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Ribocharin: A Nuclear M_r 40,000 Protein Specific to Precursor Particles of the Large Ribosomal Subunit

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Summary

Using a monoclonal antibody (No-194) we have identified, in Xenopus laevis and other amphibia, an acidic protein of M_r 40,000 (ribocharin) which is specifically associated with the granular component of the nucleolus and nucleoplasmic 65S particles. These particles contain the nuclear 28S rRNA and apparently represent the precursor to the large ribosomal subunit in nucleocytoplasmic transit. By immunoelectron microscopy ribocharin has been localized in the granular component of the nucleolus and in interchromatin granules. During mitosis ribocharin-containing particles are associated with surfaces of chromosomes and are recollected in the reconstituting nucleoli in late telophase. We suggest that ribocharin is a specific component of precursor particles of the large ribosomal subunit, which dissociates from the 65S particle before passage through the nuclear envelope, and is reutilized in ribosome biogenesis.

Introduction

Our current concept of ribosome biogenesis in eukaryotic cells is relatively far advanced in describing transcription of rRNA genes and the subsequent processing of pre-rRNA (e.g., Perry, 1967; Loening et al., 1969; Busch and Smetana, 1970; Sollner-Webb et al., 1982; Reeder and Roan, 1984; for a recent review see Hadjiolov, 1984). Although early intermediates in the cascade of pre-rRNA processing have been identified, the later steps, i.e., those immediately preceding the appearance of mature cytoplasmic 18S and 28S rRNAs, are largely unknown, as is the intranuclear location of these events. In fact, precursor forms, but no mature 28S rRNA molecules, have been detected in nuclear fractions and subfractions from various mammalian cells (Penman et al., 1966; Hadjiolova et al., 1984) as well as in manually isolated intact nuclei and nuclear envelopes from amphibian oocytes (Franke and Scheer, 1974a).

In contrast, knowledge of the proteins associated with pre-rRNA, the various intermediate products of processing, and the possible roles of these products in ribosome formation and nucleocytoplasmic transport is still very fragmentary (for review see Hadjiolov, 1984). Various species of so-called preribosomal particles have been isolated from nucleoli and have been reported to contain certain ribosomal proteins as well as other proteins that are absent from cytoplasmic ribosomes (Kumar and Warner, 1972; Prestayko et al., 1974; Kumar and Subramanian, 1975; Auger-Buendia and Longuet, 1978; Lastick, 1980; Todorov et al., 1983; Busch, 1984). Positive localization to distinct nucleolar structures in situ has so far been achieved only for ribosomal proteins S1 (Hügle et al., 1985), L4 and S14 (Chooi and Leiby, 1981), and two non-ribosomal proteins termed B23 and C23 (Daskal et al., 1980; Busch, 1984).

Presently available evidence suggests that the final maturation steps, which result in the formation of the definitive ribosomal subunits, are spatially and temporarily coupled to their export from the nucleolus into the cytoplasm. The existence of extranucleolar preribosomal particles in the nucleoplasm has been postulated in most models of ribosome formation and nucleocytoplasmic transport (summarized by Hadjiolov, 1984). However, no such particles have as yet been identified, apparently because of their low steady state concentrations in most kinds of cells. Therefore, in order to examine protein–RNA relationships in ribosome formation, notably in particles in nucleocytoplasmic transit, we have combined two experimental strategies: the production of monoclonal antibodies to proteins associated with preribosomal particles, and the biochemical characterization of extranucleolar particles containing precursors to rRNAs. We have utilized vitellogenic oocytes of Xenopus laevis because they contain an extraordinarily high number of amplified pre-rRNA genes (Brown and Dawid, 1968) that are transcribed with nearly maximum efficiency (Miller and Beatty, 1969; Scheer et al., 1977), resulting in an exceedingly high net production of ribosomes ($\geq 3 \times 10^5$ per sec; Scheer, 1973).

The use of this cell system has enabled us to identify two new nucleoplasmic preribosomal particles sedimenting at 65S and 40S, as well as an abundant protein of M_r 40,000, which is found in specific association with the granular component of the nucleolus and the 65S preribosomal particles, but is absent from 40S preribosomal particles and from mature cytoplasmic ribosomes. Our results indicate that this protein, for which the name ribocharin is proposed, is specifically associated with precursors to the large ribosomal subunit until they leave the nucleus. These data represent the first experimental evidence of a substantial difference in the protein pattern between the large ribosomal subunit in the cytoplasm and the corresponding precursor particle in the nucleoplasm, suggesting that rearrangements of the rRNA-associated proteins take place during nucleocytoplasmic translocation.

Results

Identification of Ribocharin in Nuclear Fractions of Xenopus Oocytes

In order to study nucleolar and other preribosomal proteins we raised monoclonal antibodies against nucleolar material from Xenopus laevis oocytes, which contain a

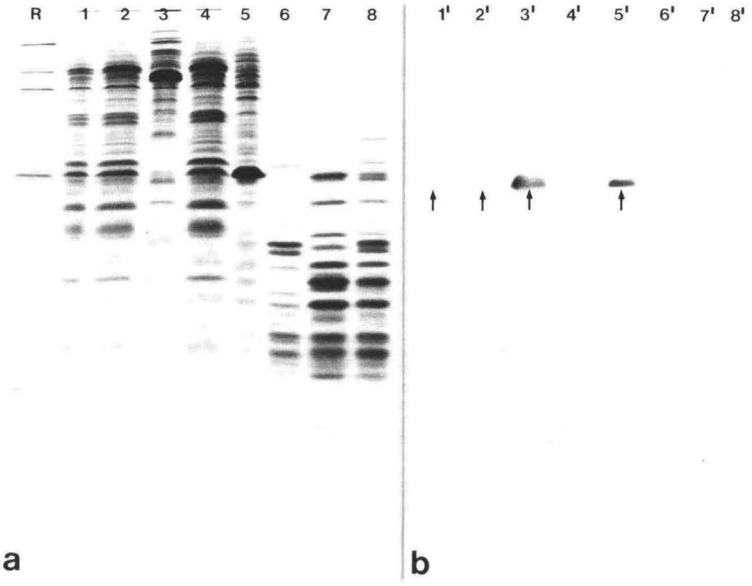


Figure 1. Identification of Ribocharin in Different Nuclear Fractions from Xenopus laevis Oocytes by Immunoblotting Using Monoclonal Antibody No-194

(a) Coomassie blue stained SDS–polyacrylamide gel (18% acrylamide) showing polypeptides of the different nuclear and ribosomal fractions. Reference proteins (R): myosin heavy chain (M_r 200,000), β-galactosidase (M_r 120,000), phosphorylase a (M_r 94,000), bovine serum albumin (M_r 68,000), actin (M_r 42,000), chymotrypsinogen (M_r 25,000), and cytochrome c (M_r 12,500). Lane 1, total proteins from 20 oocyte nuclei; lane 2, supernatant fraction after low speed (3500 × g) centrifugation of nuclear homogenates; lane 3, proteins of low speed (3500 × g) pellet of nuclear homogenates; lane 4, proteins of supernatant fraction after high speed centrifugation (1 hr; 100,000 × g) of nuclear homogenate; lane 5, high speed pellet (100,000 × g) of nuclear homogenate; lanes 6–8, cytoplasmic ribosomal proteins from Xenopus ovaries (lane 6, 40S ribosomal subunit; lane 7, 60S ribosomal subunit; lane 8, 80S ribosomes).

(b) Corresponding immunoperoxidase blot with monoclonal antibody No-194. The antibody reacts selectively with a polypeptide of M_r 40,000 (arrows) which is enriched in low speed (lane 3′) and high speed (lane 5′) pellets of nuclear homogenate. The antigen is also detected among the proteins of unfractionated nuclei (lane 1′) and in the low speed supernatant fraction (lane 2′). Amounts present in the high speed supernatant fraction (lane 4′) are too low to be detected by this immunoblotting technique. Note absence of the antigen in cytoplasmic ribosomes (lanes 6′–8′).

large number of extrachromosomal nucleoli all very active in transcription (for references see Introduction). We used a monoclonal antibody (No-194; IgM) against a so far unknown preribosome-associated protein (ribocharin) for the specific identification and localization of precursor particles of the large ribosomal subunit.

In immunoblotting experiments, antibody No-194 reacted exclusively with one polypeptide of M_r 40,000 (Figures 1 and 2). Immunoblot analysis of proteins separated by two-dimensional gel electrophoresis showed that this polypeptide was acidic (Figures 2a, 2b), with an isoelectric pH of approximately 5.8 as determined by isoelectric focusing in 9.5 M urea (data not shown).

Different nuclear fractions from Xenopus oocytes were analyzed for the presence of the antigenic polypeptide, using the immunoblotting technique. As shown in Figure 1, ribocharin was enriched in nucleoli-containing low speed pellets (lanes 3, 3′), as well as in 100,000 × g pellets containing the nucleoplasmic preribosomal particles (lanes 5, 5′). In addition, ribocharin was found to be present, at relatively low concentrations, in total nuclei (lanes 1, 1′) and in the low speed supernatant fraction of nuclear homogenates (lanes 2, 2′). Ribocharin was not found, by immunoblotting, in the 100,000 × g supernatant fraction (lanes 4, 4′). However, when larger amounts of the soluble nuclear fraction were carefully examined by sucrose gradient centrifugation and ELISA assays using monoclonal antibody No-194 (see Experimental Procedures), soluble forms of ribocharin sedimented at 6.5S and 12S (data not shown; a detailed biochemical characterization of these soluble forms will be published elsewhere). Antibody No-194 did not react with any polypeptide component present in cytoplasmic ribosomes (Figure 1, lanes 6–8 and 6′–8′). This result was confirmed by experiments in which manually isolated oocyte nuclei and cytoplasms of Xenopus oocytes were analyzed for the presence of ribocharin by the ELISA technique. In these experiments 100% of the ELISA activity was recovered in the isolated nuclei (data not shown).

