Association of Nucleoplasmin with Transcription Products
As Revealed by Immunolocalization in the Amphibian Oocyte

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Abstract. The oocyte nucleus of Pleurodeles waltlii contains a major 32,000-mol-wt acidic protein which is called nucleoplasmin. Rabbit antibodies were raised against total nuclear proteins from Pleurodeles oocytes. Affinity-purified antibodies directed against nucleoplasmin were prepared using antigens bound to nitrocellulose paper. The specificity of the antibody was controlled on two-dimensional electrophoretic gels of nuclear proteins. The intranuclear distribution of nucleoplasmin was analyzed by indirect immunofluorescence and the immunogold technique in light and electron microscopy. The antibody was tested (a) on a spread of the nuclear content prepared in the presence of calcium, (b) on the nuclear content spread in the presence of phalloidin so that an actin network appeared, and (c) on a spread of nuclei from oocytes previously treated by actinomycin D. In all cases, nucleoplasmin appeared to be localized on the lampbrush loops, i.e., on the sites of transcription and, more specifically, on the ribonucleoprotein (RNP) particles; this protein was also associated with the RNP particles of the nuclear sap (free or inserted in the actin network). Nucleoplasmin was localized on large RNP particles that appeared when transcription was blocked. We never found this protein on the chromosome axis. These results suggest that nucleoplasmin may play a role in transcriptional activity.

Nucleoplasmin is a nuclear protein, the most abundant in the amphibian oocyte. Its concentration in the soluble phase of the Xenopus oocyte is 10% (18, 24) and 17% for the Pleurodeles waltlii oocyte nucleus (7). This karyophilic protein has a molecular weight of ~32,000 with slight variations in different amphibian species; it is acidic and its isoelectric pH value covers a broad range from 4.5 to 5.3 (18, 19). Nucleoplasmin is also phosphorylated (18, 19) and thermostable (24).

This protein rapidly enters the nucleus after being injected into oocyte cytoplasm (24). In vitro, it may promote nucleosome assembly from purified histones and DNA (23); it may also have a role in maintaining an ionic environment conducive to chromatin assembly (9). In the living cell, at least a part of nucleoplasmin is found associated in a large complex (7S) containing four kinds of histones: H2A, H2B, H3, H4 (16). Previous reports on the localization of nucleoplasmin have been performed on sections of oocyte either by immunological probes observed with the light microscope. The latter case, nucleoplasmin did not seem to be associated with nuclear structures containing DNA: nucleoli and lambrush chromosomes (18, 19, 24).

In the present study, we describe the localization of this protein on the spread contents of nuclei of oocytes of the amphibian Pleurodeles waltlii using the light and electron microscope in normal and in actinomycin D-treated cells. The involvement of nucleoplasmin in transcriptional activity and in transport of transcription products is discussed.

Materials and Methods

Animal and Tissues
Full grown oocytes were isolated from Pleurodeles waltlii (Amphibia, Urodela) and from Xenopus laevis raised in our laboratory.

Preparation of an Oocyte Fraction Enriched in Nucleoplasmin
Ovary was prepared as described by Laskey et al. (23); the 145,000 g supernatant was made up to 55% in (NH4)2SO4, stored overnight at 4°C, and centrifuged at 100,000 g for 30 min. The pellet was discarded and the supernatant was dialyzed 12 h against 20 mM Tris-HCl (pH 7.5) containing 120 mM KCl, 2 mM MgCl2. This fraction was then treated by heat as described by Laskey et al. (23) in order to obtain one major thermostable polypeptide corresponding to nucleoplasmin.

Isolation of Nuclei
Oocyte nuclei were isolated for a lapse of time of 20 s (per nucleus) manually in a physiological medium (buffer A): 10 mM Tris-HCl (pH 7.2) containing 75 mM KCl, 25 mM NaCl, 1 mM MgCl2 and 5 × 10⁻³ mM CaCl2 (5, 11).

Nuclei used for immunization were kept in a minimum of buffer A at 4°C. Nuclei used for electrophoresis of nuclear proteins were transferred in 20 mM Tris-HCl (pH 7.4) containing 10 mM MgCl2, absolute ethanol...
Spread Nuclear Preparation

For preparing spreads of nuclear content, manually isolated nuclei were transferred to an observation chamber in buffer A, then opened with a needle and tweezers. The nuclear content was allowed to disperse at 4°C. The observation chambers were centrifuged for 30 min at 2,200 g as already described by Old et al. (27). After centrifugation, the lampbrush chromosomes were briefly fixed (15 min) by 70% ethanol and kept in phosphate-buffered saline (PBS) (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 7 mM NaH2PO4, and 1.5 mM KH2PO4.

