Ultrastructural Distribution of Nuclear Ribonucleoproteins as Visualized by Immunocytochemistry on Thin Sections

STANISLAV FAKAN,* GEORGE LESER, and TERENCE E. MARTIN

*Center of Electron Microscopy, University of Lausanne, 1101 Lausanne, Switzerland, and Swiss Institute for Experimental Cancer Research, 1066 Epalinges, Switzerland; and Department of Biology, University of Chicago, Chicago, Illinois 60637

ABSTRACT The ultrastructural distribution of nuclear ribonucleoproteins (RNP) has been investigated by incubation of thin sections of mouse or rat liver, embedded in Lowicryl K4M or prepared by cryoultramicrotomy, with antibodies specific for RNP. The antibodies were localized by means of a protein A-colloidal gold complex. Anti-small nuclear (sn)RNP antibodies, specific for determinants of the nucleoplasmic snRNP species containing U_1, U_2, U_4, U_5, and U_6 RNAs, were found associated preferentially with perichromatin fibrils, interchromatin granules, and coiled bodies. This indicates an early association of snRNP with structural constituents containing newly synthesized heterogeneous nuclear RNA. It also suggests a possible structural role of some snRNPs in nuclear architecture.

Antibodies against the core proteins of heterogeneous nuclear RNP particles associate preferentially with the border regions of condensed chromatin, and in particular with perichromatin fibrils and some perichromatin granules. These results are discussed in view of recent knowledge about the possible role of nucleoplasmic RNP-containing components in the functions of the cell nucleus.

The fine structural distribution of nuclear ribonucleoproteins (RNP)[1] has been extensively studied during the last two decades by techniques of ultrastructural cytochemistry and autoradiography (for review see reference 10). These investigations have allowed the characterization of nucleoplasmic structural components such as perichromatin granules, interchromatin granules, and coiled bodies. In addition, the introduction of a new differential staining technique for nuclear nucleoproteins (4) enabled Monneron and Bernard (21) to describe perichromatin fibrils, nuclear RNP constituents that could not be visualized by standard double fixation and double staining procedures. These latter structures have been shown to represent the in situ morphological counterpart of newly synthesized heterogeneous nuclear (hn)RNP (2, 11), and kinetic analysis of the distribution of radioactive RNA demonstrated that these constituents are formed within areas at the periphery of condensed chromatin, and that at least a part of them then migrates towards the interchromatin space (11, 24; for review see reference 6). Similar experiments revealed only a low level of [^{3}H]uridine labeling in the clusters of interchromatin granules, even after prolonged periods of chase (7, 8), and suggested the presence of rather stable RNA species within these nuclear components.

Although the above methodological approaches provide good evidence about the RNA nature of various nuclear structural constituents, we have no direct information about the species of RNA and proteins contained in these elements. Ultrastructural immunocytochemistry using specific anti-RNP antibodies as probes appears to be a good tool with which to approach this problem. In recent years information regarding some of the components of biochemically isolated small nuclear (sn)RNP and hnRNP has been obtained (summaries in references 12, 18) and antibodies to snRNP and hnRNP protein determinants are now available.

We have made use of a simple technique permitting direct immunolocalization of cellular constituents on ultrathin sections employing specific antibodies, which are then visualized in the electron microscope by means of a complex of colloidal gold with protein A (28). The distribution of nucleoplasmic snRNPs as well as of hnRNPs within nuclear structural components has been analyzed.

MATERIALS AND METHODS

Material: Mouse or rat livers were fixed for 1 h with 0.5 or 1% glutaraldehyde in Sorensen phosphate buffer (0.1 M), pH 7.3, at 4°C. The pieces of tissue were then thoroughly washed in the same buffer. One part of the material was dehydrated through a series of ethanol concentrations at low temperature and embedded in the resin Lowicryl K4M (1, 14). Another part of the tissue was subjected to cryoultramicrotomy (32). The following antibodies were used for immunolabeling: (*a*) total human immunoglobulin from an anti-Sm (for definitions see 31, 12) obtained from a patient suffering from mixed connective tissue disease (courtesy of Dr. H.-J. Lakomek, Medizinische Klinik and Poliklinik, Universität Düsseldorf). This Ig precipitates U_1, U_2, U_4, U_5, and U_6 snRNAs from cell extracts by virtue of protein determinants bound to these RNAs. The Ig binds to several polypeptides of molecular weight 25,000–35,000 on immunoblots of electrophoretically-separated cellular proteins; (*b*) mouse

[1] *Abbreviation used in this paper:* RNP, ribonucleoproteins; sn, small nuclear RNA; hn, heterogeneous nuclear RNA.