The solubility behavior of ribocharin was examined by treating low speed and high speed pellets from nuclear homogenates of oocytes with various buffers of low and high ionic strengths, using antibody No-194 to detect ribocharin in the extracts by ELISA assays. Virtually no ribocharin was released by low salt buffers whereas 90% was extracted from the pellets by treatment with 0.5 M NaCl (data not shown).

As indicated from our blotting experiments using different nuclear fractions (Figure 1), ribocharin was associated with some larger structures, probably nucleoli and nucleoplasmic preribosomal particles. Fractionation of the 100,000 × g pellet containing the nucleoplasmic preribosomal particles by sucrose gradient centrifugation allowed us to identify two distinct particles of 40S and 65S

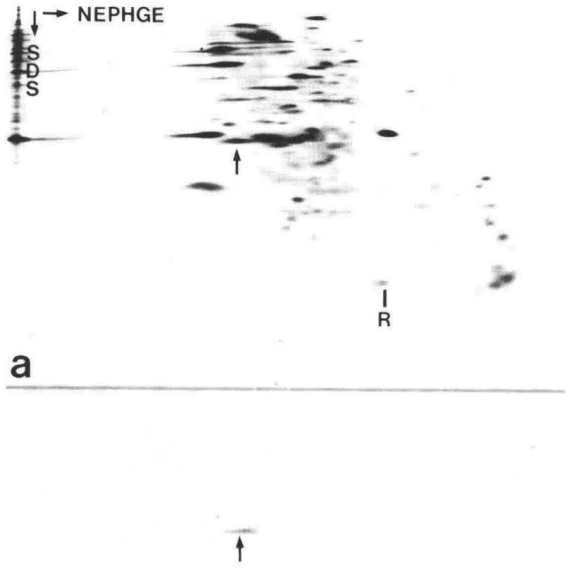


Figure 2. Characterization of Ribocharin by Two-Dimensional Gel Electrophoresis and Immunoblotting Using Monoclonal Antibody No-194

(a) Polypeptides of the high speed pellet (1 hr; 100,000 × g) from nuclear homogenate of Xenopus oocytes were separated by two-dimensional gel electrophoresis (first dimension, nonequilibrium pH gradient, NEPHGE; second dimension, electrophoresis in 18% polyacrylamide gels in the presence of sodium dodecyl sulfate) and stained with Coomassie blue. R denotes added RNAase.

(b) Corresponding immunoperoxidase blot to the gel shown in (a). Monoclonal antibody No-194 reacts specifically with only one polypeptide of M_r 40,000 denoted by arrows in (a) and (b).

(Figure 3a). When the individual fractions were assayed for the presence of ribocharin, binding activity of antibody No-194 corresponded to the peak fractions sedimenting at 65S, and immunoblotting analyses of these fractions revealed the presence of ribocharin in the 65S particles (arrows in Figures 3b and 3c). On the other hand, controls using monoclonal antibody RS1-105 directed against ribosomal protein S1 (Hügle et al., 1985) showed a specific reaction with the small 40S peak, thus identifying this moiety as a free nucleoplasmic precursor to the small ribosomal subunit (data not shown). We also examined total low speed supernatant fractions from homogenates of mass-isolated as well as manually isolated nuclei obtained after gentle disruption of the nuclei in a pipette (see Experimental Procedures). Using sucrose gradient centrifugation and the same immunological techniques as described above, we were able to detect ribocharin in fractions containing the 65S particles (data not shown). Moreover, ribocharin was found in 65S particles after recentrifugation of isolated preribosomal particles in sucrose gradients. We examined the possibility that the 65S particles might originate artificially from nucleolar frag-

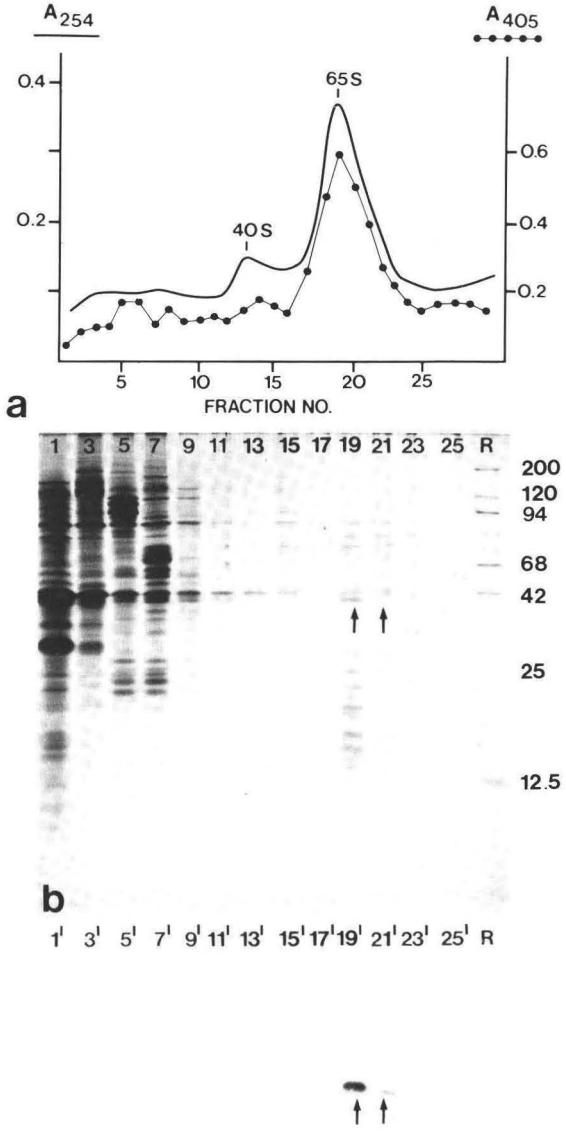


Figure 3. Immunological Identification of Ribocharin as a Component of Nucleoplasmic 65S Preribosomal Particles

(a) Material of high speed pellet (1 hr; 100,000 × g) from oocyte nuclear homogenate was redispersed and fractionated by sucrose gradient centrifugation. Preribosomal particles have sedimentation coefficients of 40S and 65S respectively (A_{254}, absorbancy at 254 nm). The antigen recognized by antibody No-194 is recovered in the 65S peak as shown by ELISA activity (A_{405}).

(b,c) Proteins of the individual sucrose fractions were analyzed by SDS–polyacrylamide gel electrophoresis and Coomassie blue staining (b). Fraction numbers are indicated on the top of the lanes. Reference proteins (lane R; molecular weights are given in 10^3 units) are from top to bottom: myosin heavy chain, β-galactosidase, phosphorylase a, BSA, actin, chymotrypsinogen, and cytochrome c. Ribocharin (arrows) is enriched in fractions 19–21 as identified by immunoperoxidase blot reaction with antibody No-194 (c).

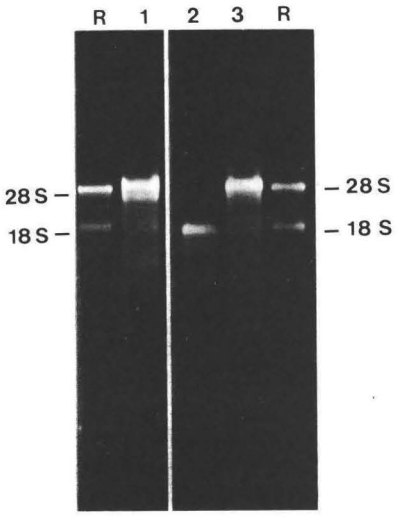


Figure 4. RNA Analysis of Nuclear Subfractions from Xenopus laevis Oocytes

RNA was separated on 1.5% agarose gels and stained with ethidium bromide. As molecular weight standards, rRNAs from ribosomes of Xenopus laevis ovaries were run in adjacent slots (R; 28S rRNA, molecular weight 1.5 × 10^6; 18S rRNA, molecular weight 0.7 × 10^6). Lane 1: RNA from the high speed pellet (100,000 × g, 1 hr) of total homogenates of mass-isolated Xenopus oocyte nuclei reveals a major band, migrating similarly to the 28S rRNA reference, and a minor band, comigrating with the 18S rRNA reference. Lane 2: RNA from isolated preribosomes sedimenting at 40S (fraction No. 13 in Figure 3a), comigrating with cytoplasmic 18S rRNA. Lane 3: 65S preribosomes (fraction No. 19–21 in Figure 3a) contain a major RNA component with an electrophoretic mobility similar to that of 28S cytoplasmic rRNA.

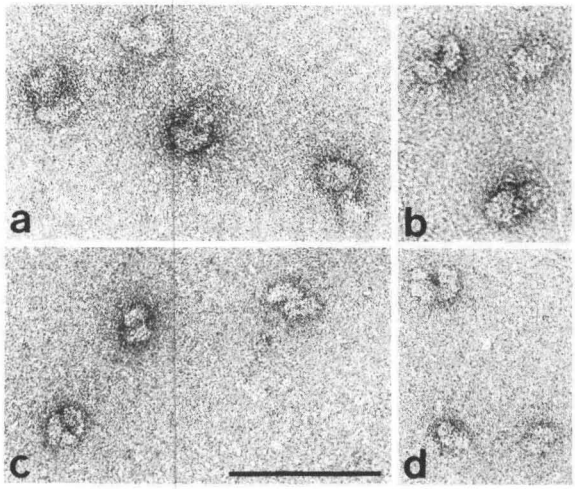


Figure 5. Electron Micrographs of Nucleoplasmic Preribosomal Particles Visualized by Negative Staining

(a,b) Preribosomes sedimenting at 65S were fixed on the grid and negatively stained with 2% uranyl acetate. They reveal a compact structure (300–400 Å) and look similar to the crown and kidney forms described for cytoplasmic 60S ribosomal subunits.

(c,d) Nucleoplasmic preribosomes sedimenting at 40S also appear as compact particles (250–300 Å) with a head-and-base structure with the typical constriction described for cytoplasmic 40S ribosomal subunits. Bar, 0.1 μm.

mentation during homogenization and resuspension of the nucleoli-containing low speed pellets (see Experimental Procedures). Under these conditions only miniscule amounts of 65S particles were released from the pellets containing nucleoli (data not shown).

