RIBONUCLEOPROTEIN COMPONENTS IN LIVER CELL NUCLEI AS VISUALIZED BY CRYOULTRAMICROTOMY

E. PUVION and W. BERNHARD

From l'Institut de Recherches Scientifiques sur le Cancer, 94800 Villejuif, France

ABSTRACT

The interphase nucleus of the normal rat hepatocyte has been studied in ultrathin frozen sections after glutaraldehyde fixation and the modification of various staining procedures known to be specific for DNA structures (Moyne's thallium stain, Gautier's osmium-ammine) or preferential for RNP carriers and basic proteins (regressive stains based on the use of EDTA or citrate, negatively charged colloidal iron). The results are comparable to those obtained after classical dehydration and embedding. Particular attention has been paid to the nucleolus and extranucleolar RNP components, such as perichromatin fibrils and granules, as well as interchromatin granules. A striking observation was the uneven size and the strongly increased number of perichromatin granules, and the appearance of a contiguous interchromatin net, containing nucleoproteins. Cryoultramicrotomy without embedding appears to be very useful for the exploration of the nucleus in thick sections which remain sufficiently transparent even with the usual accelerating voltages.

Cryoultramicrotomy still has not found the widespread application it merits in spite of the fact that commercially available instruments and a basic technology exist, and papers demonstrating its useful complementarity to the classically applied methods have been published (1–4, 6, 7, 14, 15, 20, 21, 23). The main purpose of this study was to examine the nuclear components with some recently developed cytochemical methods, in order to determine whether these stains could also be adapted to ultrathin frozen sections, which was, according to our experience with other cytochemical applications, by no means certain. Furthermore, we wanted to see if any additional information compared to the already known ultrastructural organization of the cell nucleus could be gained and, therefore, the advantage of cryosectioning demonstrated. The ultrastructural investigation of nuclear ribonucleoprotein (RNP) components is still too much neglected compared to the numerous studies in molecular biology on transcription and processing phenomena of RNP in eukaryotic cells. Practically no progress has been made during the last 5 yr in localizing the various species of RNA in situ, and in ascribing a definite function to half a dozen different nuclear RNP carriers already identified in the electron microscope (19). The purification of the extranucleolar RNP components by means of density gradients has so far failed and seems very difficult to achieve, as they are closely linked with the chromatin fibers. It was therefore tempting to use cytochemical procedures from which new information could be expected in order to contribute to the necessary convergence of biochemical and ultrastructural data.
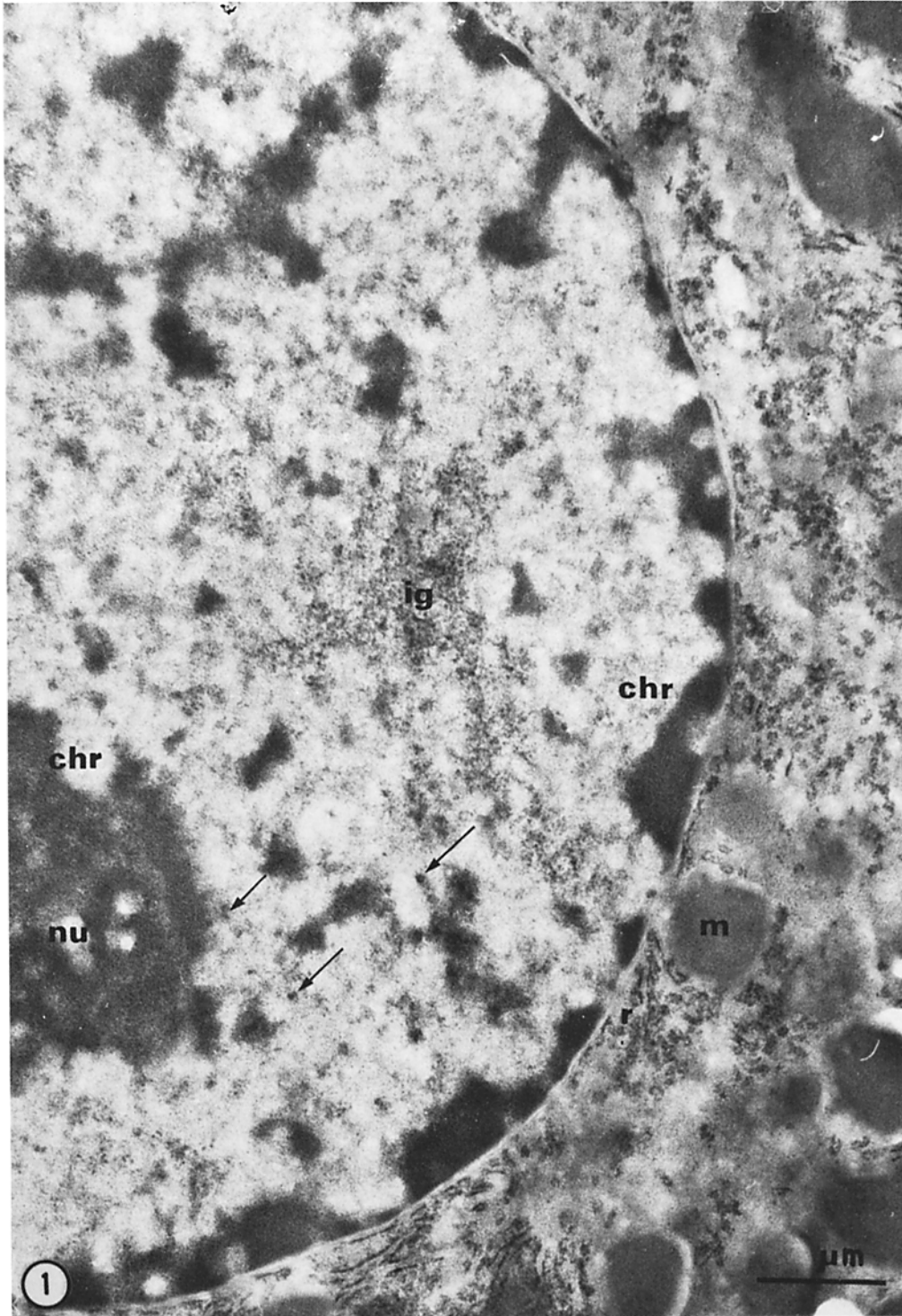
MATERIALS AND METHODS