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 JANUARY 1984 358-363

monoclonal IgG of anti-Sm type from a hybridoma derived from an autoimmune mouse (courtesy of Drs. Joan Steitz and Charles Janeway, Yale University, New Haven, CT). This antibody has been characterized by Lerner et al. (16) and precipitates snRNP containing U_1, U_2, U_4, U_5 and U_6 snRNAs. The antibody used in these experiments was obtained from ascites fluid; (*c*) Mouse monoclonal IgM from a hybridoma formed from spleen cells of a mouse immunized with chicken 30S hnRNP subcomplexes and specific for the hnRNP core group polypeptides of molecular weight 34,000–40,000 (17; and Leser, Escara-Wilke, and Martin, submitted for publication). This monoclonal IgM binds protein A when associated with its antigen, and in terms of polypeptide specificity and localization of antigens by immunofluorescence is essentially identical to the previously reported polyclonal anti-hnRNP antibodies (13, 19).

Experimental Procedures: Ultrathin sections of Lowicryl-embedded liver were placed on nickel grids and allowed to dry. They were then preincubated in 1% ovalbumin in PBS for 15 min at room temperature, and the excess of liquid was carefully removed and the grid was floated on a drop of the incubation mixture consisting of diluted antibody (1:200–1:1,000, i.e., 50–10 μg protein/ml) in PBS with 1% ovalbumin. The incubation usually takes place at 4°C, for 17 h, in a humid chamber. When human anti-Sm immunoglobulin was used, herring sperm DNA (Sigma Chemical Co., St. Louis, MO, 500 μg/ml) was added to adsorb traces of anti-DNA activity often present in these sera. The preparations were then thoroughly washed with PBS and postincubated for 1 h with the protein A-colloidal gold complex (mean particle diameter 15 nm) at room temperature. After another wash with PBS, the grids were rinsed with distilled H_2O and dried. This procedure corresponds essentially to that described by Roth (28). The sections were then stained with uranyl acetate (2.5% aqueous) for 5 min and counterstained for 30 s with lead citrate.

When ultrathin cryosections were used, the nickel grids with sections were first washed with phosphate buffer to remove sucrose. They were briefly soaked with PBS-0.01 M glycine, incubated for 10 min with 5% BSA in PBS-glycine, and then rinsed with PBS-glycine. The immunolabeling with the antibody (1:200) was performed in PBS for 20 min at room temperature. The grids were then extensively washed with PBS-glycine, PBS alone, and then floated on the protein A-gold solution for 20 min. After this step, the preparations were again thoroughly washed with PBS then with distilled H_2O and stained directly with uranyl acetate and lead citrate. Some grids were stained by the EDTA procedure (4), as modified for cryosections (11).

Control experiments were carried out by incubating the sections with antibodies preadsorbed for 30 min with the appropriate antigens: a ~10-S nuclear fraction for anti-Sm antibodies and a 30-S RNP fraction for the anti-hnRNP core protein antibody isolated from sucrose density gradients essentially as in (26). In addition, some sections were also incubated with the protein A-gold complex alone. All the preparations were observed in a Philips EM 400 electron microscope at 60 or 80 kV using a 40-μm objective aperture.

A quantitative evaluation of the distribution of labeling was performed on the resin sections incubated with the human anti-Sm serum as well as on cryosections labeled with anti-hnRNP monoclonal antibody. The surface area of the nucleolus, condensed chromatin regions, nucleoplasm, and cytoplasm surrounding the nucleus, were measured on 10 randomly chosen cells in each series of experiments. The number of gold particles in each cell compartment was counted and the labeling density expressed as number of particles per square micron.

RESULTS

The analysis of sections after Lowicryl embedding is favored because they display, after a simple uranyl-lead staining, a contrast comparable with that of Epon sections stained by the differential EDTA method for nuclear nucleoproteins (4). Consequently, chromatin appears grey-clear, whereas the RNP-containing constituents exhibit high contrast and are easily recognizable. The nuclear morphology is very well preserved compared with routinely-used plastic embedding media.