From these results we conclude that ribocharin is specifically associated with free 65S particles of the nucleoplasm. Moreover, the relative intensities of polypeptides stained with Coomassie blue, present in the 65S particle fraction (e.g. Figure 3b), indicate that ribocharin is abundant in these particles, in amounts similar to those of ribosomal proteins of the large subunit.

Characterization of Nucleoplasmic Preribosomal Particles by RNA Analysis and Electron Microscopy

We analyzed the RNA content of total 100,000 × g pellets from a homogenate of mass-isolated Xenopus oocyte nuclei and from 40S and 65S peak fractions obtained after sucrose gradient centrifugation. As shown in Figure 4, the total 100,000 × g pellet of oocyte nuclei revealed a major RNA component with an electrophoretic mobility similar to that of the cytoplasmic 28S rRNA and a minor component comigrating with cytoplasmic 18S rRNA (Figure 4, lanes R and 1). When 100,000 × g pellets from homogenates of mass-isolated oocyte nuclei were fractionated by sucrose gradient centrifugation, as shown in Figure 3a, the fraction sedimenting at 40S revealed RNA comigrating with cytoplasmic 18S rRNA (Figure 4, lane 2). The 65S peak fractions contained only one major RNA component, which migrated similarly to the 28S cytoplasmic rRNA reference (Figure 4, lane 3). These results indicate that the 40S and 65S particles correspond to extranucleolar precursor forms of the small and large ribosomal subunits, respectively.

The structure of isolated nucleoplasmic preribosomal particles was also examined in the electron microscope, using negative staining with uranyl acetate. The 65S preribosomal particles with diameters of 30–40 nm showed a compact structure with a contour morphology similar to that of the crown and kidney forms described for the cytoplasmic 60S ribosomal subunit (Figures 5a, 5b; compare Lake et al., 1974; for recent review see Bielka, 1982). Nucleoplasmic preribosomal particles sedimenting at 40S (Figures 5c, 5d) also appeared as compact particles of diameter 25–30 nm and often revealed the head-and-base structure with the typical constriction for mature small ribosomal subunits (Lake et al., 1974; Lutsch et al., 1979; Bielka, 1982).

Distribution of Ribocharin-Containing Particles in Eggs

During meiotic maturation of oocytes the nuclear envelope breaks down, resulting in a mixing of nuclear and cytoplasmic proteins and particles (cf. Laskey et al., 1978; Forbes et al., 1983). In unfertilized eggs of Xenopus laevis the bulk of ribocharin was found in association with 65S particles (Figure 6), and no considerable increase of soluble ribocharin was observed. These results indicate that ribocharin did not readily dissociate from the 65S precursor particles, now exposed to a common ooplasm, and also that no exchange of ribocharin between 65S preribosomal particles and mature ribosomes took place after the breakdown of the nuclear envelope during meiosis.

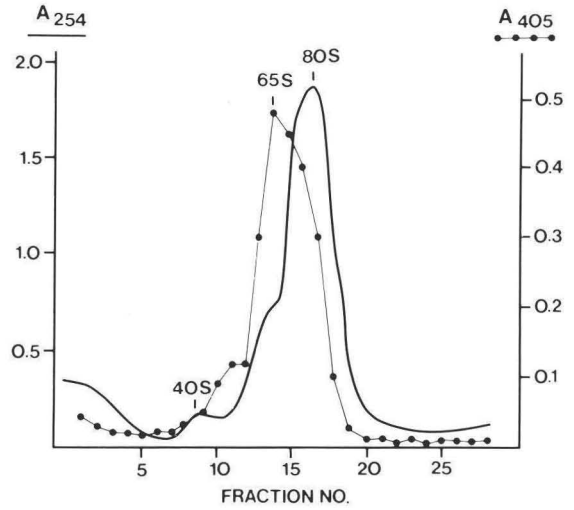


Figure 6. Ribocharin-Containing 65S Preribosomes in Eggs of Xenopus laevis

The homogenate from 200 unfertilized eggs was centrifuged at 3500 × g and the resulting supernatant was again centrifuged for 1 hr at 100,000 × g. The pellet obtained was resuspended and fractionated by linear sucrose gradient centrifugation and individual fractions were analyzed for the presence of ribocharin by ELISA technique using monoclonal antibody No-194. As shown by ELISA activity (A_{405}) ribocharin is associated with particles sedimenting at 65S. Endogenous ribosomes (80S) and free ribosomal subunits (A_{254}) serve as s-value references.

Ribocharin in Somatic Cells

In order to determine whether ribocharin is oocyte-specific, we analyzed particle fractions from total cell homogenates of cultured Xenopus laevis kidney cells (XLKE-A_6) by sucrose gradient centrifugation, followed by ELISA assays of the fractions using monoclonal antibody No-194. As shown in Figure 7a, the peak of ELISA activity corresponded to a fraction sedimenting at 65S. Immunoblotting experiments (Figure 7b) revealed that the reacting polypeptide had the same molecular weight (M_r 40,000; Figure 7b, lanes 2 and 2′) as in oocyte nuclei (lanes 1 and 1′). Similar results were obtained from analyses of kidney and liver tissues, and embryonic stages (not shown).

Localization of Ribocharin in Amphibian Tissues by Immunofluorescence Microscopy

By immunofluorescence microscopy, using monoclonal antibody No-194 on frozen sections through Xenopus laevis ovaries (Figures 8a–8d), intensive staining was found in the amplified nucleoli of previtellogenic (Figures 8a, 8b) and vitellogenic (Figures 8c, 8d) oocytes, and in several distinct small granules in the nucleoplasm (Figures 8a–8d). No staining was seen in the cytoplasm. Distinct staining was also observed in nucleoli of cells of the ovarian follicle epithelium and of interstitial connective tissue (Figures 8a and 8b). Similarly, a diversity of other somatic tissues examined showed distinct fluorescence of nucleoli and certain nucleoplasmic dots in the various amphibia tested, including urodelan species. Figure 9a presents an example of Triturus liver. In tissues that contain erythrocytes, such as myocardiac tissue (Figures 9b and 9c), the nonerythroid cell nuclei were strongly positive, whereas the erythrocyte nuclei were negative (arrows in Figure 9b). Thus, the residual nucleolar structures present in amphibian erythrocytes (cf. Schmidt-Zachmann et al., 1984) apparently are devoid of ribocharin, in agreement with reports of an absence of rDNA transcription and ribosome synthesis in these structures (Hentschel and Tata, 1978). In the testis of Xenopus (Figures 9d and 9e) and other amphibia (not shown), nucleoli and small nucleoplasmic granules were detected in Sertoli cells, spermatogonia, and spermatocytes, but not in spermatids and testicular spermatozoa.

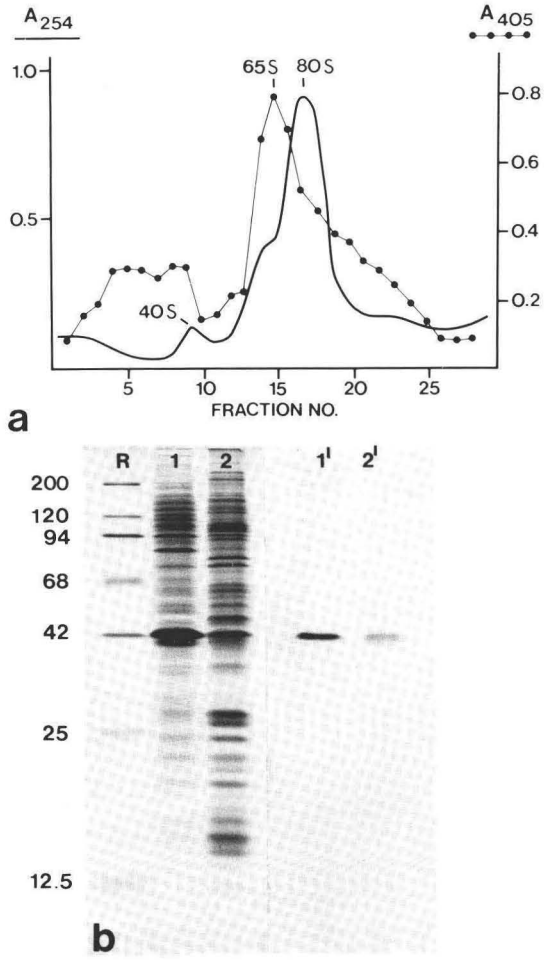


Figure 7. Immunological Identification of Ribocharin in Cultured Kidney Epithelial Cells of Xenopus laevis

(a) Approximately 1 × 10^7 XLKE-A_6 cells were homogenized and fractionated by differential centrifugation and sucrose gradients as described in the legend of Figure 6. As shown by ELISA technique using antibody No-194 (A_{405}), ribocharin is enriched in fractions sedimenting at 65S.

(b) Detection of ribocharin in the 100,000 × g pellet of a XLKE cell extract by the immunoblotting technique. Lane 2, Coomassie blue staining of proteins present in 100,000 × g pellet after separation by SDS–polyacrylamide gel electrophoresis (18% acrylamide); lane 2′, corresponding immunoblot after incubation with antibody No-194. For comparison the corresponding fraction (100,000 × g pellet) from a nuclear homogenate of Xenopus oocytes was analyzed in adjacent slots (lanes 1, 1′). Positive reaction for a M_r 40,000 protein is seen in both cell types. Reference proteins (R; M_r values are indicated in 10^3 units on the left margin) are as in Figure 1a.

