recognize all these snRNP species, while anti-RNP antibodies react only with U1 snRNP (1). Several studies have been done to determine the protein composition of the U snRNPs, using anti-Sm and anti-RNP antibodies as probes (1-8), or nonimmunological fractionation procedures (9, 10), and the results vary substantially among different laboratories. Previously, the snRNP polypeptides were detected either by labeling whole cells with one radioactive amino acid or by staining the proteins with Coomassie brilliant blue or silver (1-10). In the present report, cells were incubated with a mixture of 15 ^{3}H-labeled amino acids, to ensure a more proportional labeling of the various U snRNP polypeptides.

METHODS: Human cell RNA was labeled with [^{3}H]uridine for 16 h (11). Proteins were labeled with a mixture of 15 ^{3}H-labeled amino acids (NET-250, New England Nuclear). Cells were incubated for 30 min in medium free of those amino acids, then for 30 min with the radioactive amino acids (70 μCi/ml), next for 1.5 h at 1/20 the standard amino acid concentration, and finally for 16 h in regular medium. The low molecular weight (≤12S) fraction of nuclear extract was prepared

* Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle; PBS, 0.14 M NaCl/10 mM phosphate, pH 7.2; SDS, sodium dodecyl sulfate.

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as described before (11). All solutions were adjusted to 1 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride shortly before use. The conditions for immunoprecipitation (1) and immunoaffinity chromatography (11) were as described earlier, except that for protein analysis 2 M LiCl/4 M urea/40 mM Tris-HCl (pH 7.4) was used as the final eluting agent, instead of 2.5 M $MgCl_2$ (pH 7.2). Similar results were obtained with either eluting agent in labeled RNA experiments. RNA was deproteinized and electrophoresed in 10% polyacrylamide-7 M urea gels (12). For protein analysis, a mixture of carrier proteins (molecular weight: 12.3-150K) was added to each fraction (40 μg/ml), followed by precipitation with 25% trichloroacetic acid (30 min on ice), centrifugation and acetone washing of the pellets, in plastic tubes that had been precoated with serum albumin (4 μg/ml). Proteins were analyzed by 12.5% polyacrylamide gel electrophoresis (13). ^3H was detected by fluorography (14). The serum from an autoimmune disease patient was chosen because it showed high titers of anti-RNP antibodies without detectable anti-Sm antibodies by passive hemagglutination, and was negative for anti-DNA by immunofluorescence. Human Ig and monoclonal anti-Sm (15) Ig were prepared as indicated previously (16).

RESULTS: We tested first the effect of increasing ionic strengths (to minimize nonspecific electrostatic interactions) on the retention of U snRNAs by monoclonal anti-Sm antibodies (15). HeLa cell RNA was labeled in vivo with [^3H]uridine, and the nuclear extract was immunoprecipitated with monoclonal anti-Sm Ig. After washing the immunoprecipitates with buffered 0.5 M and 1 M NaCl, the majority of the U snRNA species remained in the pellet (Fig. 1A). The specificity of the reaction can be appreciated from the absence of RNA in the immunoprecipitate of mouse nonimmune Ig (Fig. 1A). Similar experiments were done with nuclear extracts from HeLa cells that had been incubated with a mixture of 15 ^3H-labeled amino acids. In this case, nuclear extract fractions were immunoprecipitated with the anti-RNP antibodies mentioned below, and the unreacted material was incubated then with monoclonal anti-Sm antibodies. In the low molecular weight range, four polypeptides, whose molecular weights were approximately 28000, 25000, 14000 and 9000 (and to which we will refer to as 28K, 25K, 14K and 9K) could be seen in the 1 M NaCl-washed monoclonal anti-Sm antibody immunoprecipitate above the background of the nonimmune Ig immunoprecipitate (Fig. 1B). In comparable experiments, mainly three polypeptides were detected at a higher molecular weight range: ~80K and ~55K; plus a fainter ~69K band (Fig. 1C). The 80K and 55K proteins seen in the 1 M NaCl-washed pellet, were not obviously apparent in the 0.5 M and 1 M NaCl washes (Fig. 1C). The protein patterns of the original supernatant from the immunoprecipitation with anti-Sm an-