The results of control experiments using preadsorption of antibodies with the corresponding antigens on one hand, and incubation with the protein-A-colloidal gold complex alone on the other hand, are both negative, leaving only a very low background.

Distribution of Anti-snRNP Antibodies

The patterns of topological distribution of the human anti-Sm and of the anti-Sm monoclonal antibodies were identical. The reactivity of the former was, however, much stronger, presumably because more determinants are recognized.

The label did not occur in the nucleolar region and within condensed chromatin areas. The antibodies were most frequently associated with the clusters of interchromatin granules (Figs. 1 and 4) and with perichromatin fibrils (Figs. 1 and 2). In the nuclei of mouse as well as rat liver, where a coiled body was observed (~10–20% of nuclear sections examined), it was usually strongly labeled as well (Fig. 3). Perichromatin granules, which are found as individual structures or in small groups, were in general only rarely labeled or remain unlabeled (Figs. 1 and 2). The labeling of the cytoplasm was generally low, except that some labeling could be detected in erythrocytes.

Distribution of the Anti-hnRNP Core Protein Antibody

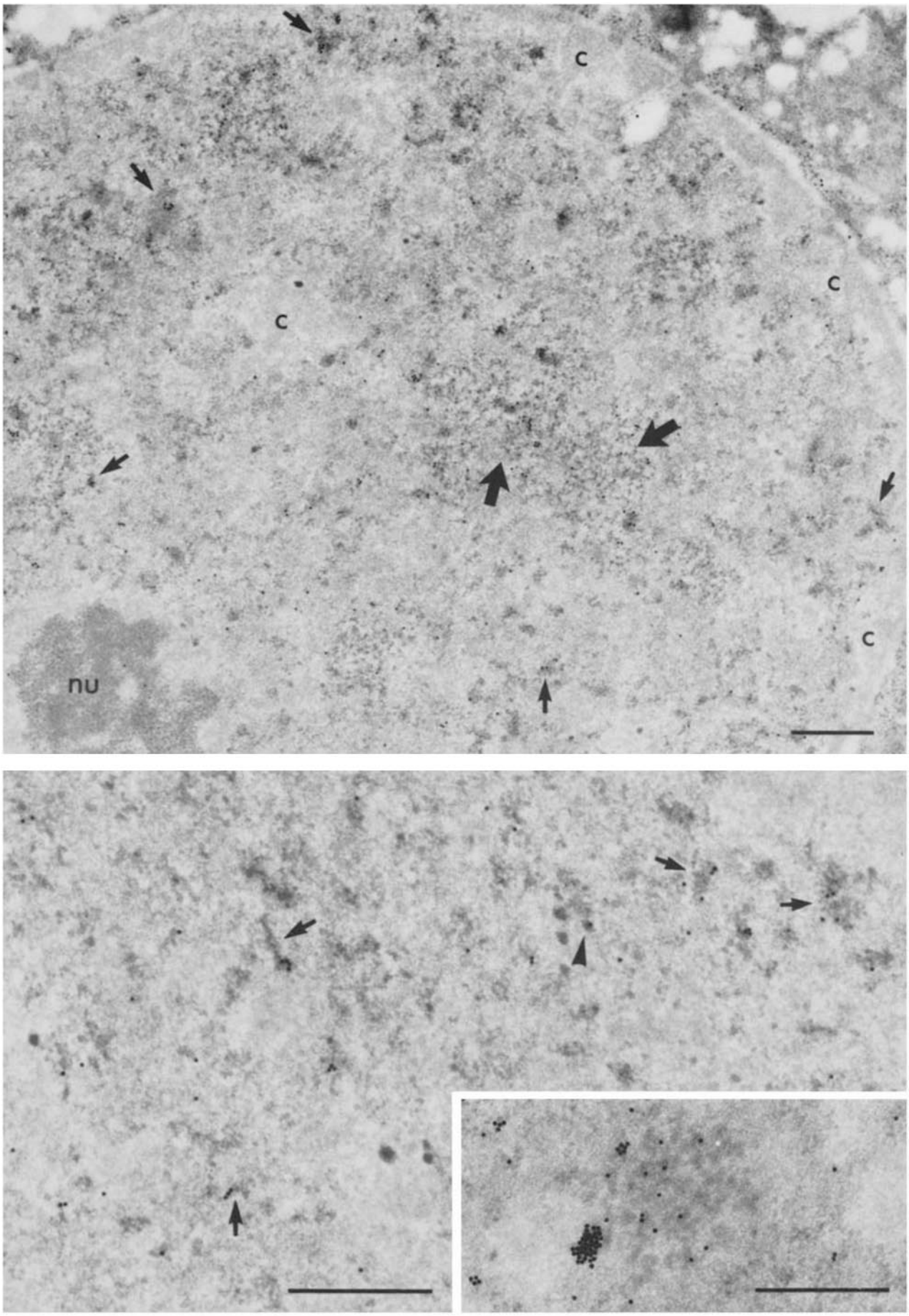
This monoclonal antibody associated preferentially with perichromatin fibrils (Fig. 6) and with perichromatin granules (Fig. 7). It reacted strongly on ultrathin frozen sections, where the gold particles decorated especially the perichromatin region (Fig. 5). In these sections, the perichromatin fibrils were, however, hardly distinguishable (11, 23). Coiled bodies often appeared labeled in both types of sections, but with lower intensity than after reaction with anti-snRNP antibodies. Clusters of interchromatin granules were occasionally labeled. The nucleoli and areas of condensed chromatin remained unlabeled. Cytoplasmic labeling was generally low with some preference for mitochondria, and some labeling could be observed in erythrocytes present in the liver tissue.