FIGURE 1   Rat liver cell. Glutaraldehyde fixation for 1 h. Ultrathin frozen section. Portion of the nucleus and cytoplasm after classical staining with uranyl acetate for 1 min and poststaining with lead citrate for 10 s. Chromatin (chr), nucleolus (nu), and interchromatin granules (ig) in the nucleoplasm. A few perichromatin granules may be detected with difficulty (→). Ribosomes (r) and mitochondria (m) are present in the cytoplasm. The two types of nucleic acid cannot be distinguished by their contrast. × 18,000.
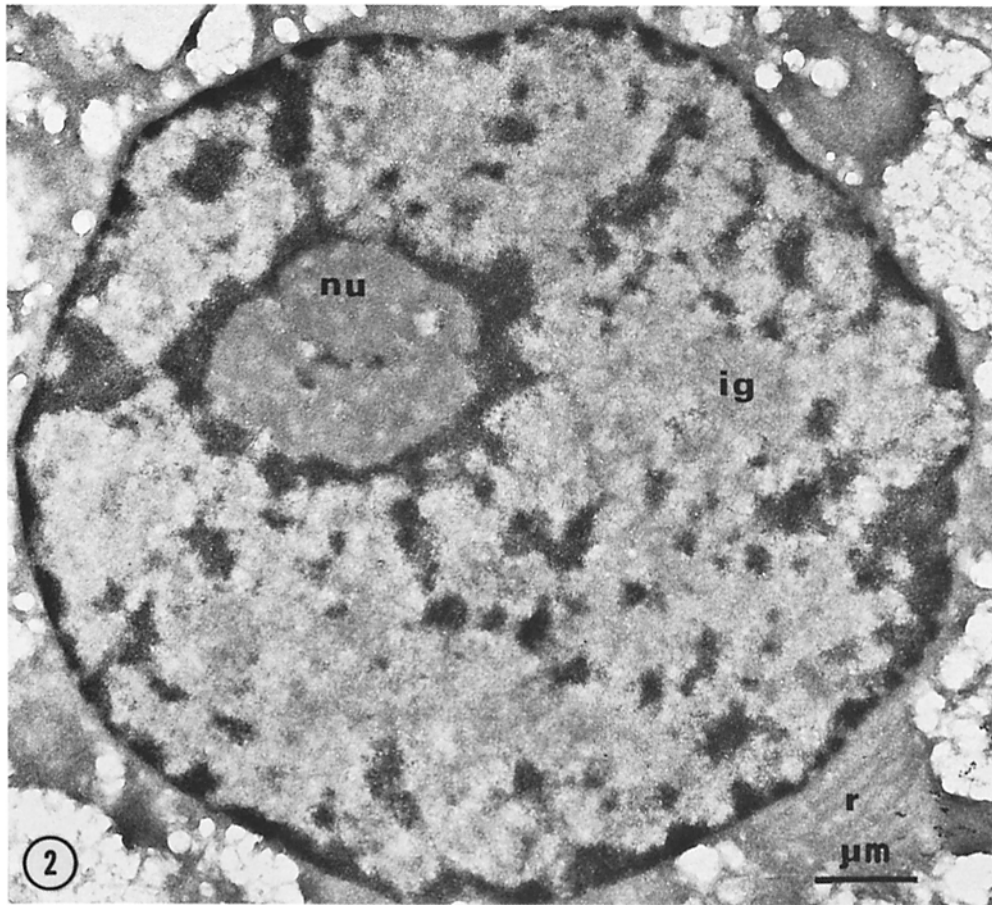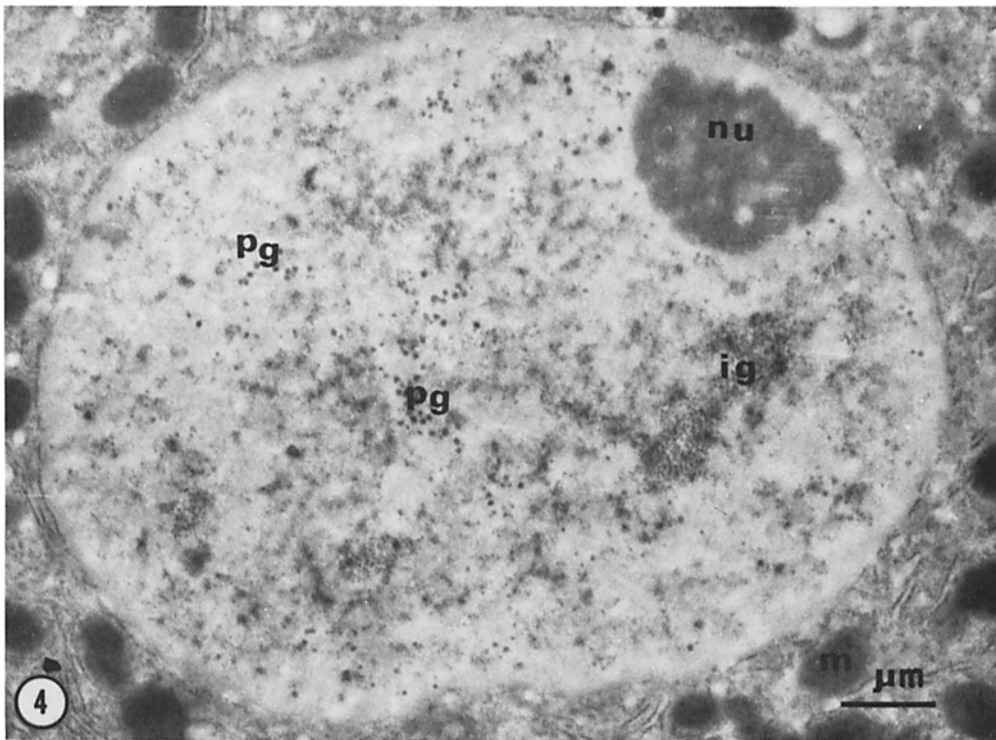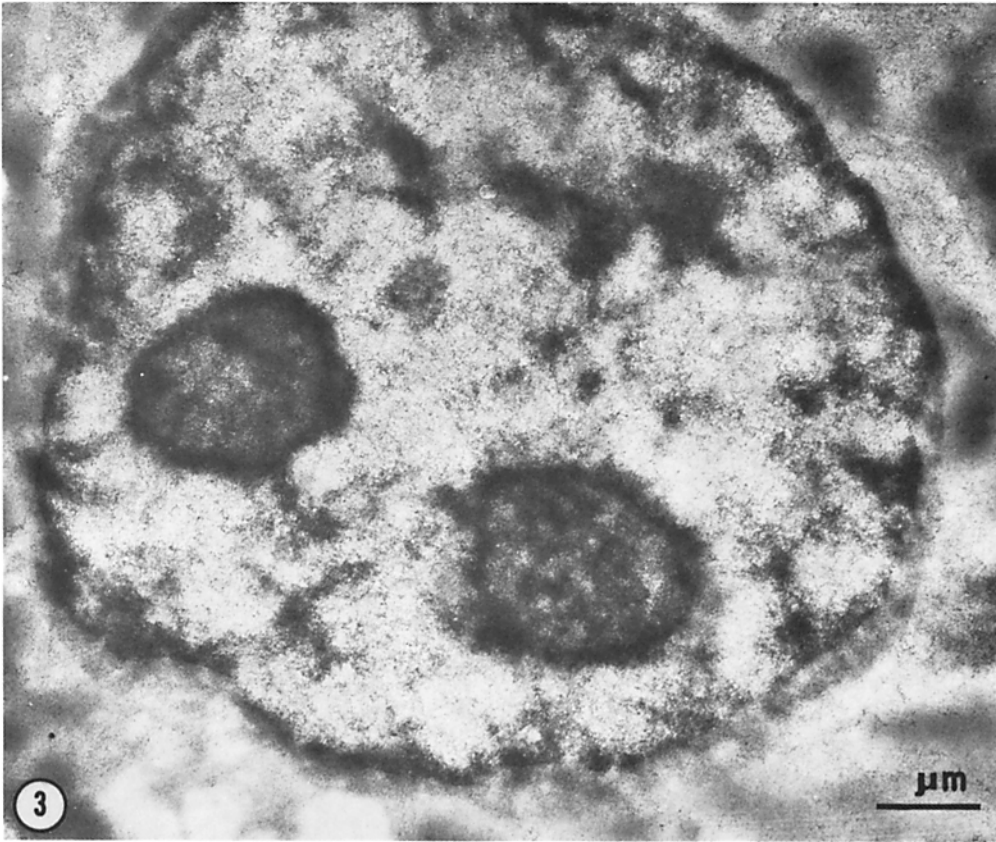
FIGURE 2 Rat liver cell nucleus. Ultrathin frozen section. Gautier's osmium-ammine stain, specific for DNA structures. Exclusive stain for the chromatin. The nucleolus (nu) and interchromatin granules (ig), as well as the ribosomes (r), remain unstained. × 12,900.

the liver was immediately removed and cut into small fragments of about 0.5-1 mm³ in 2.5% glutaraldehyde in cacodylate buffer, pH 7.2-7.4. The total fixation time was usually 1 h, but some specimens were fixed for 5 min or 15 min only, without noticeable difference in the fine structure of the nucleus. However, the tissue was easier to cut when fixed for a longer period. In some experiments, the tissue blocks were encapsulated for 5 min in 20%

gelatin at 37°C and subsequently soaked for 15 min in 30% glycerol to avoid ice-crystal formation, before quick freezing in liquid nitrogen. The method was similar to that described by Bernhard and Viron (6). In other experiments, the liver fragments were neither encapsulated nor treated with an antifreeze agent before being frozen in liquid nitrogen. The blocks were then mounted on the specimen holder and cut at about −70°C with a

FIGURE 3 Rat liver cell nucleus. Ultrathin frozen section. Puvion and Blanquet's colloidal iron stain for visualizing basic proteins. Both the chromatin and nuclear RNA-containing components, in particular the nucleolus, are stained. × 12,000.