To minimize loss of the proteins, the time of preparation of the nuclear spread until the fixation stage was as short as possible. When treated by phallolidin, the nucleus was ruptured in an observation chamber containing 10^-4 M of the drug and then processed as described above.

Electron Microscopy on Thin Sections

Lampbrush chromosomes prepared as described above were briefly fixed in a solution of 0.01 M phosphate (pH 7.2) containing 1% glutaraldehyde (vol/vol), postfixed in a solution of 1% OsO4, and dehydrated by ethanol. Coverslip preparations were flat-embedded in araldite according to Gounon and Karsenti (12). After polymerization (60°C, 48 h), the coverslips were removed by freezing in liquid nitrogen. Thin sections were stained in a solution of uranyl acetate in 50% ethanol, then in lead citrate. They were examined in a Philips 201 operating at 80 kV.

Gel Electrophoresis

One-dimensional SDS polyacrylamide gel electrophoresis was performed as described by Laemmli (22). Two-dimensional electrophoresis was performed with equilibrium pH gradient electrophoresis (26) using ampholines pH 3-11 in the first dimension, followed, in the second dimension, by electrophoresis on an SDS 10-15% polyacrylamide slab gel gradient (22). Gels were stained with Coomassie Blue or by the silver nitrate staining method of Morrissey (25).

Nuclear Protein Antiserum

Rabbits were injected twice in the muscle of the thigh at a 20-d interval with 100 nuclei in complete Freund's adjuvant, followed 2 mo later by a booster injection. Actin antiserum was prepared as previously described (12).

Ribonucleoprotein (RNP) Antiserum

RNP antibody tiers of sera obtained from patients with systemic lupus erythematosus and other related disorders termed "mixed connective tissue disease" were determined by passive hemagglutination as described by Tan and Pebbels (31). Several sera with anti-RNP specificities were obtained from Dr. R. Lahtia (The Rockefeller University, New York) and are similar to those described by Conner et al. (6). It has been known for some time that antigens recognized by these antibodies are present in the nuclei of a wide variety of cells (30). We used this property to find a serum which cross-reacts with RNP of amphibians. The sera were tested on isolated nuclei of Xenopus or Pleurodeles as described previously for anti-antibodies (12). Those that stained RNP aggregates (for description, see Gounon and Karsenti [12]) were kept for further studies. The antigens were detected by antibody binding on blots. Nuclei of Pleurodeles oocytes prepared manually were analyzed on 10% SDS polyacrylamide gels. The proteins were then transferred onto nitrocellulose sheets by diffusion and the detection of antigens on blots by antisera was carried out according to Towbin et al. (32) using peroxidase-conjugated antibodies and 4 chloro-l-naphthol as substrate. The M. J. serum presented in this paper stains a 54,000-mol-wt protein that is one of the major polypeptides found in the RNP of amphibian oocytes (for further details, see Klotz et al. [17]).

Affinity Purification

Monospecific antibodies were purified according to Olmsted (28) and Krohne et al. (20), slightly modified as follows. The nuclear proteins from thirty nuclei were separated by two-dimensional electrophoresis. They were transferred onto nitrocellulose sheets at 500 mA for 2 h in 25 mM Tris, 0.192 M glycine. The nitrocellulose sheet was stained with 0.2% Ponceau red in 0.3% trichloroacetic acid (TCA), then destained in 0.3% TCA. The spot corresponding to nucleoplasmin was cut out and incubated for 1 h at 40°C in PBS containing 1% bovine serum albumin (BSA). Ten spots were collected corresponding to 300 nuclei. The nitrocellulose was then treated with the antiserum as described by Krohne et al. (20).

Immunoblotting

The one-dimensional electrophoretically separated polypeptides were transferred by diffusion (2) for 24 h. The polypeptides separated by two-dimensional electrophoresis were blotted at 500 mA for 2 h in 25 mM Tris, 0.192 M glycine. The nitrocellulose sheet was incubated for 1 h in 3% BSA in PBS at 40°C, followed by 45 min at room temperature with the affinity-purified nucleoplasmin antibody and then washed, with several changes, with 1% PBS-Tween 20. Peroxidase-conjugated sheep anti-rabbit IgG (Institut Pasteur Production) was added for 45 min. After several washes in 1% PBS-Tween 20, the peroxidase activity was revealed using 4-chloro-l-naphthol as substrate.