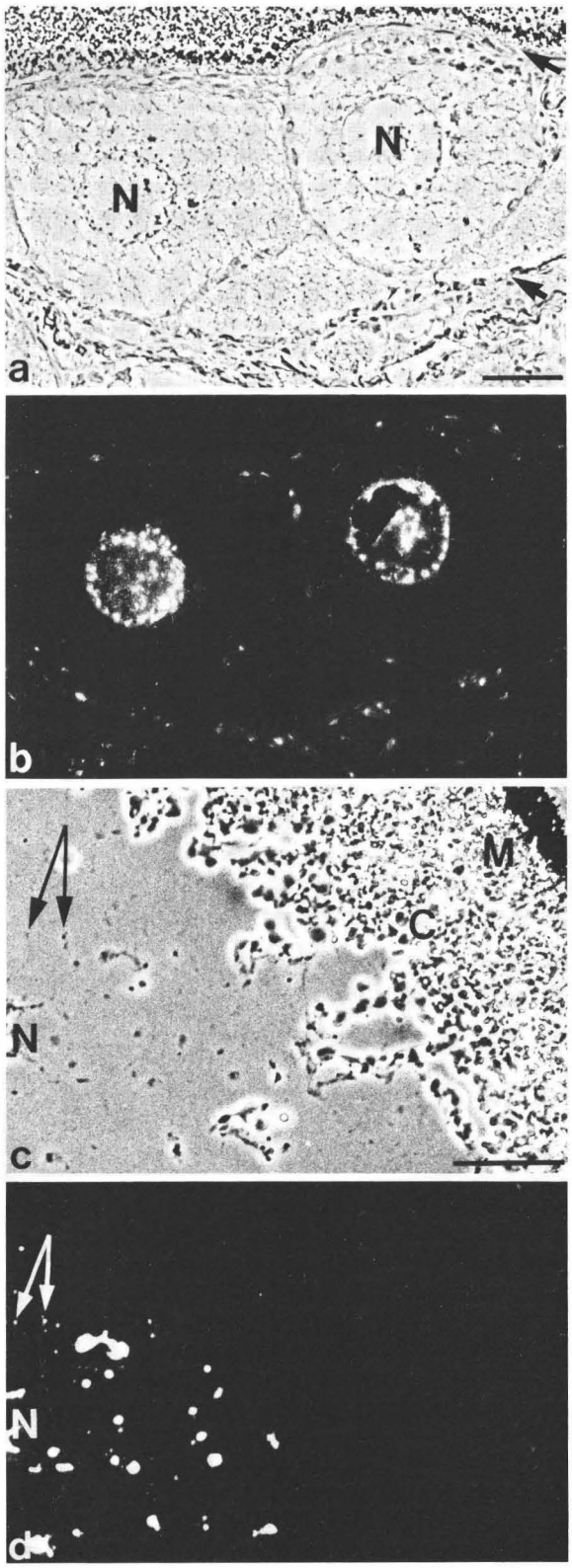


Figure 8. Localization of Ribocharin in Previtellogenic and Vitellogenic Oocytes of Xenopus laevis by Immunofluorescence Microscopy

(a,b) Frozen section of an ovary of Xenopus laevis showing previtellogenic oocytes in phase contrast (a) and epifluorescence (b) optics after incubation with monoclonal antibody No-194. In the nuclei (N) numerous amplified nucleoli, most of them in the nuclear cortex, as well as some smaller nucleoplasmic entities are fluorescing. In addition, the nucleoli of the surrounding follicle epithelial cell layer (arrows) are also stained.

(c,d) Vitellogenic oocytes in phase contrast (c) and epifluorescence optics (d) after reaction with antibody No-194. In the very large nuclei (N) intense staining is observed on nucleoli and the numerous small nucleoplasmic dots (some are denoted by arrows). C, cytoplasm; M, melanin granules of cortex. Bars, 50 μm.

Monoclonal antibody No-194 did not react with tissues from chicken and mammals (rat, cow, man).

Localization of Ribocharin in Cultured Interphase Cells and during Mitosis

In cultured cells of Xenopus laevis (XLKE-A_6) the distribution of ribocharin in interphase and mitosis showed remarkable changes. By immunofluorescence microscopy using antibody No-194, ribocharin-positive fluorescence was localized in interphase cells over nucleoli and certain distinct granules scattered throughout the nucleoplasm (Figures 10a and 10a′), whereas the cytoplasm was ribocharin-negative. In preprophase and prophase (Figures 10b and 10b′) the nucleoli began to disintegrate, and antibody No-194 stained nuclear granules of various sizes, including what appeared to be fragments derived from the disintegrated nucleolus. In metaphase (Figures 10c and 10c′) and anaphase (Figures 10d and 10d′), strong and rather uniform fluorescence was observed on the surfaces of all chromosomes, including those without nucleolus organizers. In late telophase (Figures 10e and 10e′), ribocharin was seen to reaccumulate into distinct granules located within the confinements of the newly formed daughter nuclei, with progressive concentration in the reconstituting nucleoli.

This mitotic distribution of ribocharin was different from that of several other nucleolar proteins examined in parallel. For example, RNA polymerase I, which is localized in the fibrillar centers of the interphase nucleolus, remained in association with the nucleolar organizer regions of mitotic chromosomes (cf. Scheer and Rose, 1984). Another nucleolar protein of M_r 180,000, which served as a marker for the dense fibrillar component of the nucleolus, was found to be uniformly distributed through the whole cytoplasm of metaphase cells (Schmidt-Zachmann et al., 1984). On the other hand, the association of ribocharin with surfaces of mitotic chromosomes resembled the mitotic distribution of ribosomal protein S1, which was located in the granular component of the interphase nucleolus and on chromosome surfaces during mitosis (Hügle et al., 1985).

Electron Microscopic Immunolocalization of Ribocharin

The subnucleolar location of ribocharin and the extranucleolar forms of ribocharin-containing material were examined at the electron microscopic level by the immunogold technique. In oocytes, the gold particles were found over most of the nucleolar body, showing some enrichment in the periphery, and were also scattered throughout most of the nucleoplasm (data not shown). In hepatocyte nucleoli of Xenopus laevis and Triturus crista-

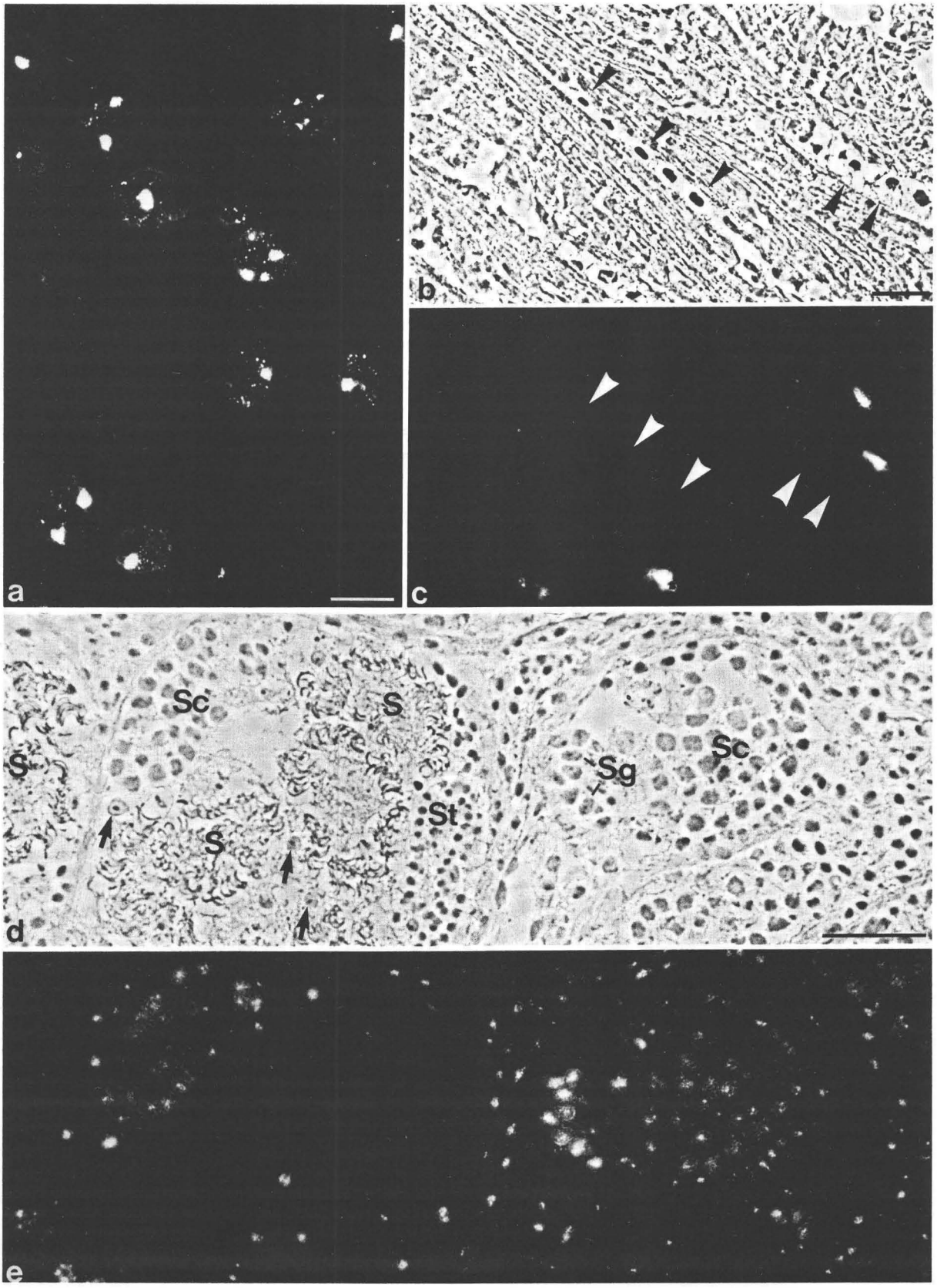


Figure 9. Localization of Ribocharin in Different Amphibian Tissues by Immunofluorescence Microscopy Using Antibody No-194

(a) Fluorescence micrograph of a frozen section of liver tissue of Triturus cristatus, showing bright fluorescence of nucleoli and finely dispersed small particles in the nucleoplasm. The cytoplasm is not stained.