Quantitative Analysis of Antibody Distribution

The quantitative evaluation of the gross labeling distribution on our cell sections reveals respectively the following gold particle densities (±SEM): (*a*) resin sections labeled with human anti-Sm serum: nucleolus, 3.56 ± 0.94; condensed chromatin, 1.54 ± 0.41; nucleoplasm, 8.84 ± 0.59; cytoplasm, 2.74 ± 0.37; (*b*) ultrathin frozen sections labeled with anti-hnRNP: monoclonal IgM, nucleolus 2.17 ± 0.31; condensed chromatin, 4.77 ± 0.53; nucleoplasm, 23.79 ± 1.45; cytoplasm, 3.22 ± 0.47. Although these determinations show the preferential labeling of the nucleoplasm by antibodies to both snRNP and hnRNP (Wilcoxon signed rank, $p < 0.01$), they do tend to underestimate the specificity of localization. In effect, most of the label was concentrated on structures occupying only a limited minor portion of the nucleoplasm, the surface of which is, e.g., for perichromatin fibrils, hardly measurable. In addition, since it has been demonstrated that, at least in the resin sections, the protein A-gold labeling is restricted to the surface of the sections, only antigenic sites exposed at the section surface were revealed. In ultrathin cryosections, some penetration of antibodies might occur, probably depending on the density of the cellular structural components (for discussion of these questions, see reference 28).

DISCUSSION

This work makes the following novel observations concerning the presence of RNP components within nucleoplasmic structures: (*a*) perichromatin fibrils contain snRNP as well as hnRNP antigens; (*b*) perichromatin granules (or at least a certain proportion of them) contain hnRNP, but only little



FIGURES 1–3 Sections of mouse liver embedded in Lowicryl K4M and labeled with human anti-Sm serum. Bar, 0.5 μm. Fig. 1: An overview of a nucleus showing label associated preferentially with perichromatin fibrils (small arrows) and clusters of interchromatin granules (large arrows). The nucleolus (*nu*) and areas of condensed chromatin (*c*) remain unlabeled. × 30,000. Fig. 2: A detail of a nucleoplasmic region with labeled perichromatin fibrils (arrows) and occasional labeled perichromatin granules (arrowhead). × 53,800. Fig. 3: A detail of a labeled coiled body. × 49,500.