FIGURE 4 Rat liver cell nucleus. Ultrathin frozen section stained with the EDTA method preferential for RNP structures in the nucleoplasm. All the chromatin is bleached. Heavy contrast of the nucleolar body (nu), the interchromatin granules (ig), and perichromatin granules (pg). Mitochondrial proteins (m) unspecifically stained. × 12,000.
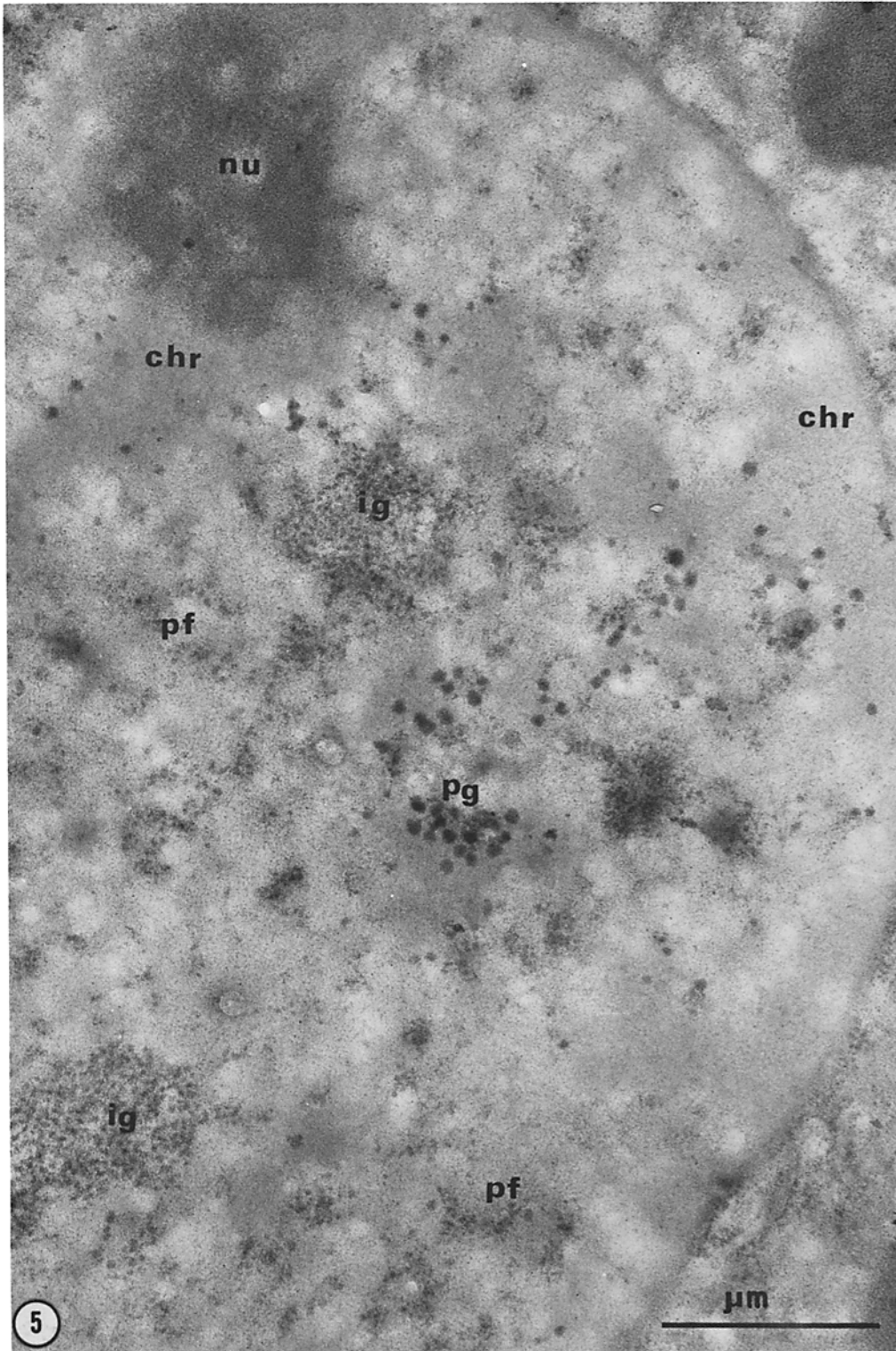
FIGURE 5   Rat liver cell nucleus. Ultrathin frozen section. Typical aspect of the nucleoplasm after EDTA stain. Most of the RNP-carrying components are easily visible: nucleolus (*nu*), interchromatin granules (*ig*), perichromatin granules (*pg*) characteristically clustered, a few perichromatin fibrils (*pf*). The chromatin (*chr*) is bleached. × 36,000.

Sorvall cryokit adapted to a Porter-Blum MT2 ultrami-crotome (8,9). The sections were either spread in a trough containing a solution of 50% DMSO at about −50°C or picked up on a toothpick with a saturated sucrose droplet according to Tokuyasu (27), or collected without spreading in a dry state by means of a needle and pressed on the grid with a copper rod (9). We never observed any difference in ultrastructural preservation among these various specimen treatments, but the yield of well-spread sections was always much higher when they were floated on the trough as indicated earlier (4).

Staining Procedures

USUAL NONSPECIFIC CONTRAST STAINING OF BOTH TYPES OF NUCLEOPROTEIN

The sections sticking on a carbon-Formvar-coated copper grid were dried in air at room temperature. For the usual controls, the classical uranyl-lead stain was applied, but the treatment had to be much shorter than for plastic-embedded tissues: aqueous uranyl acetate 5%, for 1 min, rinsing in distilled water, then lead citrate for 10 s, rinsing, and drying at room temperature.

SPECIFIC DNA STAINS

MOYNE'S SCHIFF-THALLIUM REACTION (22): The principle of this method is similar to that of the classic Feulgen reaction, and it enhances specifically the contrast not only of chromatin but also of any other type of DNA-carrying structure, including viruses. After several years of application, we have found no exception for plastic-embedded material (Epon, GMA). The main technical steps of the reaction are as follows: glutaraldehyde fixation, 1 h; prolonged rinsing in buffer solution; ultrathin frozen sections collected on gold grids with double-coated carbon-Formvar membrane; acetylation of the sections overnight at 45°C with pyrimidine (60 vol)-acetic acid anhydride (40 vol); hydrolysis with HCl 5 N at 20°C for 30 min; floating of the grids on Schiff's reagent for 30 min; air drying of the sections; staining with a solution of thallium ethylate 1 mg/ml.

GAUTIER'S OSMIUM-AMMINE STAIN (13): The principle of this method is also based on the Feulgen reaction: hydrolysis with HCl and reaction of apurinic acid with the new stain, osmium-ammine, which reacts with its pseudoaldehyde groups. The technique is carried out as follows. The sections are fixed in 2.5% buffered glutaraldehyde for 1 h as usual. The frozen sections are collected on gold grids coated with a carbon-Formvar membrane. Hydrolysis in HCl 5 N is carried out at 20°C for 25 min. The grids are floated upside down for 30 min on the osmium-ammine solution 0.1% which has been previously treated with SO_2 for 30 min. More technical details, in particular the synthesis of osmium-ammine, are given elsewhere (10, 13).

DEMONSTRATION OF RNP COMPONENTS

The EDTA technique, previously described elsewhere (5), is now well standardized for plastic-embedded tissues. However, it had to be considerably modified to obtain reproducible results with frozen sections. The following adaptations are recommended. (a) Before drying, the sections are "stabilized" for 10 s with 5% aqueous uranyl acetate. Disintegration of the tissue is thus reduced, probably because of a fixation effect of the uranyl ions. (b) Prolonged drying (overnight) is an indispensable step which produces increased dehydration and also oxidizes the components of the biological material. (c) In order to enhance oxidation, the sections may be treated, in addition, with a solution of H_2O_2 2% for 90 s, which leads to enhancement of the contrast. (d) The sections are floated on an EDTA solution 0.2 M at pH 7 for 15–20 s. The time limits are very critical. This is not the case if EDTA is replaced by sodium citrate as chelating agent. We can therefore recommend, as an alternative method, staining in a mixture of aqueous uranyl acetate 2.5% and sodium citrate 0.1 M, pH 5.5, for 4 min. In this case, the time of treatment is less critical, and the penetration by this solution is more regular and is less influenced by the thickness of the section. (e) The sections are poststained with lead citrate for 60 s.

VISUALIZING OF BASIC PROTEINS

The principle and the technical steps of this stain have been described by Puvion and Blanquet (25). The method can be compared to the fast green method. Negatively charged colloidal iron particles suspended in a solution at pH 10.5 are fixed on NH_2 groups of the proteins. Fixation of the tissue is carried out in formaldehyde 4% for 2 h, followed by rinsing in a phosphate buffer solution, pH 7.3, overnight. DNase treatment is indispensable to remove the negatively charged DNA and to liberate the NH_2 groups of the histones and other basic proteins. Staining with the colloidal iron solution is carried out for 2 h at room temperature.