(b,c) Frozen section of heart muscle of Xenopus laevis in phase contrast (b) and epifluorescence (c) optics, showing strong reaction on nuclei of myocardiac and other mesenchymal cells, but absence of staining on erythrocyte nuclei (some are denoted by arrows).

(d,e) In frozen sections of Xenopus laevis testis (d, phase contrast; e, epifluorescence optics) antibody No-194 reacts with nucleoli and certain distinct nucleoplasmic granules of spermatogonia (Sg), spermatocytes (Sc) and Sertoli cells (arrows), whereas spermatids (St) and spermatozoa (S) are negative. Bars denote 20 μm (a,b,c) and 50 μm (d,e).

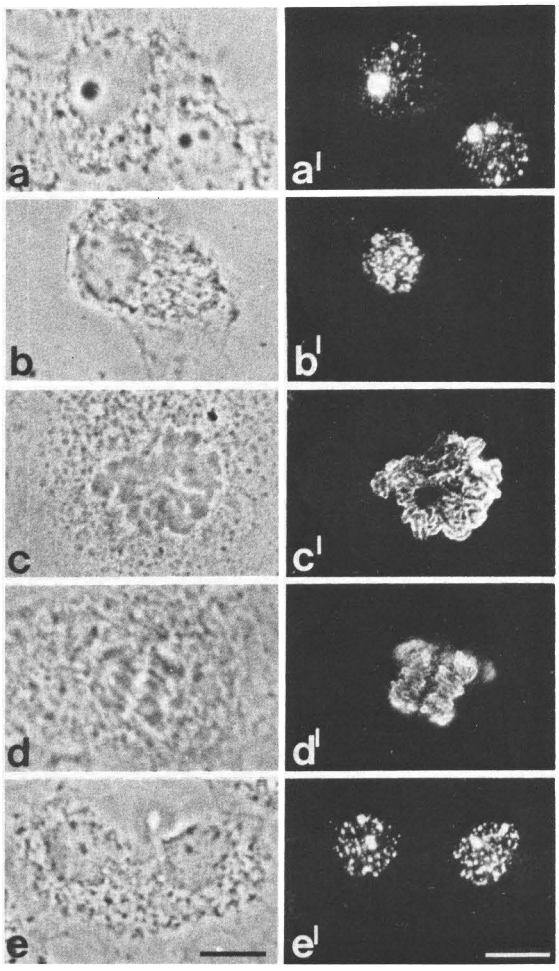


Figure 10. Distribution of Ribocharin during Mitosis of Cultured Xenopus laevis Cells as Shown by Immunofluorescence Microscopy Using Antibody No-194

Phase contrast images are shown in (a–e), the corresponding immunofluorescence micrographs in (a′–e′). In interphase cells (a, a′), ribocharin is localized in nucleoli and surrounding nucleoplasmic granules, but is absent from the cytoplasm. In preprophase and prophase (b, b′), fragments of the disintegrating nucleoli and numerous small entities are stained. In metaphase (c, c′) and anaphase (d, d′), uniform fluorescence of the chromosomal surfaces is observed. In late telophase (e, e′), the reforming nucleoli are stained as well as numerous nucleoplasmic entities. Bar, 10 μm.

tus, better structural preservation was obtained and allowed a finer localization. In these cells, the granular component of the nucleolus was specifically labeled by gold particles. Figure 11a presents an example of Triturus, Figure 11d one for Xenopus. Other nucleolar subcompartments—dense fibrillar component and fibrillar centers—were not decorated by this antibody. Outside of the nucleolus, certain distinct extranucleolar entities were found to be conspicuously decorated by the antibody-binding gold particles (arrows in Figure 11a). These structures, which appeared irregularly scattered throughout the nucleoplasm, appeared as roundish aggregates of variable diameters (0.1–0.3 μm) and revealed granular substructures of diameters 20–35 nm (Figures 11b and 11c). The morphology of these clustered particles closely resembles that of typical interchromatin granules (Swift, 1963; Monneron and Bernhard, 1969; for review see Puvion and Moyne, 1981). Obviously, these aggregates of 20–35 nm granules seen in the electron microscope correspond to the small fluorescent nucleoplasmic dots observed by light microscopic immunolocalization (compare Figures 9a and 10a′ with Figures 11a–11d).

In control experiments performed in parallel, antibodies against a M_r 180,000 protein specifically labeled the dense fibrillar component of the nucleolus (cf. Schmidt-Zachmann et al., 1984). This illustrated the accessibility of this nucleolar substructure for immunoglobulins under the conditions used, thus emphasizing the specificity of the localization of ribocharin in the granular part of the nucleolus and the nucleoplasmic particles.

Discussion

In the present study we have identified ribocharin, an acidic nuclear protein of M_r 40,000 exhibiting a very specific location. Although a certain proportion of this protein is found as a soluble molecule in the nucleoplasmic sap (data will be presented elsewhere), most of it is stably associated with the particles of the granular component of the nucleolus and with the extranucleolar precursor particles to the large ribosomal subunit distributed throughout the nucleoplasm. Ribocharin is absent from cytoplasmic ribosomes. Within the nucleolus, the protein colocalizes with some authentic ribosomal proteins, such as the largest protein of the small ribosomal subunit, termed S1.

Ribocharin appears to be different from all hitherto described nuclear and ribosomal proteins. Some of its properties resemble those reported for protein B23, a nucleolar protein of rat liver and hepatoma cells (for review see Busch, 1984) to which a M_r value of 37,000 and a considerably lower isoelectric pH value of approximately 5.0 have been assigned. In contrast to ribocharin, protein B23 has been found exclusively in nucleoli and not in nucleoplasmic bodies. In addition to being found in the granular component of the nucleolus, it has also been

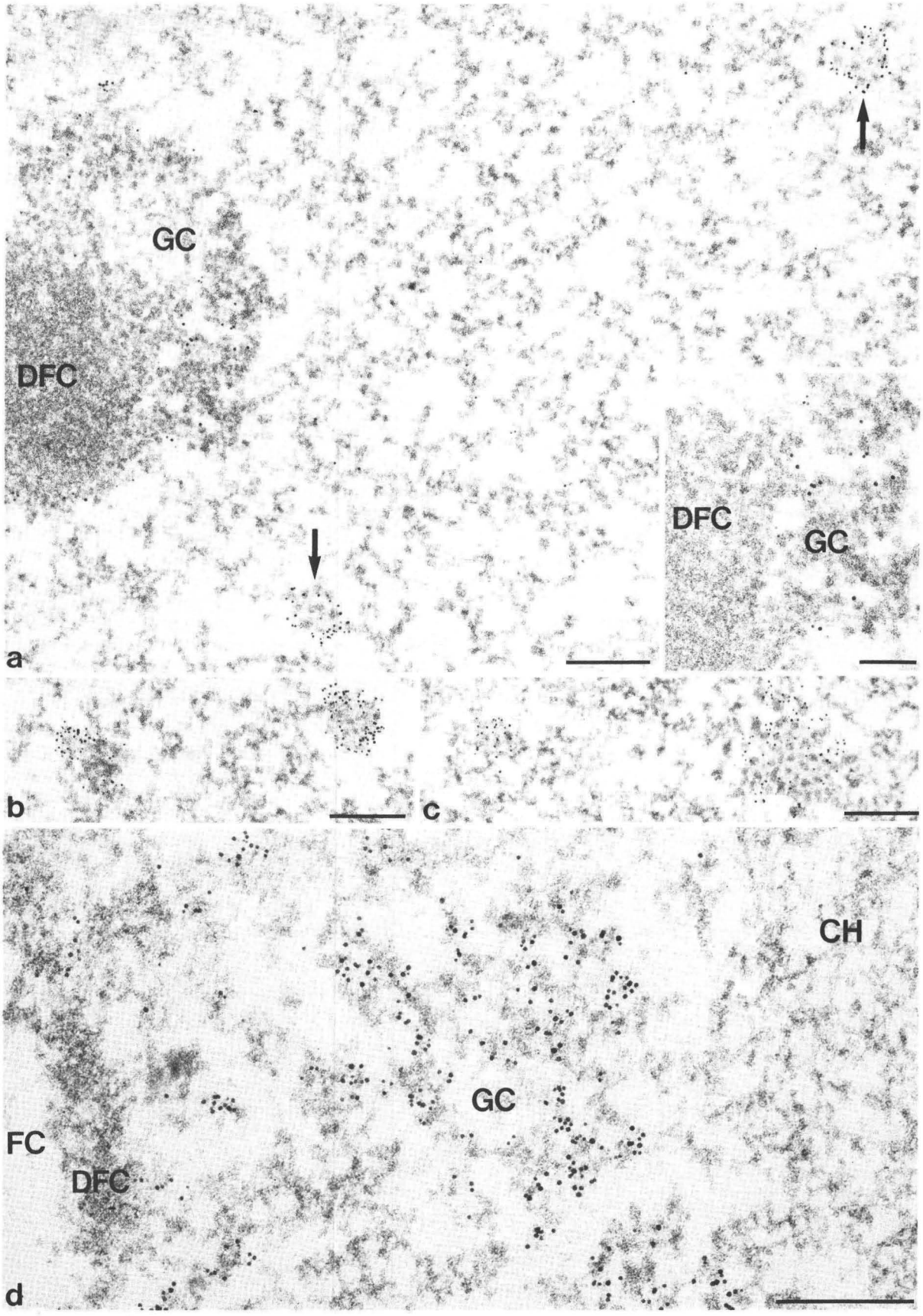
Figure 11. Electron Microscopic Localization of Ribocharin by the Immunogold Technique

Frozen sections of liver tissue of Triturus (a–c) or Xenopus (d) were incubated with antibody No-194, followed by secondary antibodies coupled to 5 nm colloidal gold particles. After the immunoreaction, tissue sections were processed for electron microscopy

(a) In hepatocyte nucleoli of Triturus, the granular component (GC) of the nucleolus is intensely decorated by gold particles (shown at higher magnification in the insert in the lower right), whereas the dense fibrillar component (DFC) and the fibrillar centers (not seen here) are not labeled. In addition, a certain type of distinct nucleoplasmic entities is also heavily labeled by gold particles (arrows).