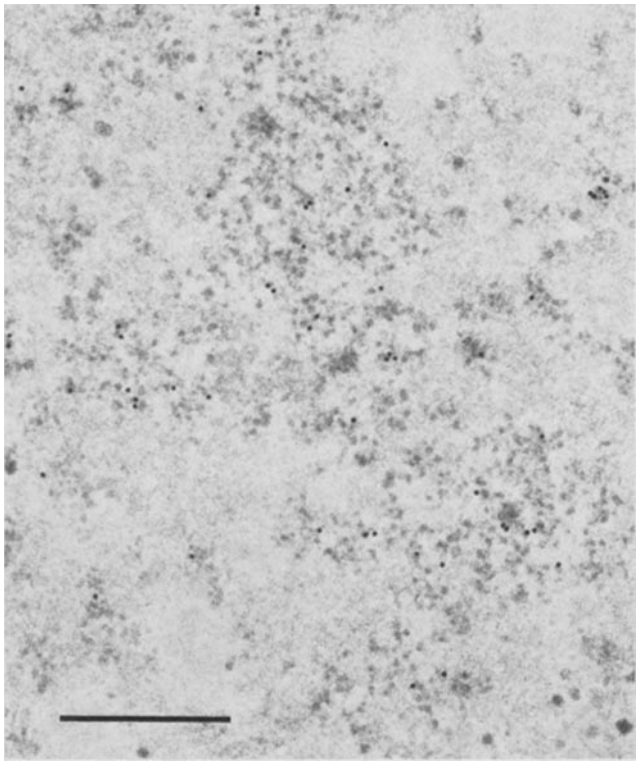


FIGURE 4 A detail of a large labeled cluster of interchromatin granules. × 45,000.

snRNP; (*c*) interchromatin granules and coiled bodies carry high amounts of snRNP. The latter constituents also contain a small quantity of hnRNP. The term "perichromatin fibrils" as used here includes all nucleoplasmic RNP fibrils regardless of whether they appear in the perichromatin areas or within the interchromatin space. Since it has been shown that the fibrils appearing within the interchromatin space result from the migration of fibrils formed in the perichromatin areas (11, 22) towards the interchromatin space (11, 24), and because all the fibrils are morphologically identical, we do not employ the term "interchromatin fibrils" used by certain authors (25), to avoid confusion.

It has been previously demonstrated that perichromatin fibrils contain newly synthesized hnRNA (2, 11). After their formation the majority of the fibrils migrate towards the interchromatin space and their RNA undergoes processing during that period (11). In addition, the frequency of perichromatin fibrils in the nucleus reflects the rate of hnRNA synthesis in different cell systems (9; for review see reference 6). The fact that the anti-hnRNP core protein antibodies label the perichromatin fibrils provides additional evidence confirming the hnRNP nature of these constituents.

As far as the perichromatin granules are concerned, their role in nuclear functions still remains obscure. Hypotheses have been proposed as to their possible role in the transport of pre-mRNA within the nucleus (21), or in its intranuclear storage (9); in addition, it is not clear whether there are several populations of perichromatin granules with regard to the type of RNA that they contain (for review see references 10 and 25). Preliminary attempts to isolate biochemically the perichromatin granules have indicated that they may contain hnRNP core proteins (5). Our labeling experiments with the anti-hnRNP protein antibody indicate the presence of hnRNP in at least some perichromatin granules in mouse and rat liver cells. An attempt to quantify this phenomenon using ultrathin frozen sections, where these granules are particularly visible, is now in progress.