Examination of the Sections

All the preparations were examined after air drying at room temperature. The electron microscope used was either a Siemens Elmiskop I at 80 kV, or a Siemens 101 at 100 kV. Both were equipped with a 50-μm objective aperture.

RESULTS

The cell nucleus is a relatively stable and resistant organelle after cryosectioning, compared to the more fragile cytoplasm. Exploration of the nucleus with this method is therefore facilitated. Furthermore, a series of cytochemical procedures can indeed be adapted to ultrathin frozen sections for visualizing specifically, or preferentially, DNA,

6

f

g

chr

0.5 μm

chr

chr

0.5 μm

7
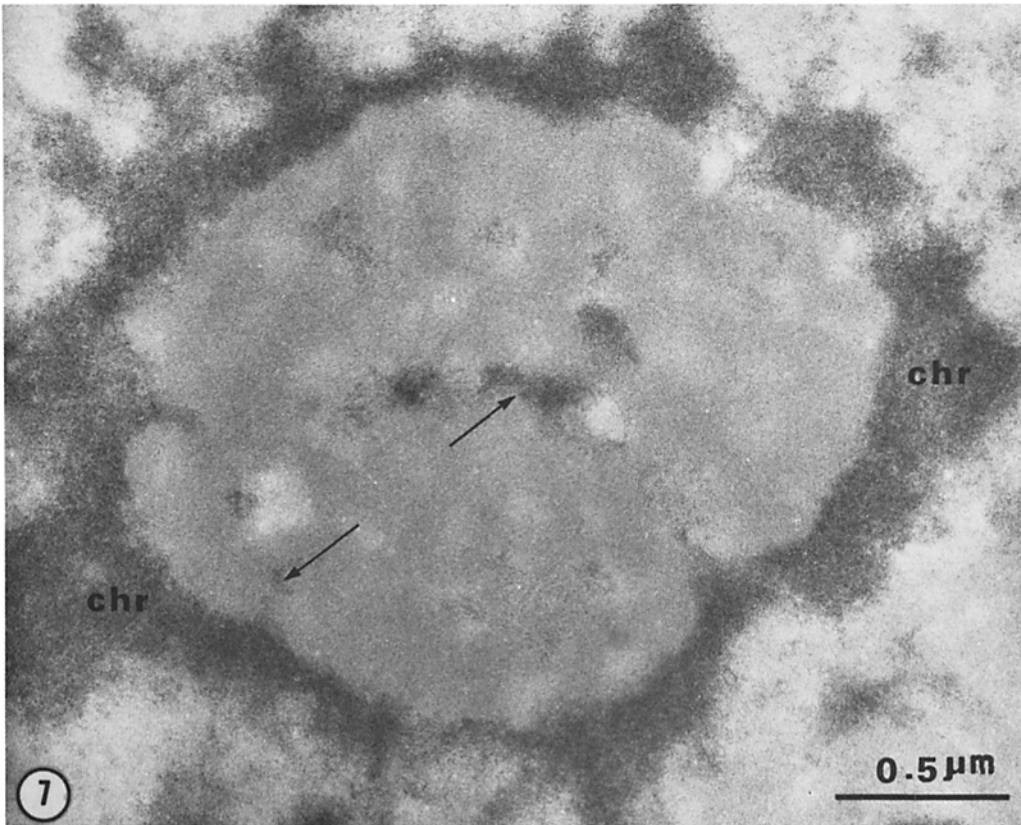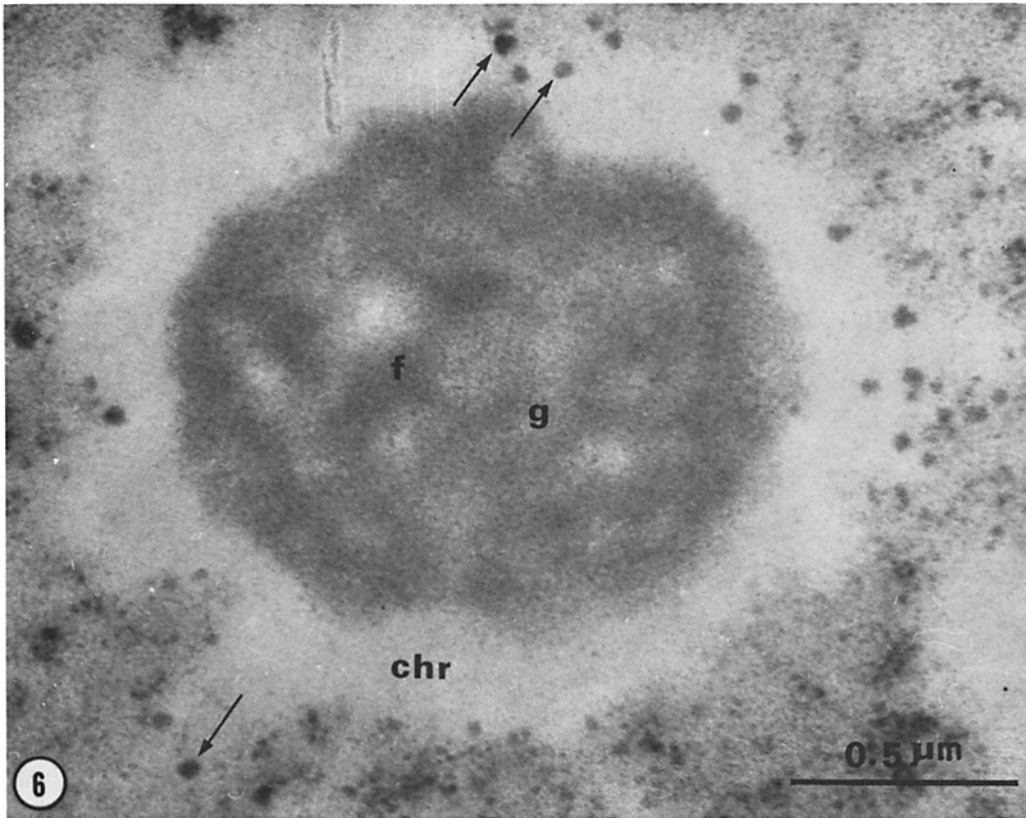
RNA, or basic proteins. Our work has focused on nuclear ribonucleoproteins, particularly extranucleolar RNP. If the usual uranyl-lead double staining is employed, the fine structure of the nucleus is very much like that observed in plastic-embedded material—at least as far as the chromatin and ribonucleoprotein carriers are concerned (Fig. 1). However, the nuclear membrane is not visible in this case, as no osmium tetroxide fixation has been used. It readily appears if negative staining is used. Concerning the RNP structures, they show practically the same contrast as chromatin, but it may be possible to identify clusters of interchromatin and single perichromatin granules.

Cytochemical procedures, applied directly to the thin sections, allow the localization of DNA by enhancing the electron opacity of these components. In particular, the use of a Feulgen-like method has been very successful. Both Moyne's and Gautier's stains seem to be specific for ultrathin frozen sections. However, frozen sections are extremely fragile even after spreading on grids, and many specimens have been lost, especially when the thallium method was applied. Unexpected difficulties of this technique have arisen with frozen sections. First of all, the fragility of unembedded material makes the transfers to different reagents difficult. In particular, acetylation frequently induces disintegration of the sections even when they are floated with the Formvar membrane on the grid, as the membrane itself is dissolved unless it is heavily coated with carbon, but in this case, the resolving power becomes rather poor. More satisfactory and regular results have been obtained with Gautier's osmium-ammine stain (10). The nuclear fine structure is well preserved, and the enhanced contrast is strictly limited to DNA-carrying structures, i.e., condensed as well as spread chromatin (Fig. 2). At higher magnification, the "cleanliness" of this stain is well revealed. No granular precipitates appear, and specifically stained fibrils of a diameter down to 30-50 Å can be visualized (Fig. 7). It is most important that neither perichromatin granules nor interchromatin granules (Fig. 2) are stained. The nucleolar body, with the exception of intranucleolar chromatin, remains equally unstained.