(b,c) Details showing that the nucleoplasmic clusters reacting with antibodies to ribocharin, are represented by roundish aggregates (0.1–0.3 μm in diameter) of 20–35 nm particles, which sometimes reveal a less intensely stained core (c).

(d) Part of a nucleolus of Xenopus hepatocyte, showing the specific reaction of antibody No-194 with the granular component (GC). The other nucleolar components (dense fibrillar component, DFC; fibrillar center, FC) and the surrounding chromatin (CH) are free of gold particles. Bars 0.2 μm (a–d) and 0.1 μm (insert in a).


GC
DFC
a
DFC
GC
b
c
CH
GC
FC
DFC
d

reported to occur in the fibrillar nucleolar component (e.g., Daskal et al., 1980; Ochs et al., 1983; Smetana et al., 1984; Spector et al., 1984). Interestingly, ribocharin accumulates on chromosomal surfaces during metaphase and anaphase, a distribution that is similar to that reported for ribosomal protein S1 (Hügle et al., 1985) and nucleolar protein B23, but that differs from the mitotic location of such proteins as the M_r 180,000 protein (Schmidt-Zachmann et al., 1984), the M_r 110,000 protein C23 (Spector et al., 1984), and RNA polymerase I (Scheer and Rose, 1984), which are located in fibrillar components of the nucleolus.

In accord with these results we conclude that ribocharin represents a new type of nucleolar, nonribosomal protein, which associates with the precursor particles containing the 28S rRNA sequence within the confinements of the nucleolus, but at some distance from the rDNA template chromatin. Although the limited interspecies cross reactivity of antibody No-194 did not allow us to identify ribocharin or related protein (or proteins) in higher vertebrates such as birds and mammals, we predict that a nucleolar protein with similar properties exists in other species as well.

In our study we have also identified a new type of free nucleoplasmic particle of 65S that contains a nuclear precursor to the 28S rRNA, several ribosomal proteins (data not shown here), and ribocharin, the latter being present in amounts apparently equimolar to those of the ribosomal components present. We hypothesize that this 65S particle is a special form of nucleoplasmic precursor to the large ribosomal subunit en route to the cytoplasm. The existence of such a particle has been postulated in most hypothetical models of nucleocytoplasmic transfer of ribosomal precursors (e.g. Perry, 1967; Prestayko et al., 1974; Hadjiolov, 1984). However, it had so far not been found, probably because of technical difficulties and the very low steady state concentrations present in nuclei of somatic cells. Our choice of the amphibian oocyte with its high ribosome production rate has certainly facilitated the discovery of this particle, as it provides a large nuclear volume and allows the isolation of structurally intact nuclei. In its properties this 65S nucleoplasmic particle is clearly distinct from the preribosomal nucleolar particles of approximately 80S, containing the pre-rRNA, and 55S, containing processing intermediates, that have been extracted with vigorous methods from isolated nucleoli or nuclei of various cells (Kumar and Warner, 1972; Prestayko et al., 1974; for review see Hadjiolov, 1984; for data from amphibian oocytes see Rogers, 1968).

Electron microscopic immunolocalization with ribocharin antibody No-194 has also identified, in diverse somatic cells, typical extranucleolar aggregates of 20–35 nm granules, the so-called interchromatin granules (Monneron and Bernhard, 1969), as distinct structures rich in ribocharin. Our findings suggest that these interchromatin granules represent aggregates of nucleoplasmic forms of precursors to the large ribosomal subunit and are equivalent to the 65S nucleoplasmic particles observed by sucrose gradient centrifugation. This interpretation of the interchromatin granules as preribosomal particles is in agreement with previous cytochemical tests suggesting a ribonucleoprotein character of these particles, and with studies of effects of drugs interfering with ribosome production (Puvion et al., 1984). It may be that interchromatin granules contain additional proteins that are not present in nucleoli (Clevenger and Epstein, 1984). In the future, we propose to use ribocharin antibodies as very selective and sensitive probes for nuclear precursors to the large ribosomal subunit and interchromatin granules.

We have also observed a smaller free nucleoplasmic particle of 40S that contains 18S rRNA and some ribosomal proteins of the small subunit, including protein S1, and that lacks ribocharin. These particles, which are present in much lower concentrations than the 65S particles (for corresponding observations of disproportionately low nuclear levels of precursors to the 18S rRNA in various cell types, amphibian oocytes included, see Penman et al., 1966; Franke and Scheer, 1974b; Eckert et al., 1978), probably represent the nucleocytoplasmic translocation form of precursors specific to the small ribosomal subunit. However, more detailed characterization of this particle is necessary to elucidate its nature.

We have shown that the large subunit of the cytoplasmic ribosome and the corresponding, slightly larger, precursor particle in the nucleoplasm differ in respect to at least one specific protein, i.e., ribocharin. This illustrates the profound compositional difference between the two related particles. It also suggests that rearrangements of 28S rRNA-associated proteins must occur very late in ribosome maturation, that is, after release from the nucleolus and during nucleocytoplasmic translocation, which could be at the level of the nuclear pore complex (for discussion see Franke and Scheer, 1974a, 1974b). In correlation with this hypothesis, previous reports show that certain ribosomal proteins, notably some of the small subunit, are not detected in nuclear and nucleolar fractions, suggesting that these proteins are added only upon release of the precursor into the cytoplasm (cf. Warner, 1979; for review see Hadjiolov et al., 1984). In this respect, the nucleocytoplasmic transport of ribosomal precursors seems to be similar to that of nuclear ribonucleoprotein particles containing mRNA sequences; drastic changes in protein composition during migration from nucleus to cytoplasm have been reported for those particles (e.g., Martin et al., 1980; Pederson and Munroe, 1981; Setyono and Greenberg, 1981).

Our finding that no exchange to cytoplasmic ribosomes takes place upon disruption of the nuclear envelope during mitosis or during meiotic divisions demonstrates the specificity and the stability of the association of ribocharin with nuclear precursor particles to the large ribosomal subunit. Moreover, the fact that during mitotic telophase, ribocharin, as well as the particles containing it, is rapidly accumulated in the reforming nuclei, points to the existence of as yet unknown mechanisms for nuclear sorting of these particles. On the other hand, one must also postulate a mechanism of specific release of ribocharin from the 65S particles late in the intranuclear maturation of the large ribosomal subunit. This release could be

related to the removal of the 5′-terminal excess sequence from the immediate precursor to the 28S rRNA (cf. Hadjiolova et al., 1984). This is currently under study in our laboratory. The released ribocharin might then contribute to the small nucleoplasmic pool of soluble 6.5S and 12S forms of ribocharin, and might be reutilized in the nucleolus. This hypothesis would be in line with previous studies of protein labeling kinetics indicating that several nonribosomal proteins were reutilized for ribosome assembly in the nucleolus (Kumar and Warner, 1972; Kumar and Subramanian, 1975; Warner, 1979). We hope that detailed protein chemical and cross-linking studies of ribocharin, as well as in vitro studies of its binding to precursor particles, will help in elucidating the function in ribosome biogenesis and nucleocytoplasmic transport of this and other nonribosomal proteins associated with ribosomal precursor particles.

Experimental Procedures

Animals and Cells

Adult male and female clawed toads (Xenopus laevis) were purchased from the South African Snake Farm (Fish Hoek, South Africa). Newts (Triturus cristatus) and salamanders (Pleurodeles waltlii) were reared in our laboratory. Frogs (Rana temporaria), chickens, and rats were purchased from a local animal dealer. Tissue samples were taken and frozen as described (Krohne et al., 1978). Cultured cells derived from Xenopus laevis kidney epithelium (XLKE cells, line A_6; American Type Culture Collection) and various nonamphibian cell lines were grown as described (Franke et al., 1979).

Monoclonal Antibodies

Monoclonal antibodies were raised essentially as described by Köhler and Milstein (1975). Balb/c-mice were immunized with the pellet obtained after low speed centrifugation (10 min; 3500 × g) of a homogenate of mass-isolated Xenopus oocyte nuclei (for procedure see Scalenghe et al., 1978; Kleinschmidt and Franke, 1982). This pellet was greatly enriched in nucleoli. Antibody-producing hybridoma cell line No-194 was selected by the method described by Schmidt-Zachmann et al. (1984). Immunoglobulin subclasses were determined by double immunodiffusion (Ouchterlony and Nilsson, 1978) using doubly concentrated hybridoma cell culture supernatant and subclass-specific antibodies to mouse immunoglobulins (Miles-Yeda, Rehovot, Israel). Production of antibody-containing ascites fluid and purification of monoclonal mouse IgM antibodies was performed as described (Hügle et al., 1985).

Preparation of Ribosomes

Ribosomes of Xenopus laevis ovaries were prepared according to Ford (1971) with some modifications that have been described in detail elsewhere (Hügle et al., 1985).

Isolation and Fractionation of Oocyte Nuclei

Large-scale isolation of nuclei from Xenopus laevis oocytes (stages IV–VI; Dumont, 1972) was carried out as essentially described by Scalenghe et al. (1978) with the modifications of Kleinschmidt and Franke (1982). Mass-isolated nuclei were gently homogenized by brief whirling in a Vortex and by sucking the suspension several times up and down in an Eppendorf plastic tip. The homogenate was centrifuged for 10 min at 3500 × g at 4°C. The resulting pellet, called a low speed pellet, was enriched in nucleoli, nuclear envelopes and chromosomes. The supernatant obtained—low speed supernatant—was centrifuged at 100,000 × g for 1 hr. Under these conditions nucleoplasmic ribosomal precursor particles were recovered in the pellet (high speed pellet) whereas soluble nuclear proteins remained in the supernatant (high speed supernatant). Manually isolated oocyte nuclei and cytoplasms (Krohne and Franke, 1983) were homogenized and fractionated by low speed centrifugation (10 min at 3500 × g).