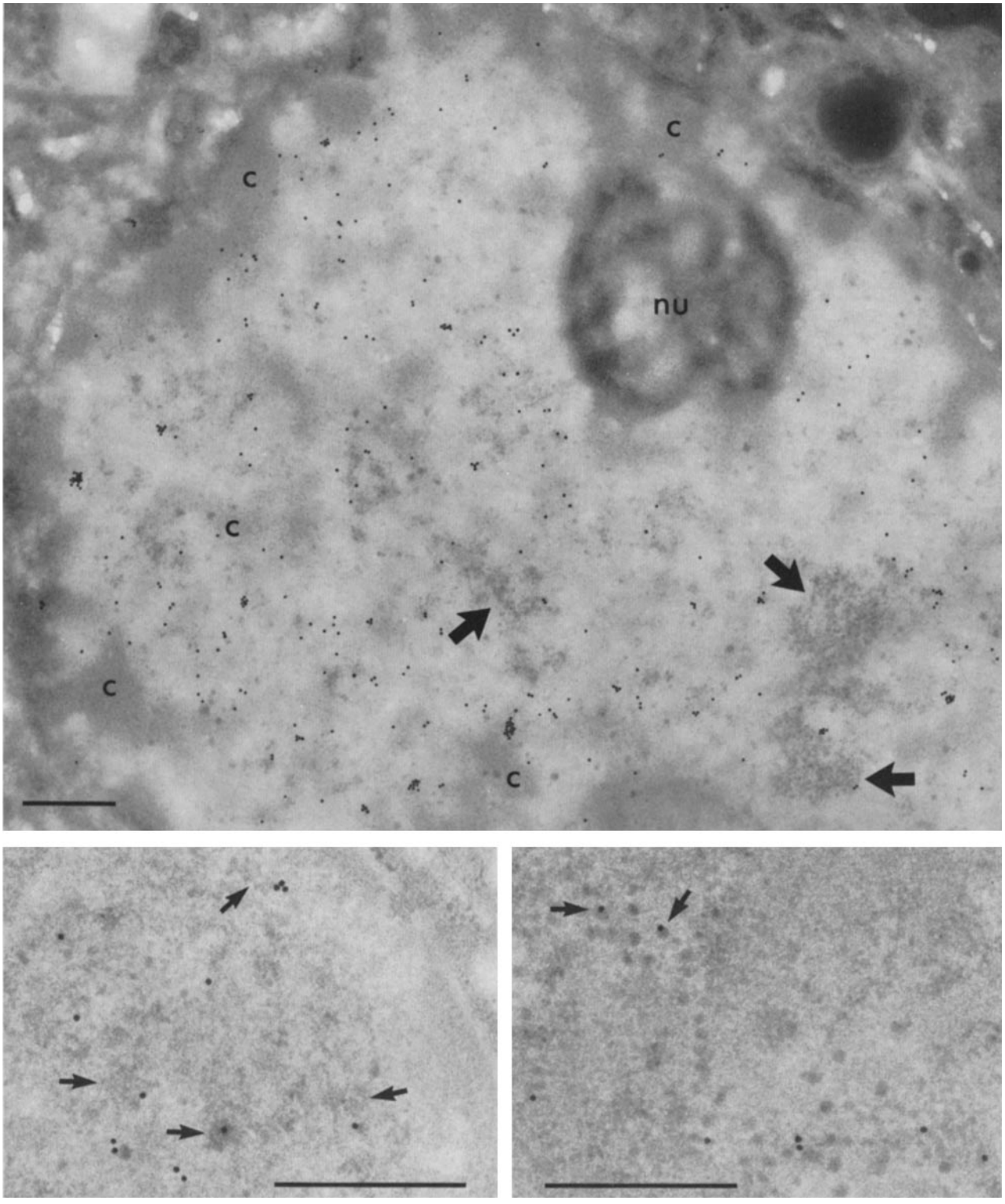
The structure of snRNAs and their role in nuclear functions have been extensively investigated in many laboratories (for review see references 27 and 29). Hypotheses have been formulated as to their possible structural role in nuclear pre-mRNP particles (30) and more recently as to their involvement in pre-mRNA splicing (15). The anti-snRNP antibodies used in this study react with five species of nucleoplasmic snRNPs (U_1, U_2, U_4, U_5, and U_6; 27). Their association with perichromatin fibrils indicates the simultaneous occurrence of at least some of these snRNPs together with newly synthesized hnRNPs in these components. The low labeling of perichromatin granules with anti-snRNP antibodies represents an interesting finding, suggesting that these granules are involved only to a very limited extent in the functions proposed by the above hypotheses. From the technical point of view, since the immunoreaction has been shown to take place only at the surface of resin sections (28), the question arises as to the possible inaccessibility of the antigenic sites in these structures. However, the fact that perichromatin granules are labeled under the same conditions with anti-hnRNP antibody that is, in general, less reactive than human anti-Sm serum, does not favor this possibility. The very rare labeling of interchromatin granules we observed would speak against the possibility that stable core proteins contributing to hnRNP structure accumulate within these granules.

The finding of snRNPs within clusters of interchromatin granules might suggest a storage role for this type of RNA in these constituents, a hypothesis in agreement with previous kinetic studies showing only a low level of [^{3}H]uridine labeling in clusters of interchromatin granules (7, 8) and suggesting the presence of rather stable RNA species. The reaction of anti-snRNP antibodies with clusters of interchromatin granules may be the source of the commonly observed speckled pattern obtained in immunofluorescence studies with autoimmune disease sera (31, 16). Since interchromatin granules are the only clearly recognizable nucleoplasmic component within the network called "the inner nuclear matrix" (3, 20), a possible structural role of the snRNPs in nuclear architecture cannot be excluded.