The picture is changed after application of a preferential stain for basic proteins (25). As expected in this case, all DNA and RNA carriers are simultaneously visualized, that is, chromatin, the nucleolar body, and interchromatin granules, and also the cytoplasmic ribosomes. Unfortunately, this method does not allow a high resolution as it is based on the use of colloidal iron particles. Figs. 1-3 are partly similar because of the predominance of chromatin in the interphase nucleus. Fig. 4 gives the opposite impression: all the chromatin is bleached with the preferential RNP-staining method, and the RNP components appear exclusively in the nucleoplasm, i.e. the nucleolar body without its associated chromatin, but with perichromatin and interchromatin granules, as expected (Fig. 5). Curiously enough perichromatin fibrils adjacent to the chromatin clumps are rather faintly stained in frozen sections, whereas in Epon-embedded material they are always easily revealed (Figs. 8, 9). Figs. 6-11 illustrate the application of the same cytochemical methods to demonstrate fine-structural details. Ultrathin frozen sections can now be obtained thin enough to visualize macromolecular structures with the same precision as with classical fixation and embedding procedures. Fig. 6 demonstrates an unusually high number of perichromatin granules in the nucleolus-associated chromatin, a picture which is never seen after classical embedding. There is another important point to be stressed. Whereas in the plastic sections the perichromatin granules are remarkably constant in size, their diameter varies considerably in frozen sections.

In analyzing the fine structure of the nucleolus, the usual RNP fibrils in the nucleolonema are

---

FIGURE 6   Details of the nucleolar area, after EDTA stain. The reticular structure of the nucleolar body is well revealed. Its RNP fibrils are always more visible (f) than the nucleolar granules (g). The nucleolus-associated chromatin (chr) is bleached, but many perichromatin granules of variable size (→) are observed at its periphery. × 60,000.

FIGURE 7   Details of the nucleolar area after Gautier's osmium-ammine stain (higher enlargement of Fig. 2). The nucleolar body with its RNP components remains totally unstained, whereas the nucleolus-associated chromatin (chr) surrounding it shows an enhanced contrast. This method also allows the visualization of minute quantities of intranucleolar DNA (→). Perichromatin granules invisible. × 45,000.
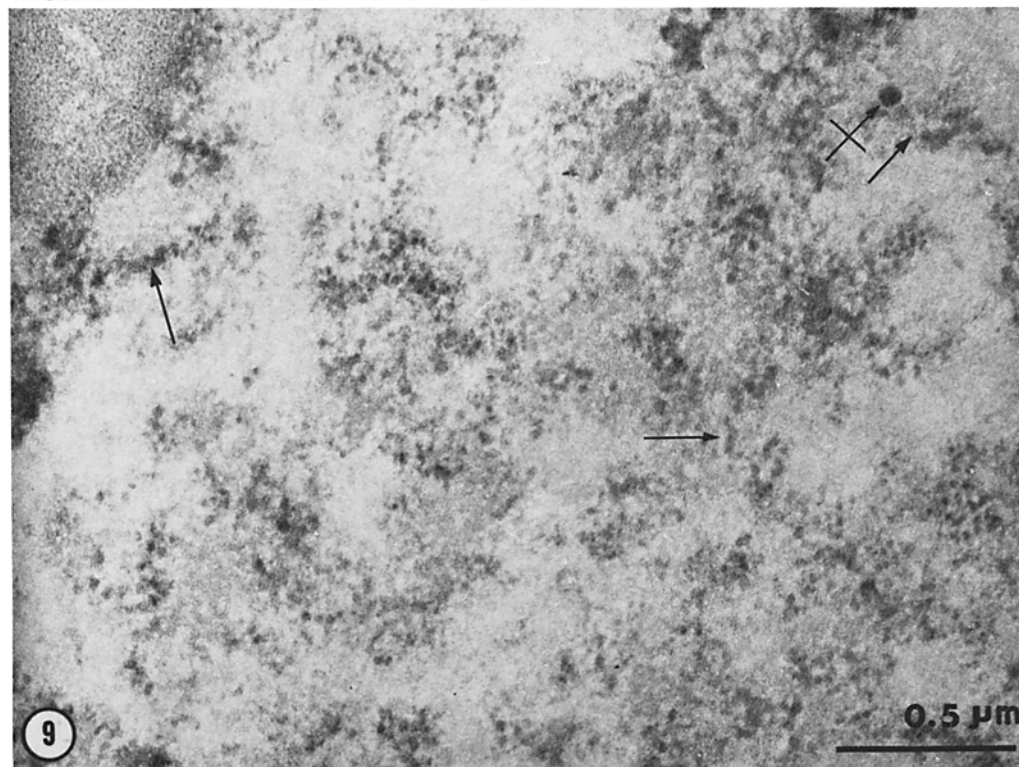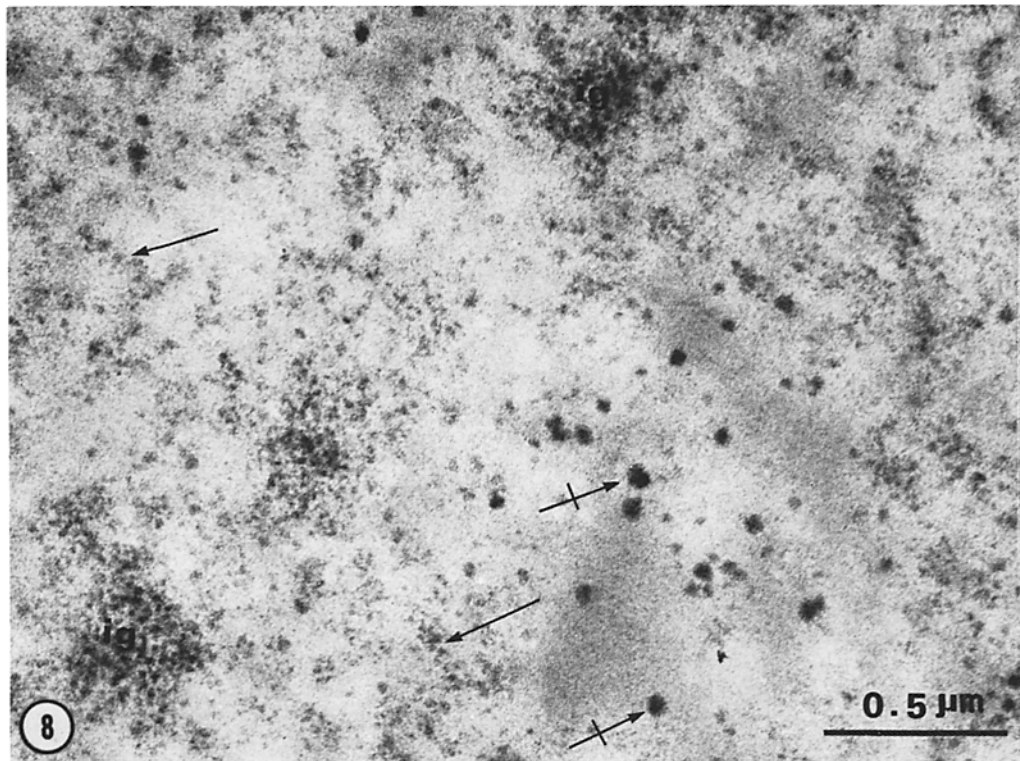
FIGURES 8 and 9   Rat liver cell nucleoplasm. Comparison of the morphologies of perichromatin fibrils in a frozen section (Fig. 8) and in Epon-embedded material (→) (Fig. 9) after EDTA stain. These RNP components are more difficult to demonstrate in frozen sections where they are mostly detached from the chromatin clumps and appear as very fine granules or helices. Interchromatin granules (ig). Abundance of perichromatin granules (→). After classical dehydration and embedding (Fig. 9), perichromatin fibrils are closely associated with the dispersed chromatin (chr) and are shown as short, irregular fibrils or granules (→). Scarcity of perichromatin granules (↔). Figs. 8, 9, × 48,000.
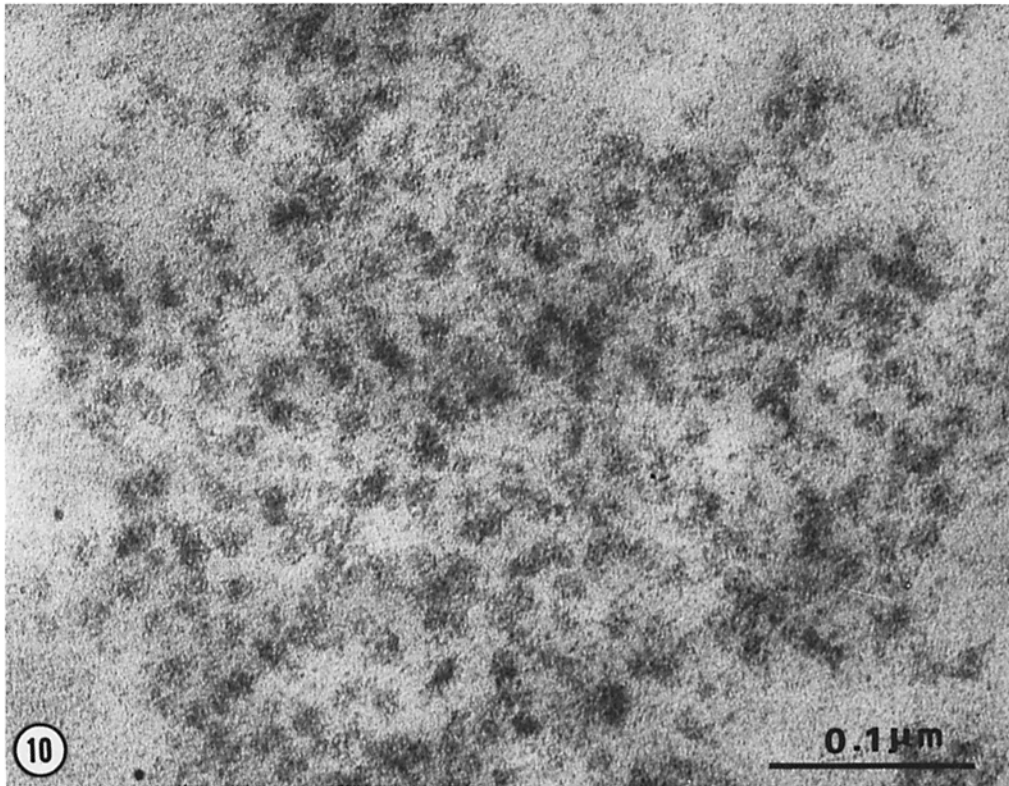
FIGURE 10   Rat liver cell. Nucleoplasm after EDTA stain demonstrating a cluster of interchromatin granules. These granules are of irregular shape and appear frequently to be interconnected with tiny fibrils. × 250,000.