Immunoprecipitation Experiments

One hundred nuclei were collected in 1 ml PBS, 2 mM PMSF. After sonication, they were centrifuged at 100,000 g for 30 min, 4°C. The supernatant was incubated with the purified antibodies at 1:10 dilution for 16 h at 4°C. Antigen-antibody complexes were recovered after incubation of the sample with 60 μl (packed bead volume) of protein A conjugated with Sepharose 4B (Pharmacia Fine Chemical AB, Uppsala, Sweden). After incubation for 2 h with agitation, the immunoadsorbent beads were washed five times with PBS. The immunoprecipitated proteins were eluted from the immunoadsorbent by incubating the Sepharose beads for 5 min at 100°C in Laemmli buffer. The solution recovered from this last step was analyzed directly on an SDS gel.

Immunofluorescence Microscopy

The preparations of lampbrush chromosomes, prepared as described above, were incubated with affinity-purified nucleoplasmin antibody in PBS at room temperature for 30 min, then washed, with several changes, with PBS. Fluorescein isothiocyanate-conjugated goat anti–rabbit IgG (Miles Scientific, Paris), used at a dilution of 1:100, was added for another 30 min. After several changes in PBS, the coverslips covered with chromosomes were removed and mounted in Mowiol.

The anti–RNP serum was applied at a dilution of 500:1. Rhodamine-conjugated goat anti–human IgG (Miles Scientific) was used at a dilution of 1:100 as the second antibody.

Immunogold Staining

For nucleoplasmin immunolocalization in electron microscopy, the antibody-binding procedure was identical to that used for immunofluorescence microscopy, except that 20-nm gold particle–labeled goat anti–rabbit IgG was used instead of the fluorescein isothiocyanate–conjugated antibody. After the antibody-binding step, chromosome preparations were flat embedded in araldite and thin section cut as described above.

Immunocytochemistry

Pieces of ovary were fixed at −20°C with Romeis fixative (25 ml saturated mercuric chloride, 20 ml trichloroacetic acid, 15 ml 37% formaldehyde) for 2 h. They were transferred to 100% ethanol for 2 h with one change and then to 50% and 100% polyethylene wax (15). Sections were overlaid with 1% PBS-BSA for 10 min, followed by the purified antibody for 30 min; they were then washed three times in PBS. Treatment with fluorescein isothiocyanate–conjugated goat anti–rabbit IgG was as described above.

Results

Obtaining of a Monospecific Antibody

An antiserum directed against nuclear proteins was used to prepare affinity-purified antibodies directed against the ma-
whole oocyte nuclei manually isolated; and (d) a 140,000 g oocyte fraction enriched with nucleoplasmin (Fig. 1).

The reaction was positive with only one 32,000-mol-wt polypeptide. This polypeptide was present (a) in the whole ovary, (b) in the nucleus, and (c) in the 140,000 g oocyte fraction enriched with nucleoplasmin (Fig. 1); it was absent from the enucleated oocyte. When the affinity-purified nucleoplasmin antibody was pretreated with the 140,000 g oocyte fraction enriched with nucleoplasmin, there was a negative reaction for each of the four cases described above (not shown).

The antibody was tested on nuclear polypeptides separated by two-dimensional electrophoresis (Fig. 2 a). Only one spot was positive, corresponding to nucleoplasmin (Fig. 2 b). The purity of the antibody preparation was also tested by immunoprecipitation experiments described in Materials and Methods. The protein precipitated by the antibody was analyzed on one-dimensional electrophoresis and stained by the silver nitrate staining method (Fig. 3). We observed a single 32,000-mol-wt band.

Specificity of the Antigen–Antibody Reaction

The monospecific antibody was characterized by its binding to electrophoretically separated and blotted polypeptides. We examined binding to (a) the 100,000 g fraction of whole ovary homogenized in PBS; (b) the 100,000 g fraction of the oocyte manually enucleated and homogenized in PBS; (c) whole oocyte nuclei manually isolated; and (d) a 140,000 g oocyte fraction enriched with nucleoplasmin (Fig. 1).

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Species Specificity

Ovaries of Pleurodeles and of Xenopus were fixed in Romeis fixative and embedded in polyester wax, as described in Materials and Methods. The sections were treated with the Pleurodeles nucleoplasmin antibody and revealed by fluorescence. The reaction was positive for Pleurodeles and negative for Xenopus (Fig. 4, a–d). In the case of Pleurodeles, the fluorescence was strictly confined to the nucleus and distributed throughout the nucleoplasm, but some structures, the lampbrush chromosomes and the nucleoli, appeared to be strongly stained (Fig. 4, b and f).

The purification technique we used thus allowed us, by means of a polypeptide separated on two-dimensional electrophoresis, to extract a monospecific antibody directed against Pleurodeles nucleoplasmin from an antiserum.

Interaction of Nucleoplasmin with Nuclear Elements

The Lampbrush Chromosomes and Nucleoli. We have described above the association of nucleoplasmin with lampbrush chromosomes and nucleoli on histological sections of ovary. To determine the exact location of the protein on such structures, immunolocalization was carried out on spread nuclei observed with both the light and electron microscopes.