Our present observations about the occurrence of snRNP and also of hnRNP antigens in the coiled body represent a new finding about this enigmatic structure, described many years ago as an RNP containing nucleoplasmic constitutent (21). The possible function of this nuclear structure remains, for the moment, completely unclear.

The authors would like to thank Miss F. Flach, Mrs. J. Fakan, Mrs. C. Kaczmarek, and Mr. S. Monsma for excellent technical assistance, Dr. T. Pexieder for help with computer analysis, and Dr. Hans Will, University of Heidelberg for assisting us in obtaining a range of human autoimmune sera. They are grateful to Drs. A. Gautier and R. Hancock for discussion and critical reading of the manuscript, to Mr. O. Gaeng for photographic work, and to Mrs. F. Nyffenegger for the preparation of the manuscript.

This work was supported by the F. Hoffmann-La Roche Foundation, the Sandoz Foundation, the Geigy Jubilaums Stiftung, and by the Fonds National Suisse de la Recherche Scientifique (grant No. 3.252-0.82) and the USPHS National Cancer Institute (CA 12550).



FIGURES 5–7 Sections labeled with anti-hnRNP core protein monoclonal antibody. Bar, 0.5 μm. Fig. 5: A general view of a rat liver cell nucleus from a section prepared by cryoultramicrotomy and stained by the EDTA technique. The contrast of condensed chromatin (*c*) is reduced. The nucleolus (*nu*) and the clusters of interchromatin granules (arrows) are unlabeled. Labeling is associated predominantly with the border of condensed chromatin areas. × 31,300. Figs. 6–7: Lowicryl sections of mouse liver showing association of label with the fine network of perichromatin fibrils (Fig. 6, arrows), and with some perichromatin granules (Fig. 7, arrows). × 64,500.

Received for publication 10 August 1983, and in revised form 17 September 1983.