easily detectable, but the granules, although present, are less prominent than in the classical pictures. This depends very much upon the staining procedure used (17). We also obtained the usual granular components indistinguishable from the well-known nucleolar fine structure. Fig. 7 is almost the exact "negative" image of Fig. 6. The only visible component is the external nucleolus-associated and internal nucleolar chromatin. Not a single perichromatin granule can be detected. However, very minute amounts of intranucleolar DNA appear not only, as usual, in dense clumps, but also as fine fibrillar areas which have not been visualized so far by other techniques.

Figs. 8 and 9 summarize in a typical way the difference between frozen (Fig. 8) and conventional (Fig. 9) sections as observed for extranucleolar RNP components. For a similar image density, the frozen section reveals many more perichromatin granules, and of different size. The Epon section presents only a few granules, all of them usually of the same size. On the other hand,

perichromatin fibrils are difficult to identify in frozen section. Transitions towards the interchromatin granules may be found. In the Epon section, on the contrary, perichromatin fibrils are adjacent to chromatin areas and cannot be confused with any other components after application of the EDTA stain.

A typical area of interchromatin granules is visualized in an exceptionally thin portion of a frozen section in Fig. 10. One notices that these granules are of irregular, angular shape and seem to be composed of fine fibrils. Furthermore, many of them are interconnected and form a chainlike network.

Fig. 11 illustrates the very striking differences in the size, shape, and spatial configuration of perichromatin and interchromatin granules. Both, however, are composed of tiny, wrapped-up fibrillar components, usually more easily seen in perichromatin granules if the sections are extremely thin (Fig. 11, inset). These granules appear as grapelike clusters much more frequently in frozen
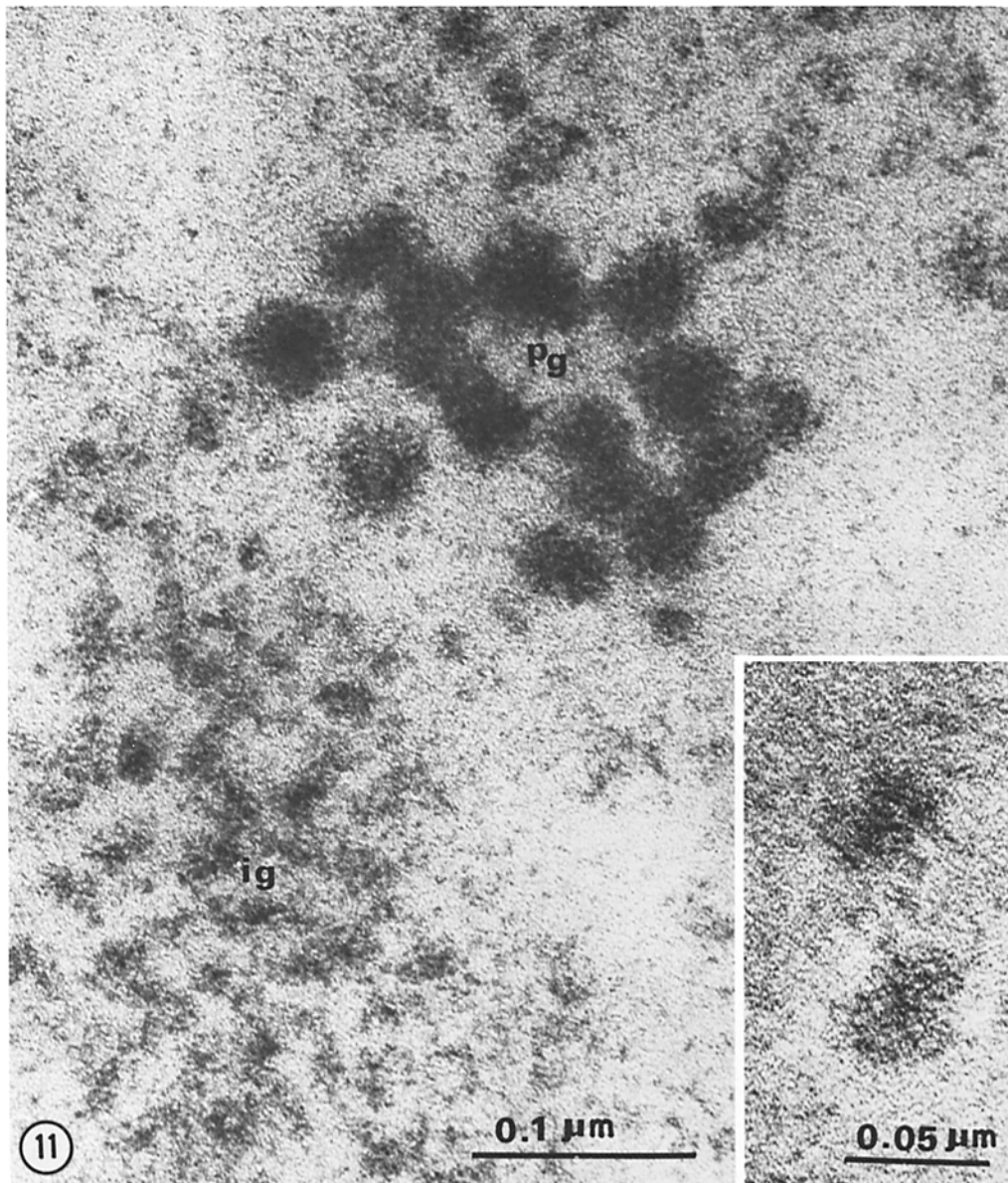
FIGURE 11 Perichromatin (*pg*) and interchromatin (*ig*) granules in the nucleoplasm of a hepatocyte after EDTA staining. The perichromatin granules are characteristically clustered and sometimes interconnected in frozen sections. In very thin specimens, they appear to be composed of irregularly coiled fibrils of about 30-Å thickness (*inset*). The fibrillar nature of interchromatin granules is also easily visible on this electron micrograph. × 300,000. *Inset*, × 400,000.

than in conventional sections. Although usually not interconnected, these clusters may sometimes be visualized as rods, as demonstrated with the classical techniques.