Sucrose Gradient Analysis

High speed pellets obtained either from 10 ml nuclear homogenates of vitellogenic oocytes, from 200 unfertilized eggs, or from 1×10^7 cultured XLKE cells were resuspended in 0.5 ml TBS (100 mM NaCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 7.4), and layered on top of a 10%–40% [w/v] linear sucrose gradient made up in TBS. Gradients were centrifuged at 21,000 rpm in a Beckman SW40 rotor for 16 hr at 4°C. For S value estimation cytoplasmic 80S ribosomes, or 40S and 60S ribosomal subunits from Xenopus laevis ovaries were examined in parallel gradients. In some experiments 10 ml low speed supernatant of oocyte nuclei, including total nucleoplasmic particles and soluble proteins, were concentrated by vacuum dialysis and fractionated by sucrose gradient centrifugation as described above. Alternatively, peak fractions obtained were pooled and recentrifuged under the same conditions.

For fractionation of soluble nuclear proteins, 3 to 5 fold concentrated high speed supernatant (final volume 0.5 ml) was analyzed on 5%–30% (w/v) linear sucrose gradients made in TBS. Gradients were centrifuged at 35,000 rpm in a Beckman SW40 rotor for 18 hr at 4°C. Reference proteins (bovine serum albumin, 4.3S; immunoglobulin G, 6.5S; catalase, 11.3S and thyroglobulin, 16.5S) were applied to parallel gradients.

Fractions of 0.4 ml were collected and either used directly for ELISA tests or the proteins were precipitated by 15% trichloroacetic acid (final concentration), washed repeatedly with acetone, and analyzed by SDS–polyacrylamide gel electrophoresis.

Gel Electrophoresis of Proteins and Immunoblotting

Gel electrophoresis in the presence of SDS was performed essentially as described by Thomas and Kornberg (1975) using acrylamide concentrations of 18%. Two-dimensional gel electrophoresis using nonequilibrium pH gradient (NEPHG) in the first dimension was carried out according to O'Farrell et al. (1977); the second dimension was performed in 18% acrylamide gels, using the gel and buffer system described by Thomas and Kornberg (1975). Before being applied to the first dimension separation, material of 100,000 × g pellets was first digested with 0.1 mg/ml pancreatic RNAase (Boehringer, Mannheim, FRG) for 15 min at 37°C in order to remove nucleic acids that might interfere with the migration of the proteins into the gel.

For immunoblotting experiments, polypeptides separated by one- or two-dimensional gel electrophoresis were partially renatured by incubation of the gel in 4 M urea, 50 mM NaCl, 2 mM EDTA, 0.1 mM dithiothreitol, 10 mM Tris-HCl (pH 7.5), for 1.5 hr at room temperature (Bowen et al., 1980). After partial renaturation, proteins were transferred electrophoretically from the gels to nitrocellulose paper according to the method of Towbin et al. (1979). Protein binding sites on nitrocellulose were saturated by 1% bovine serum albumin (BSA) in 140 mM NaCl, 10 mM Tris-HCl (pH 7.4) for 2–3 hr followed by overnight incubation at 4°C with culture supernatant of antibody-producing hybridoma cells. After several washes with 1 M NaCl in 10 mM Tris-HCl (pH 7.4), nitrocellulose sheets were incubated for 2 hr at room temperature with horseradish peroxidase coupled goat anti-mouse IgG/IgM (Medac, Hamburg, FRG) diluted 1:1000 in 1% BSA. Nitrocellulose sheets were extensively washed with 1 M NaCl and 10 mM Tris-HCl (pH 7.4), followed by several washes with 140 mM NaCl, 10 mM Tris-HCl (pH 7.4) containing 0.1% Triton X-100. Bound antibody was visualized by enzyme reaction using 4-chloro-1-naphtol and H_2O_2 as substrate (Hawkes et al., 1982).

Enzyme-Linked Immunosorbent Antibody Assays (ELISA)

The ELISA assay was performed as described by Hautanen and Linder (1981). Activated microtiter plates (Flow Laboratories, Meckenheim, FRG) were coated with either aliquots of sucrose gradient fractions or fractions containing nuclear proteins (50 μl/well). After adsorption of the antigen, plates were incubated with undiluted tissue culture supernatant of hybridoma clone No-194. As secondary antibodies, alkaline phosphatase coupled goat antibodies to mouse IgM (Medac, Hamburg, FRG) were used at a dilution of 1:1000. Specifically bound antibodies were visualized by enzyme reaction, and absorbance was measured at 405 nm by a Titertek Multiscan photometer (Flow Laboratories, Meckenheim, FRG). For controls, cell culture supernatant of non-Ig-producing hybridoma cells was used instead of the primary antibody.

Immunolocalization

Light microscopic immunolocalization, using frozen sections (5 μm thickness) or cultured cells grown on coverslips, was performed as described (Hügle et al., 1985). Incubation times of primary and secondary antibodies were kept short (normally 15 min each) in order to minimize artificial elution of the antigen during preparation for immunofluorescence. For electron microscopic immunolocalization, cryostat sections through ovary and liver tissue from Xenopus laevis or Triturus cristatus were dehydrated for 10 min in acetone (−20°C), air dried, and incubated for 20 min with the primary antibody No-194 (50 μg IgM/ml). After brief washing with PBS, sections were postfixed with 2% formaldehyde (in PBS) for 10 min and treated with 1% Triton X-100 (in PBS) for 5 min. Specimens were then washed in PBS and incubated overnight with goat anti-mouse immunoglobulin coupled to 5 nm colloidal gold particles (Janssen Life Sciences, Beerse, Belgium), which was diluted 1:5 or 1:10 in PBS. Sections were thoroughly washed in PBS, fixed in 2.5% glutaraldehyde, and processed for electron microscopy as described (Scheer and Rose, 1984). Micrographs were taken with a Zeiss EM 10 electron microscope.

Negative Staining of Ribosomes and Preribosomal Particles

Peak fractions from sucrose gradients were dialyzed against TBS, and 20 μl were directly applied to carbon coated grids activated by glow discharge. After 1 min of incubation, particles were fixed on the grid for 30 sec with 0.2% glutaraldehyde. After removing the fixative, particles were stained with 2% uranyl acetate for 1 min, air dried, and examined in a Zeiss EM 10 electron microscope.

RNA Analysis

Pellets obtained after centrifugation (100,000 × g) of mass-isolated Xenopus laevis oocyte nuclei were incubated with 50 mM Tris-HCl (pH 8.4), 20 mM EDTA, and 1% Sarkosyl NL-97, containing 0.5 mg/ml proteinase K (Boehringer, Mannheim, FRG; preincubated for 30 min at 37°C). After 2–6 hr, RNA was precipitated by the magnesium phosphate method as described by Dessev and Grancharov (1973). RNA from sucrose gradient fractions was first precipitated by the same method, then digested with proteinase K, and again precipitated by addition of 2.5 vol ice-cold ethanol. RNA was analyzed on horizontal 1.5% agarose slab gels as described by Franke et al. (1981).

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References

Auger-Buendia, M.-A., and Longuet, M. (1978). Characterization of proteins from nucleolar preribosomes of mouse leukemia cells by two-dimensional polyacrylamide gel electrophoresis. Eur. J. Biochem. *85*, 105–114.

Bielka, H. (1982). The Eukaryotic Ribosome. (Berlin, Heidelberg, New York: Springer-Verlag), pp. 1–338.

Bowen, B., Steinberg, J., Laemmli, U. K., and Weintraub, H. (1980). The detection of DNA-binding proteins by protein blotting. Nucl. Acids Res. *8*, 1–20.

Brown, D. D., and Dawid, I. B. (1968). Specific gene amplification in oocytes. Science *160*, 272–280.

Busch, H. (1984). Nucleolar proteins: purification, isolation and functional analyses. In Chromosomal Nonhistone Proteins IV: Structural Associations, L. S. Hnilica ed. (Boca Raton, Florida: CRC Press), pp. 233–286.

Busch, H., and Smetana, K. (1970). The Nucleolus. (New York: Academic Press), pp. 1–626.

Chooi, W. Y., and Leiby, K. R. (1981). An electron microscopic method for localization of ribosomal proteins during transcription of ribosomal DNA: a method for studying protein assembly. Proc. Natl. Acad. Sci. USA *78*, 4823–4827.

Clevenger, C. V., and Epstein, A. L. (1984). Identification of a nuclear protein component of interchromatin granules using a monoclonal antibody and immunogold electron microscopy. Exp. Cell Res. *151*, 194–207.

Daskal, Y., Smetana, K., and Busch, H. (1980). Evidence from studies on segregated nucleoli that nucleolar silver staining proteins C23 and B23 are in the fibrillar component. Exp. Cell Res. *127*, 285–291.

Dessev, G. N., and Grancharov, K. (1973). Precipitation of RNA, DNA and nucleo-protein particles from very dilute solutions. Anal. Biochem. *53*, 269–271.

Dumont, J. N. (1972). Oogenesis in Xenopus laevis (Daudin). I. Stages of oocyte development in laboratory-maintained animals. J. Morphol. *136*, 153–164.

Eckert, W. A., Kaffenberger, W., Krohne, G., and Franke, W. W. (1978). Introduction of hidden breaks during rRNA maturation and ageing in Tetrahymena pyriformis. Eur. J. Biochem. *87*, 607–616.

Forbes, D. J., Kirschner, M. W., and Newport, J. W. (1983). Spontaneous formation of nucleus-like structures around bacteriophage DNA microinjected into Xenopus eggs. Cell *34*, 13–23.

Ford, P. J. (1971). The proteins of Xenopus ovary ribosomes. Biochem. J. *125*, 1091–1107.

Franke, W. W., and Scheer, U. (1974a). Pathways of nucleocytoplasmic translocation of ribonucleoproteins. Symp. Soc. Exp. Biol. *28*, 249–282.

Franke, W. W., and Scheer, U. (1974b). Structures and functions of the nuclear envelope. In The Cell Nucleus, Vol. 1, H. Busch ed. (New York: Academic Press), pp. 219–347.