REFERENCES

1. Armbruster, B. L., E. Carlemalm, R. Chiovetti, R. M. Garavito, J. A. Hobot, and E. Kellenberger. 1982. Specimen preparation for electron microscopy using low temperature embedding resins. J. Microsc. (Oxf.) 126:77–85.
2. Bachellerie, J. P., E. Puvion, and J. P. Zalta. 1975. Ultrastructural organization and biochemical characterization of chromatin RNA-protein complexes isolated from mammalian cell nuclei. *Eur. J. Biochem.* 58:327–337.
3. Berezney, R., and D. S. Coffey. 1977. Nuclear matrix. Isolation and characterization of a framework structure from rat liver nuclei. *J. Cell Biol.* 73:616–637.
4. Bernhard, W. 1969. A new staining procedure for electron microscopical cytology. *J. Ultrastruct. Res.* 27:250–265.
5. Daskal, Y., L. Komaromy, and H. Busch. 1980. Isolation and partial characterization of perichromatin granules. *Exp. Cell Res.* 126:39–46.
6. Fakan, S. 1978. High resolution autoradiography studies on chromatin functions. *In* The Cell Nucleus. Harris Busch, editor. Academic Press, Inc., New York, 5:3–53.
7. Fakan, S., and W. Bernhard. 1973. Nuclear labelling after prolonged ^{3}H-uridine incorporation as visualized by high resolution autoradiography. *Exp. Cell Res.* 79:431–444.
8. Fakan, S., and P. Nobis. 1978. Ultrastructural localization of transcription sites and of RNA distribution during the cell cycle of synchronized CHO cells. *Exp. Cell Res.* 113:327–337.
9. Fakan, S., and N. Odartchenko. 1980. Ultrastructural organization of the cell nucleus in early mouse embryos. *Biol. Cell.* 37:211–218.
10. Fakan, S., and E. Puvion. 1980. The ultrastructural visualization of nucleolar and extranucleolar RNA synthesis and distribution. *Int. Rev. Cytol.* 65:255–299.
11. Fakan, S., E. Puvion, and G. Spohr. 1976. Localization and characterization of newly synthesized nuclear RNA in isolated rat hepatocytes. *Exp. Cell Res.* 99:155–164.
12. Hinterberger, M., I. Pettersson, and J. A. Steitz. 1983. Isolation of small nuclear ribonucleoproteins containing U_1, U_2, U_4, U_5, and U_6 RNAs. *J. Biol. Chem.* 258:2604–2613.
13. Jones, R. E., C. S. Okamura, and T. E. Martin. 1980. Immunofluorescent localization of the proteins of nuclear ribonucleoprotein complexes. *J. Cell Biol.* 86:235–243.
14. Kellenberger, E., E. Carlemalm, W. Villiger, J. Roth, and R. M. Garavito. 1980. Low denaturation embedding for electron microscopy of thin sections. *Chemische Werke Lowi GmbH (Waldkraiburg, Federal Republic of Germany).* 1–59.
15. Lerner, M. R., J. A. Boyle, S. M. Mount, S. L. Wolin, and J. A. Steitz. 1980. Are snRNPs involved in splicing? *Nature (Lond.).* 283:220–224.
16. Lerner, E. A., M. R. Lerner, C. A. Janeway, Jr., and J. A. Steitz. 1981. Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology and autoimmune disease. *Proc. Natl. Acad. Sci. USA.* 78:2737–2741.
17. Leser, G. P., J. Escara-Wilke, and T. E. Martin. 1982. Studies of 30S hnRNP core proteins using monoclonal antibodies. *J. Cell Biol.* 95(2, Pt. 2):472*a*. (Abstr.)
18. Martin, T. E., J. M. Pullman, and M. D. McMullen. 1980. Structure and function of nuclear and cytoplasmic ribonucleoprotein complexes. *In* Cell Biology. A Comprehensive Treatise. D. M. Prescott and L. Goldstein, editors. Academic Press, Inc., New York. 4:137–174.
19. Martin, T. E., and C. S. Okamura. 1981. Immunocytochemistry of nuclear hnRNP complexes. *In* The Cell Nucleus. Harris Busch, editor. Academic Press, Inc., New York. 9:119–144.
20. Miller, T. E., C. Y. Huang, and A. O. Pogo. 1978. Rat liver nuclear skeleton and ribonucleoprotein complexes containing hnRNA. *J. Cell Biol.* 76:675–691.
21. Monneron, A., and W. Bernhard. 1969. Fine structural organization of the interphase nucleus in some mammalian cell. *J. Ultrastruct. Res.* 27:266–288.
22. Nash, R. E., E. Puvion, and W. Bernhard. 1975. Perichromatin fibrils as components of rapidly labeled extranucleolar RNA. *J. Ultrastruct. Res.* 53:395–405.
23. Puvion, E., and W. Bernhard. 1975. Ribonucleoprotein components in liver cell nuclei as visualized by cryioultramicrotomy. *J. Cell Biol.* 67:200–214.
24. Puvion, E., and G. Moyne. 1978. Intranuclear migration of newly synthesized extranucleolar ribonucleoproteins. *Exp. Cell Res.* 115:79–88.
25. Puvion, E., and G. Moyne. 1981. In situ localization of RNA structures. *In* The Cell Nucleus. Harris Busch, editor. Academic Press, Inc., New York. 8:59–115.
26. Quinland, T. J., P. B. Billings, and T. E. Martin. 1974. Nuclear ribonucleoprotein complexes containing polyadenylate from mouse ascites cells. *Proc. Natl. Acad. Sci. USA.* 71:2632–2636.
27. Reddy, R., and H. Busch. 1981. U snRNA's of nuclear snRNP's. *In* The Cell Nucleus. Harris Busch, editor. Academic Press, Inc., New York. 8:261–306.
28. Roth, J. 1982. The protein A-gold (pAg) technique—a qualitative and quantitative approach for antigen localization on thin sections. *In* Techniques in Immunocytochemistry. G. R. Bullock and P. Petrusz, editors. Academic Press, Inc., New York. 1:107–133.
29. Sekeris, C. E., and A. Guialis. 1981. Low-molecular weight nuclear ribonucleoprotein particles. *In* The Cell Nucleus. Harris Busch, editor. Academic Press, Inc., New York. 8:247–259.
30. Sekeris, C. E., and J. Niessing. 1975. Evidence for the existence of a structural RNA component in the nuclear ribonucleoprotein particles containing heterogeneous RNA. *Biochem. Biophys. Res. Commun.* 62:642–650.
31. Tan, E. M. 1979. Autoimmunity to nuclear antigens. *In* The Cell Nucleus. Harris Busch, editor. Academic Press, Inc., New York. 8:475–477.
32. Tokuyasu, K. T. 1973. A technique for ultracryotomy of cell suspensions and tissues. *J. Cell Biol.* 57:551–565.