Fig. 12 has been chosen to indicate a hitherto unexploited possibility of examining thick frozen sections (up to 3 μm) without using high voltage microscopy. However, this technique is meaningful only if some specific contrast is obtained for a given cell organelle. In our case, a thick section of a liver cell has been treated with the preferential RNP staining method. Only RNP-carrying struc-

tures are electron dense. All the chromatin is bleached. One notices that most extranucleolar RNP is concentrated within a contiguous netlike space, for which we proposed the term "interchromatin net." But a very high number of perichromatin granules are seen at the periphery of the bleached chromatin clumps. It would be interesting to make stereoscopic electron micrographs of such specimens. Preliminary studies started in our laboratory with a tilting device have allowed us to visualize the spatial contiguity of the "net."

DISCUSSION

Our investigations have clearly demonstrated that it is possible to obtain from ultrathin frozen sections information comparable or even superior to that gained from classical techniques for study-

ing the fine structural organization of the cell nucleus. The quality of the sections may be in all respects comparable to that of plastic-embedded material, provided prefixation with aldehydes is used. The general preservation of the nuclear fine structure is then excellent, and some areas may be as thin as after Epon embedding, allowing very high resolution. The important fact is that these results are obtained with a technique that is totally different from those used in most other laboratories. Cryosectioning is not meant to replace those techniques, but to allow verification of results that could be artifacts due to osmium tetroxide fixation, chemical dehydration, or extraction by plastic embedding. Our electron micrographs of the cell nucleus are remarkably similar to the classical pictures, as far as fine structures is concerned,
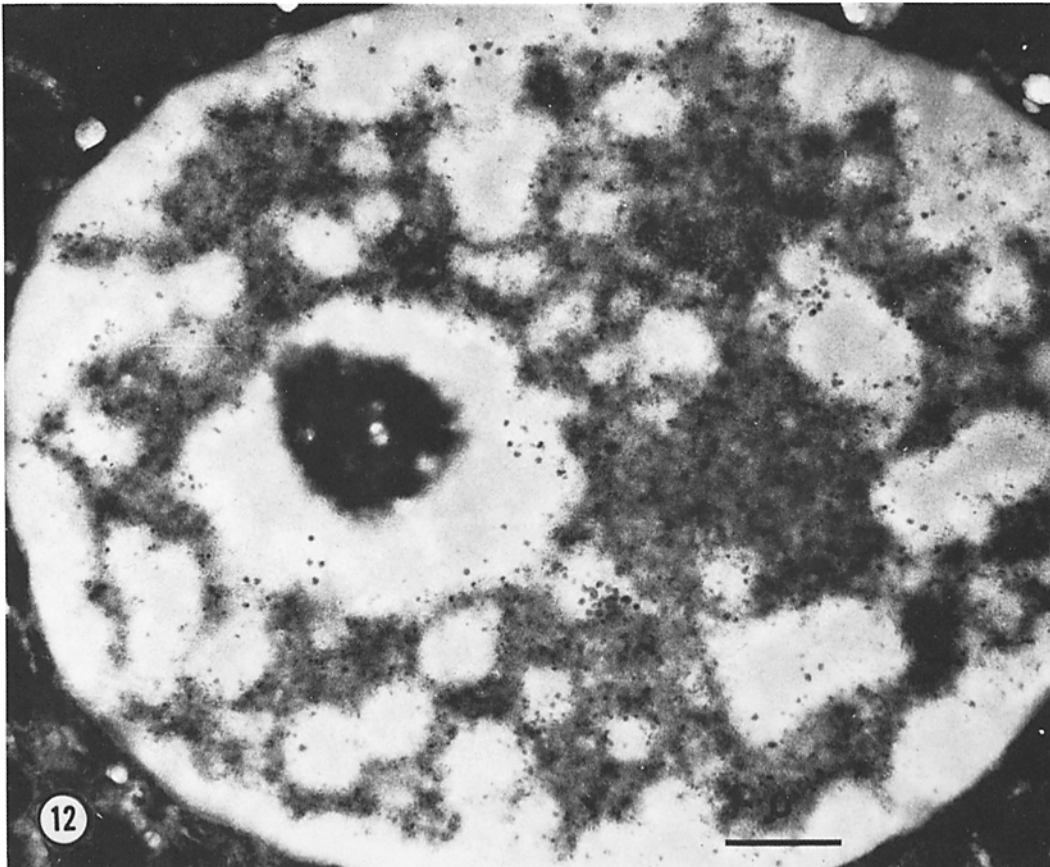

FIGURE 12   Rat liver cell nucleus, after regressive stain with citrate-uranyl solution applied to a very thick, frozen section (about 2–3 μm). Examination at 100 kV; exposure time 2.5 s. This document demonstrates the possibility of examining unusually thick specimens with the ordinary electron microscope. The periphery of the bleached chromatin contains an abundance of perichromatin granules, but most of the RNP components are found inside the heavily stained "interchromatin net." × 16,000.

although in frozen sections the general matrix of the cell appears much denser. We conclude that a considerable number of smaller molecules and lipids are preserved, and this may be important for future cytochemical studies or microprobe analysis. It is also very likely that the biochemical and immunochemical reactivity of frozen sections is higher in our case, but we are still quite ignorant concerning such applications. Indeed, certain easily diffusible enzymes, such as acid phosphatase, may need prolonged fixation to be immobilized for visualization *in situ*, and the classical methods may then be superior (16). We are only at the beginning of cytochemical investigation by means of ultrathin frozen sections, and it appears that any stain already used in ultrastructural cytochemistry has to be adapted, as many of the parameters that play a role in the staining reaction (penetration, diffusion, adsorption, degree, and type of fixation, total amount of the reactive molecules, etc.) are entirely different, and as the reaction can be observed directly, without intermediate steps, on the section surface.

This is true for the demonstration of enzymes, and of DNA and RNA. As we have seen, Moyne's thallium method, excellent for plastic-embedded material, is not recommended for frozen sections, whereas Gautier's osmium-ammine stains DNA beautifully, with absolute specificity, and without any granular precipitate, thus allowing the demonstration of minute quantities, perhaps even of single nucleohistone molecules. On the other hand, the EDTA method for visualizing the presence of RNA, as described in the original papers (5, 19), does not induce any reaction in frozen sections unless the procedure is considerably modified. The chelating agent may be replaced by sodium citrate added directly to the aqueous uranyl solution. In this case, the results are practically identical in the Epon-embedded and frozen sections, except for three details: the nucleolar RNP granules appear smaller; the perichromatin fibrils are more difficult to demonstrate; and the perichromatin granules are of variable size and increased number. Finally, for the localization of basic proteins by means of colloidal iron, the staining reaction also works in ultrathin frozen sections with a high degree of specificity, although the granularity of the reaction product does not allow high resolution.

Concerning the second goal of our investigation —gaining additional knowledge of the fine structure of nuclear components and their functional

significance—we cannot add much to what has already been described (19). An intriguing question arises from the difficulty of demonstrating perichromatin fibrils after cryosectioning. This is precisely the component we are now particularly studying with other techniques to verify our original hypothesis, according to which these fibrils are the structural substrate of the newly transcribed extranucleolar RNA, in particular of heterogeneous nuclear RNA (HnRNA) (19, 24). Combined biochemical, autoradiographic, and cytochemical investigations tend to corroborate this interpretation (11). Thus, it is now possible to localize RNA synthesis in certain areas of chromatin. In other words, gene activity can be traced and visualized in thin Epon sections in the electron microscope, and, concomitantly, transcriptional complexes of such interphase nuclei can be isolated in selected fractions of nucleohistones.[1] The difficulties of showing perichromatin fibrils in frozen sections are probably linked to the different speeds of differentiation of the regressive stain in classical and frozen sections. The optimal staining time for perichromatin fibrils may be much shorter than that for the other RNP components, perhaps because of the different amounts of associated proteins. On the other hand, this method demonstrates nuclear RNP. So far, we do not know any exception for the nucleoplasm; in the cytoplasm, however, various components other than ribosomes may keep their contrast (5) (see mitochondria in Fig. 4).