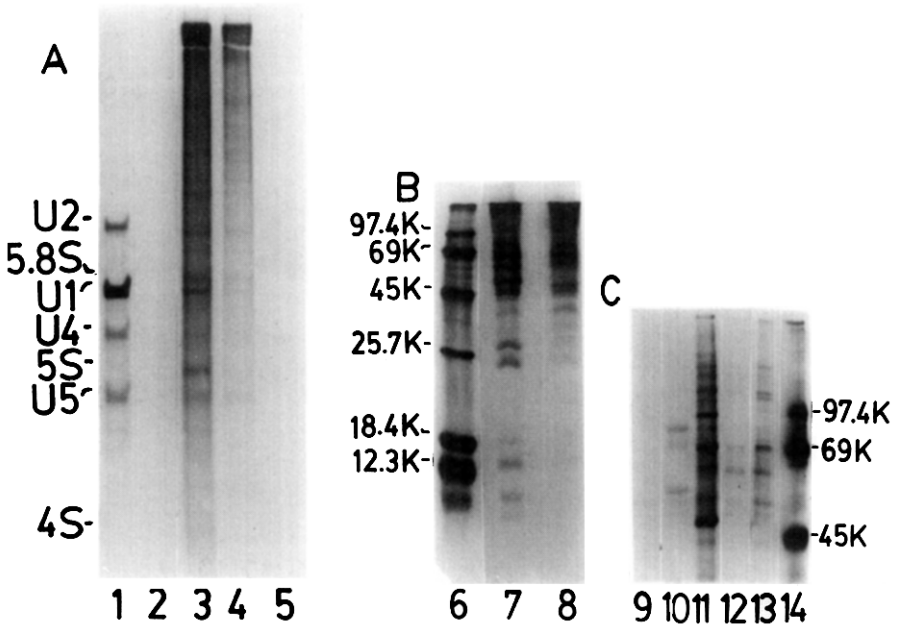

Fig. 1. RNA species (A) and polypeptides (B and C) immunoprecipitated with monoclonal anti-Sm antibodies, visualized by fluorography after polyacrylamide gel electrophoresis. A, B and C are separate electrophoreses. In A the lanes are: washes of the immunoprecipitate with buffered 0.15 M NaCl (3), 0.5 M NaCl (4) and 1 M NaCl (5), and the washed pellet (1) after immunoprecipitation with monoclonal anti-Sm antibodies; and the final pellet obtained with mouse nonimmune Ig (2). Comparable portions of each fraction are displayed. In B and C the ≤12S fraction of the nuclear extract was the starting material. Final washed pellets after immunoprecipitation with monoclonal anti-Sm (7 and 10) or mouse nonimmune (8 and 9) Ig. First supernatant (11), buffered 0.5 M NaCl wash (12) and 1 M NaCl wash (13) after immunoprecipitation with monoclonal anti-Sm Ig. ^{14}C-labeled proteins were used as molecular weight standards (6 and 14). Similar proportions of each fraction are displayed in lanes 7 and 8, as well as 9, 10 and 12. The X-ray film exposure in lane 13 is ~5.5-fold longer than the rest in C.

tibodies and of the immunoprecipitate of mouse nonimmune Ig (Fig. 1C) suggest specificity of the reaction.

The nuclear extract from KB cells that had been labeled overnight with [^{3}H]uridine was loaded next on a Sepharose 4B column of anti-RNP Ig. The great majority of the retained RNA remained in the column in the presence of 1 M NaCl (Fig. 2A). U1 RNA was essentially the only RNA species present in the final 2.5 M $MgCl_2$ eluate, and little U1 RNA was washed off the column with up to 1 M NaCl (Fig. 2A). The titer of our anti-RNP antibodies was high, since virtually all the U1 snRNP loaded was retained by the antibody column. In similar experiments, cells were incubated with a mixture of 15 ^{3}H-labeled amino acids. After