Franke, W. W., Schmid, E., Winter, S., Osborn, M., and Weber, K. (1979). Widespread occurrence of intermediate-sized filaments of vimentin-type in cultured cells from diverse vertebrates. Exp. Cell Res. *123*, 633–654.

Franke, W. W., Kleinschmidt, J. A., Spring, H., Krohne, G., Grund, C., Trendelenburg, M. F., Stoehr, M., and Scheer, U. (1981). A nucleolar skeleton of protein filaments demonstrated in amplified nucleoli of Xenopus laevis. J. Cell Biol. *90*, 289–299.

Hadjiolov, A. A. (1984). The Nucleolus and Ribosome Biogenesis. (Berlin, Heidelberg, New York: Springer-Verlag), pp. 1–268.

Hadjiolova, K. V., Georgiev, O. I., and Hadjiolov, A. A. (1984). Excess 5′-terminal sequences in the rat nucleolar 28 S ribosomal RNA. Exp. Cell Res. *153*, 266–269.

Hautanen, A., and Linder, E. (1981). C3c-binding ELISA for detection of immunoconglutins and immunoglobulin aggregates. Meth. Enzymol. *74*, 588–607.

Hawkes, R., Niday, E., and Gordon, J. (1982). A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. *119*, 142–147.

Hentschel, C. C., and Tata, J. R. (1978). Template-engaged and free RNA polymerases during Xenopus erythroid cell maturation. Dev. Biol. *65*, 496–507.

Hügle, B., Hazan, R., Scheer, U., and Franke, W. W. (1985). Localization of ribosomal protein S1 in the granular component of the interphase nucleolus and its distribution during mitosis. J. Cell Biol. *100*, 873–886.

Kleinschmidt, J. A., and Franke, W. W. (1982). Soluble acidic complexes containing histones H3 and H4 in nuclei in Xenopus laevis oocytes. Cell *29*, 799–809.

Köhler, G., and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature *256*, 495–497.

Krohne, G., and Franke, W. W. (1983). Proteins of pore complex-lamina structures from nuclei and nuclear membranes. Meth. Enzymol. *96*, 597–608.

Krohne, G., Franke, W. W., Ely, S., D'Arcy, A., and Jost, E. (1978). Localization of a nuclear envelope-associated protein by indirect im-

munofluorescence microscopy using antibodies against a major polypeptide from rat liver fractions enriched in nuclear envelope associated material. Cytobiologie *18*, 22–38.

Kumar, A., and Subramanian, A. R. (1975). Ribosome assembly in HeLa cells: labelling pattern of ribosomal proteins by two-dimensional resolution. J. Mol. Biol. *94*, 409–423.

Kumar, A., and Warner, J. R. (1972). Characterization of ribosomal precursor particles from HeLa cell nucleoli. J. Mol. Biol. *63*, 233–246.

Lake, J. A., Sabatini, D. D., and Nomura, Y. (1974). Ribosome structure as studied by electron microscopy. In Ribosomes, M. Nomura, A. Tissières, P. Lengyel, eds. (Cold Spring Harbor Laboratory), pp. 543–557.

Laskey, R. A., Honda, B. M., Mills, A. D., Morris, N. R., Wyllie, A. H., Mertz, J. E., De Robertis, E. M., and Gurdon, J. B. (1978). Chromatin assembly and transcription in eggs and oocytes of Xenopus laevis. Cold Spring Harbor Symp. Quant. Biol. *42*, 171–178.

Lastick, S. M. (1980). The assembly of ribosomes in HeLa cell nucleoli. Eur. J. Biochem. *113*, 175–182.

Loening, U. E., Jones, K. W., and Birnstiel, M. L. (1969). Properties of the ribosomal RNA precursor in Xenopus laevis; comparison to the precursor in mammals and in plants. J. Mol. Biol. *45*, 353–366.

Lutsch, G., Noll, F., Theise, H., Enzmann, G., and Bielka, H. (1979). Localization of proteins S1, S2, S16 and S23 on the surface of small subunits of rat liver ribosomes by immune electron microscopy. Molec. Gen. Genet. *176*, 281–291.

Martin, T. E., Pullman, J. M., and McMullen, M. D. (1980). Structure and function of nuclear and cytoplasmic ribonucleoprotein complexes. In Cell Biology, Vol. 4, D. M. Prescott and L. Goldstein, eds. (New York: Academic Press), pp. 137–174.

Miller, O. L., and Beatty, B. R. (1969). Visualization of nucleolar genes. Science *164*, 955–957.

Monneron, A., and Bernhard, W. (1969). Fine structural organization of the interphase nucleus in some mammalian cells. J. Ultrastr. Res. *27*, 266–288.

Ochs, R., Lischwe, M., O'Leary, P., and Busch, H. (1983). Localization of nucleolar phosphoproteins B23 and C23 during mitosis. Exp. Cell Res. *146*, 139–149.

O'Farrell, P. Z., Goodman, H. M., and O'Farrell, P. H. (1977). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell *12*, 1133–1142.

Olson, M. O. J., Rivers, Z. M., Thompson, B. A., Kao, W.-Y., and Case, S. T. (1983). Interaction of nucleolar phosphoprotein C23 with cloned segments of rat ribosomal deoxyribonucleic acid. Biochemistry *22*, 3345–3351.

Ouchterlony, Ö., and Nilsson, L.-Å. (1978). Immunodiffusion and immunoelectrophoresis. In Handbook of Experimental Immunology, D. M. Weir, ed. (London: Blackwell Scientific Publications), pp. 1:19.1–19.44.

Pederson, T., and Munroe, S. H. (1981). Ribonucleoprotein organization of eukaryotic RNA. XV: different nucleoprotein structures of globin messenger RNA sequences in nuclear and polyribosomal ribonucleoprotein particles. J. Mol. Biol. *150*, 509–524.

Penman, S., Smith, J., and Holtzman, E. (1966). Ribosomal RNA synthesis and processing in a particulate site in the HeLa nucleus. Science *154*, 786–789.

Perry, R. P. (1967). The nucleolus and the synthesis of ribosomes. Progr. Nucleic Acid Res. Mol. Biol. *6*, 219–257.

Prestayko, A. W., Klomp, G. R., Schmoll, D. J., and Busch, H. (1974). Comparison of proteins of ribosomal subunits and nucleolar preribosomal particles from Novikoff hepatoma ascites cells by two-dimensional polyacrylamide gel electrophoresis. Biochemistry *13*, 1945–1951.

Puvion, E., and Moyne, G. (1981). In situ localization of RNA structures. In The Cell Nucleus, Vol. 8, H. Busch ed. (New York: Academic Press), pp. 59–115.

Puvion, E., Viron, A., and Xu, F. X. (1984). High resolution autoradiographical detection of RNA in the interchromatin granules of DRB-treated cells. Exp. Cell Res. *152*, 357–367.

Reeder, R. H., and Roan, J. G. (1984). The mechanism of nucleolar dominance in Xenopus hybrids. Cell *38*, 39–44.

Rogers, M. E. (1968). Ribonucleoprotein particles in the amphibian oocyte nucleus. J. Cell Biol. *36*, 421–432.

Scalenghe, F., Buscaglia, M., Steinheil, C., and Crippa, M. (1978). Large scale isolation of nuclei and nucleoli from vitellogenic oocytes in Xenopus laevis. Chromosoma *66*, 299–308.

Scheer, U. (1973). Nuclear pore flow rate of ribosomal RNA and chain growth rate of its precursor during oogenesis of Xenopus laevis. Dev. Biol. *30*, 13–28.

Scheer, U., and Rose, K. M. (1984). Localization of RNA polymerase I in interphase cells and mitotic chromosomes by light and electron microscopic immunocytochemistry. Proc. Natl. Acad. Sci. USA *81*, 1431–1435.

Scheer, U., Trendelenburg, M. F., Krohne, G., and Franke, W. W. (1977). Lengths and patterns of transcriptional units in the amplified nucleoli of oocytes of Xenopus laevis. Chromosoma (Berlin) *60*, 147–167.

Schmidt-Zachmann, M. S., Hügle, B., Scheer, U., and Franke, W. W. (1984). Identification and localization of a novel nucleolar protein of high molecular weight by a monoclonal antibody. Exp. Cell Res. *153*, 327–346.

Setyono, B., and Greenberg, J. R. (1981). Proteins associated with poly(A) and other regions of mRNA and hnRNA molecules as investigated by crosslinking. Cell *24*, 775–783.

Smetana, K., Ochs, R., Lischwe, M. A., Gyorkey, F., Freireich, E., Chudomel, V., and Busch, H. (1984). Immunofluorescence studies on proteins B23 and C23 in nucleoli of human lymphocytes. Exp. Cell Res. *152*, 195–203.

Sollner-Webb, B., Wilkinson, J. K., and Miller, K. G. (1982). Transcription of Xenopus ribosomal RNA genes. In The Cell Nucleus. Vol. 12, H. Busch and L. Rothblum, eds. (New York: Academic Press), pp. 31–67.

Spector, D. L., Ochs, R. L., and Busch, H. (1984). Silver staining, immunofluorescence, and immunoelectron microscopic localization of nucleolar phosphoproteins B23 and C23. Chromosoma (Berlin) *90*, 139–148.

Swift, H. (1963). Cytochemical studies on nuclear fine structure. Exp. Cell Res. Suppl. *9*, 54–67.

Thomas, J. O., and Kornberg, R. D. (1975). An octamer of histones in chromatin and free in solution. Proc. Natl. Acad. Sci. USA *72*, 2626–2630.

Todorov, I. T., Noll, F., and Hadjiolov, A. A. (1983). The sequential addition of ribosomal proteins during the formation of the small ribosomal subunit in Friend erythroleukemia cells. Eur. J. Biochem. *131*, 271–275.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA *76*, 4350–4354.

Warner, J. R. (1979). Distribution of newly formed ribosomal proteins in HeLa cell fractions. J. Cell Biol. *80*, 767–772.