As for the perichromatin granules, their study is greatly facilitated in frozen sections because of their abundance, their tendency for clustering, and the appearance of many granules of much smaller size than usual. These smaller sized granules are interpreted as forms intermediate between perichromatin fibrils and the "mature" perichromatin granules. There is no doubt now that the latter include a small protion of the newly synthesized extranucleolar RNA. Our original hypothesis that they represent a storage or transport form of mRNA (19) still seems valid, but the technique used has not added any new information to confirm it. They arise, without any doubt, from messenger-like RNA fibrils. As most of the perichromatin granules are surrounded by nucleohis-

[1] Bachellerie, J. P., E. Puvion, and J. P. Zalta. 1975. Ultrastructural organization and biochemical characterization of chromatin-RNP complexes isolated from mammalian cell nuclei. *Eur. J. Biochem.* (1975). In press.

tones, their isolation in density gradients in sufficient number to allow biochemical analysis seems rather hopeless, unless they accumulate as large inclusions after treatment of the cells with certain drugs (18).[2] Their morphological similarity to Balbiani ring granules of giant Dipteran chromosomes still remains another important argument in favor of our hypothesis (28).

Our study has also shown that in thick sections interchromatin granules are located within a more or less contiguous network and are not isolated as clusters as they appear in very thin sections. Although it is very likely that they contain small amounts of RNA (19), this RNA must then have a very low turnover, as even prolonged treatment with tritiated uridine does not label these areas significantly, whereas the regions of perichromatin fibrils are intensively radioactive (12).

Finally, it should be stressed that even very thick sections can be examined with the usual accelerating voltage and may reveal the relationship between organelles in their spatial arrangement (Fig. 12). As no embedding materials is used, there is much less electron scattering than in conventional plastic sections. Therefore, both the brightness of the image and the resolving power are very satisfactory even for sections of 1-3-μm thickness. It may thus be expected also that chromosomes treated with one of the DNA-staining methods in thick sections will reveal details of their ultrastructure in the conventional microscope, without the use of very high voltages (26).

CONCLUSION

In conclusion, it is demonstrated that cryoultramicrotomy can be used as a new tool for the exploration of the interphase nucleus. The quality of the morphological preservation of all its components is equal to the best results obtained with classical techniques. But, in addition, very little or no extraction of low molecular weight material occurs, and it may be that the expected cytochemical and immunochemical reactivity of the molecules is better preserved, allowing further functional and *in situ* studies of nuclear structures.

---

[2] Puvion, E., and G. Moyne. Manuscript in preparation.

REFERENCES

1. BABAI, F., and W. BERNHARD. 1971. Detection cytochimique par l'acide phosphotungstique de certains polysaccharides sur coupes à congélation. *J. Ultrastruct. Res.* **37**:601.
2. BAUER, H., and E. SIGARLAKIE. 1972. Ultrathin frozen sections of yeast cells. *J. Ultrastruct. Res.* **40**:197.
3. BENICHOU, J. C., and A. RYTER. 1973. Mise au point de la technique de coupes à congélation pour les bactéries Gram+ et Gram−. *J. Microsc. (Paris)*. **17**:227.
4. BERNHARD, W., and E. H. LEDUC. 1967. Ultrathin frozen sections. I. Methods and ultrastructural preservation. *J. Cell Biol.* **34**:757.
5. BERNHARD, W. 1969. A new staining procedure for electron microscopical cytology. *J. Ultrastruct. Res.* **27**:250.
6. BERNHARD, W., and A. VIRON, 1971. Improved techniques for preparation of ultrathin frozen sections. *J. Cell Biol.* **49**:731.
7. BERNIER, R., R. IGLESIAS, and R. SIMARD. 1972. Detection of DNA by tritiated actinomycin D on ultrathin frozen sections. *J. Cell Biol.* **53**:798.
8. CHRISTENSEN, A. K. 1969. A way to prepare frozen thin sections of fresh tissue for electron microscopy. *In* Autoradiography of Diffusible Substances. L. J. Roth and W. E. Stumpf, editors. Academic Press, Inc., New York. 349.
9. CHRISTENSEN, A. K. 1971. Frozen thin sections of fresh tissue for electron microscopy with a description of pancreas and liver. *J. Cell Biol.* **51**:772.
10. COGLIATI, R., and A. GAUTIER. 1973. Mise en évidence de l'ADN et des polysaccharides à l'aide d'un nouveau réactif "de type Schiff." *C. R. Hebd. Séances Acad. Sci.* **276**:3041.
11. FAKAN, S., and W. BERNHARD. 1971. Localization of rapidly and slowly labeled nuclear RNA as visualized by high resolution autoradiography. *Exp. Cell Res.* **67**:129.
12. FAKAN, S., and W. BERNHARD. 1973. Nuclear labelling after prolonged ³H-uridine incorporation as visualized by high resolution autoradiography. *Exp. Cell Res.* **79**:431.
13. GAUTIER, A., R. COGLIATI, M. SCHREYER, and J. FAKAN. 1973. "Feulgen-type" and "PAS-type" reactions at the ultrastructural level. *In* Electron Microscopy and Cytochemistry. E. Wisse, W. T. Daems, I. Molenaar, and P. Van Duyn, editors. North Holland Publishing Company, Amsterdam. 271.
14. IGLESIAS, R., R. BERNIER, and R. SIMARD. 1971 Ultracryotomy: a routine procedure. *J. Ultrastruct. Res.* **36**:271.

15. KOLEMAINEN-SEVEUS, L. 1970. Frozen ultrathin sections. *In* Microscopie Electronique. P. Favard, editor. Société Française de Microscopie Electronique, Paris. Vol. I. 432.

16. LEDUC, E. H., W. BERNHARD, S. J. HOLT, and J. P. TRANZER. 1967. Ultrathin frozen sections. II. Demonstration of enzymatic activity. *J. Cell Biol.* **34**:773.

17. MARINOZZI, V. 1964. Cytochimie ultrastructurale du nucléole: RNA et protéines intranucléolaires. *J. Ultrastruct. Res.* **10**:433.

18. MONNERON, A., C. LAFARGE, and C. FRAYSSINET. 1968. Induction par l'Aflatoxine et la Lasiocarpine d'amas de grains périchromatiniens dans le foie de rat. *C. R. Hebd. Séances Acad. Sci.* **267**:2053.

19. MONNERON, A., and W. BERNHARD. 1969. Fine structural organization of the interphase nucleus in some mammalian cells. *J. Ultrastruct. Res.* **27**:226.

20. MORGENSTERN, E., K. NEUMANN, and G. WERNER. 1971. Das elektronenmikroskopische Bild von Blutzellen in ultradünnen Gefrierschnitten. *Cytobiologie.* **5**:101.

21. MORGENSTERN, E., K. NEUMANN, and G. WERNER. 1973. Die Gefrierultramikrotomie als elektronen mikroskopische Schnellmethode. *Mikroskopie.* **29**:163.

22. MOYNE, G. 1973. Feulgen-derived techniques for electron microscopical cytochemistry of DNA. *J. Ultrastruct. Res.* **45**:102.

23. PAINTER, R. G., K. T. TOKUYASU, and S. J. SINGER. 1973. Immunoferritin localization of intracellular antigens: the use of ultracryotomy for direct immunoferritin staining. *Proc. Natl. Acad. Sci. U.S.A.* **70**:1649.

24. PETROV, P., and W. BERNHARD. 1971. Experimentally induced changes of extranucleolar ribonucleoprotein components of the interphase nucleus. *J. Ultrastruct. Res.* **35**:386.

25. PUVION, E., and P. BLANQUET. 1971. Cytochimie ultrastructurale des groupements de point isoionique élevé. Application à la mise en évidence des protéines basiques. *J. Microsc. (Paris).* **12**:171.

26. RIS, H. 1974. High voltage electron microscopy in the analysis of chromosome organization. *In* Electron Microscopy. Abstracts of papers presented to the Eighth International Congress on Electron Microscopy. Canberra. J. V. Sanders and D. J. Goodchild, editors. The Australian Academy of Sciences, Canberra. Vol II. 250.

27. TOKUYASU, K. T. 1973. A technique for ultracryotomy of cell suspension and tissues. *J. Cell Biol.* **57**:551.

28. VAZQUEZ-NIN, G., and W. BERNHARD. 1971. Comparative ultrastructural study of perichromatin and Balbiani ring granules. *J. Ultrastruct. Res.* **36**:842.