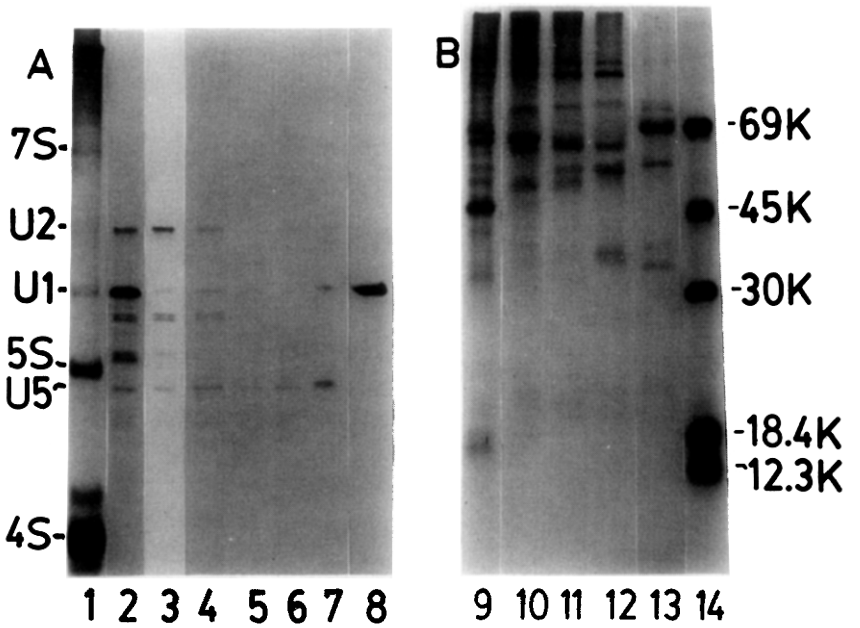

Fig. 2. RNA (A) and polypeptide (B) gel electrophoresis patterns from immunosorbent affinity chromatography fractions using polyclonal anti-RNP antibodies. The samples in the lanes shown in A are: material loaded on the column (≲12S fraction of the nuclear extract) (2); flow-through (3); buffered 0.3 M NaCl (4), 0.5 M NaCl (5), 1 M NaCl (6) and 1% sodium deoxycholate/1% Triton X-100/1 M NaCl (7) washes; and 2.5 M $MgCl_2$ eluate (8) from the anti-RNP antibody column. Comparable portions of each fraction are displayed in lanes 4-8. The X-ray film exposure in lane 3 is ~2.5-fold longer than the rest. Cytoplasmic RNA species (1) were used as standards. In B the samples in the lanes shown are: flow-through (9); buffered 0.3 M NaCl (10), 0.5 M NaCl (11) and 1 M NaCl (12) washes, and buffered 2 M LiCl/4 M urea eluate (13) from the anti-RNP antibody column. Similar proportions of each fraction are displayed in lanes 10-13. ^{14}C-labeled proteins were used as molecular weight standards (14).

the loaded antibody column was washed with buffered 1 M NaCl, three major protein bands were detected in the final eluate: ~69K; ~58K and ~35K; plus a fainter ~39K band (Fig. 2B). These anti-(U1)RNP antibodies react directly with the 69K (or 70K) protein in protein gel immunoblots (16). In the final eluate from the anti-(U1)RNP antibody column, the fainter bands larger than 69K do not appear to be specific, since they were seen also in the eluate from nonimmune human Ig columns. The 69K, 58K and 35K bands were not readily detectable in the 0.3 M-1 M NaCl washes (Fig. 2B). X-ray film overexposure did not reveal any bands in the ≲19K range in the final eluate from the anti-(U1)RNP columns. The high intensity of the 69K band with respect to the 58K and 35K bands in the eluate from the anti-(U1)RNP column (Fig. 2B) may be artifactual, since our nonimmune Ig columns sometimes retained some material migrating as ~69K.

DISCUSSION: In this study, the polypeptides immunoprecipitated with monoclonal anti-Sm antibodies were mainly six: 80K; 55K; 28K; 25K; 14K and 9K. Three of them (25K, 14K and 9K) may be equivalent to those observed in some laboratories (1, 6, 7, 9, 10). The monoclonal anti-Sm antibodies react directly (in immunoblots) with the 28K protein (17, 16). We assume that the 80K, 55K, 25K, 14K and 9K proteins bind directly or indirectly to the 28K polypeptide; this association is stable in the presence of 1 M NaCl. There is no precedent in the literature for the 80K and 55K proteins in this report. In order to lower their U1 snRNP content, the nuclear extract fractions were immunoprecipitated with anti-(U1)RNP antibodies before incubation with monoclonal anti-Sm antibodies. This is why the polypeptide pattern of our anti-Sm immunoprecipitates did not exhibit substantial levels of the proteins immunoprecipitated by the anti-(U1)RNP antibodies. Our anti-(U1)RNP antibody columns retained mainly three polypeptides: 69K; 58K and 35K. The same batch of antibodies reacted with the 69K (or 70K) protein in immunoblots (16), as do some other anti-(U1)RNP sera (17). The presence of a ~69K protein in U1 snRNP is in agreement with several previous reports (18, 8, 9). The 35K protein in the present study could be equivalent to the 33K protein observed by others (1, 9). There is no precedent for our finding of a 58K protein associated with U1 snRNP. Five of the proteins reported as components of U1 snRNP isolated by biochemical fractionation (28K, 16K, 13K, 12K and 11K) (9) were not seen in the final eluate from our anti-(U1)RNP antibody columns. It does not appear that these proteins were retained by the columns and then removed with the washes at increasing ionic strength, since they were not detected in those washes either (Fig. 2). The absence of low molecular weight proteins from the antigen-antibody complex only occurred using anti-(U1)RNP antibodies, but not with anti-Sm antibodies, suggesting that it was not the result of trivial losses during our handling procedure.

The patterns of the apparently major proteins retained by anti-RNP and anti-Sm antibodies starting from crude extracts vary substantially among several laboratories (1-8). The patterns that we obtained in turn differ from those reported by others. The reasons for these apparent differences are not known.

However, a few points should be considered. Either protein staining with Coomassie brilliant blue or silver, or incorporation of a radioactive amino acid were used in all previous reports (1-10). Estimation of the relative concentration of various polypeptides based on these methods could be potentially erroneous, because of differences in staining capacity among various proteins or in amino acid composition. We have used a mixture of 15 radiolabeled amino acids which should result in very proportional labeling of different polypeptides. It seems unlikely that our results would reflect protein aggregation because the samples were incubated in boiling water in the presence of 2% SDS and 5% 2-mercaptoethanol immediately before electrophoresis. It is possible that the presence of apparently new high molecular weight polypeptides and absence of small polypeptides could represent lack of degradation, since, among other precautions, we used freshly dissolved phenylmethylsulfonyl fluoride in our buffers. Some precautions were taken to minimize the possibility of loss of small polypeptides during handling. Plastic tubes were used that had been precoated with protein. Carrier proteins (40 μg/ml) were included in the only precipitation step (using cold 25% trichloroacetic acid). Moreover, similar results were obtained when there were no measurable losses during handling. We have verified that the majority of the U snRNA present in nuclear extracts sedimented with the low molecular weight fractions used in this report. Therefore, the bulk of the U snRNP population was analyzed in the present study, instead of a small subpopulation. The concentration in the particle of a protein whose turnover rate in particle form was high would be underestimated with our experiments, since pulse-chase radiolabeling conditions were used. However, it would be expected that most U snRNP proteins should have long metabolic half lives, as do U snRNAs.

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REFERENCES

1. Lerner, M.R. and Steitz, J.A. (1979) Proc. Nat. Acad. Sci. USA 76, 5495-5499.

2. Douvas, A.S., Stumph, W.E., Reyes, P., and Tan, E.M. (1979) J. Biol. Chem. 254, 3608-3616.
3. White, P.J., Gardner, W.D., and Hoch, S.O. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 626-630.
4. Takano, M., Golden, S.S., Sharp, G.C., and Agris, P.F. (1981) Biochemistry 21, 5929-5936.
5. Barque, J.P., Yeni, P., Peraudeau, L., Danon, F., and Larsen, C.J. (1981) Biochem. Biophys. Res. Comm. 99, 284-291.
6. Kinlaw, C.S., Dusing-Swartz, S.K., and Berget, S.M. (1982) Mol. Cell. Biol. 2, 1159-1166.
7. Matter, L., Schopfer, K., Wilhelm, J.A., Nyffenegger, T., Parisot, R.F., and De Robertis, E.M. (1982) Arthritis Rheum. 25, 1278-1283.
8. Wooley, J.C., Zukerberg, L.R., and Chung, S.-Y. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 5208-5212.
9. Hinterberger, M., Pettersson, I., and Steitz, J.A. (1983) J. Biol. Chem. 258, 2604-2613.
10. Kinlaw, C.S., Robberson, B.L., and Berget, S.M. (1983) J. Biol. Chem. 258, 7181-7189.
11. Smith, J.H. and Eliceiri, G.L. (1983) J. Biol. Chem. 258, 4636-4640.
12. Chandrasekharappa, S.C., Smith, J.H., and Eliceiri, G.L. (1983) J. Cell. Physiol. 117, 169-174.
13. Laemmli, U.K. (1970) Nature 227, 680-685.
14. Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83-88.
15. Lerner, E.A., Lerner, M.R., Janeway C.A., Jr., and Steitz, J.A. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 2737-2741.
16. Symington, J., Gurney, T., Jr., and Eliceiri, G.L. (1984) Biochem. Biophys. Res. Comm. 120, 81-87.
17. Steitz, J.A., Wolin, S.L., Rinke, J., Pettersson, I., Mount, S.M., Lerner, E.A., Hinterberger, M., and Gottlieb, E. (1982) Cold Spring Harbor Symp. Quant. Biol. 47, 893-900.
18. White, P.J. and Hoch, S.O. (1981) Biochem. Biophys. Res. Comm. 102, 365-371.