When spread in the presence of calcium, the nuclear content was fluid and contained many particles, such as nucleoli and RNP particles, surrounding lampbrush chromosomes with large loops. The loops extended laterally from the chromosome axis. The chromatin of the axis was in a condensed state (chromomeres), whereas that of the loops was in a decondensed state and was the site of intense transcriptional activity. The axis of the loops was embedded in an RNP matrix composed of nascent RNA chains associated with proteins. Alongside the numerous normal loops (3, 4), some loops had a particular feature due to the morphology of their matrix; these were used as landmarks to identify some bivalents.

In this type of preparation, observed by immunofluorescence with the light microscope, nucleoplasmin antibody labeled normal and landmark loops of the chromosome and the nucleoli (Fig. 5). The chromosome axis was masked by the loop insertions, so that the chromomeres appeared to be labeled, but the resolution level did not allow us to confirm this.

At the ultrastructural level, on thin sections of the chromosomal area after immunogold staining, the antibody appeared only on loops of the chromosome, and chromomeres were never labeled (Fig. 6). The loops were labeled over their entire length. The protein appeared to be associated...
Immunofluorescence labeling of a part of a bivalent from *Pleurodeles* by purified nucleoplasmin antibody. All normal loops are stained, as are landmark loops (arrowhead). In the nuclear sap surrounding the chromosomes, numerous particles are stained, as are nucleoli (N). Bar, 20 μm.

with RNP particles of the loops. For the globular loops (Fig. 7) homogeneous labeling of all globules was noted.

**Nucleoskeleton and RNP Particles of the Nuclear Sap.**

To more easily observe the nucleoskeleton, we used phalloidin, a drug which stabilizes F-actin in vivo and in vitro. Under these conditions, the nuclear sap appeared to consist of a network of intricate fibrils covered with granular material. This network was composed mainly of actin cables, as clearly shown by staining with anti-actin antibodies (12). In addition, many individual microfilaments and particles, the size of which varied from 20 to 300 nm, were observed. These particles disappeared when the preparations were treated by RNase (Gounon, P., personal communication) and were labeled with an anti-RNP antibody (see below), strongly suggesting their RNP nature.

We applied the immunogold technique using nucleoplasmin antibody to thin sections of this nuclear network. The gold particles were localized on RNP aggregates inside and outside of the actin cables, while unlabeled microfilaments were observed (Fig. 8).

These results were therefore in agreement with the hypothesis of an association of nucleoplasmin with the nascent transcription products of the loops.

**Figure 6.** Binding of nucleoplasmin antibody to lambrush chromosome as revealed by immunogold staining. Electron micrograph of one thin-sectioned bivalent. An intense labeling with 20-nm gold particles is observed all along the loops. The axis of the chromosome (A) is not labeled. Bar, 1 μm.

**Figure 7.** Immunogold staining of globular loop using nucleoplasmin antibody. Electron micrograph of one thin-sectioned globular loop. The labeling with 20-nm gold particles is observed on each granule of the loop. The axis of the chromosome is unlabeled. Bar, 1 μm.
Immunolocalization When Transcription Was Inhibited

Full-grown oocytes were incubated for 24 h at 19°C in TC-199 medium (Flubio) 20% diluted, containing 10 μg/ml actinomycin D. In this case, all lateral loops retracted; this retraction was accompanied by extensive foreshortening of the chromosomal axis. Some globular loops seemed to be less sensitive to the drug; their retraction was slow and was always the last to occur. Thus there existed a phase characterized by the disappearance of the normal loops, except for some residual structures, and the persistence of globular loops. In these persisting globular loops, or residual loop structures, transcription images could be observed (Penrad-Mobayed, M., E. N'Da, and N. Angelier, manuscript in preparation). We used this state of retraction in our experiments.

In the nuclear sap, a meshwork developed which was denser than in the control. This meshwork was composed of actin and was analogous to that obtained with phalloidin (29). The density of RNP particles increased, and a striking heterogeneity in their size was observed; in particular, numerous large particles 200 nm in diameter appeared.

Thin sections of the chromosomal area pretreated with actinomycin D were incubated with nucleoplasmin antibody and immunogold stained (Fig. 9). The labeling was localized exclusively on the residual structures of loops, and on globules of persisting globular loops. The chromosomal axis was never labeled.

Discussion

Based on these observations, and particularly on the analysis of the distribution of nucleoplasmin at the ultrastructural level, it appears that nucleoplasmin is not randomly distributed in the oocyte nucleus. We noted an association with nucleoli, with the chromosome loops on transcription products and with the RNP particles of the nuclear sap.

It might be hypothesized that the disposition of the protein in the nucleus which we have described was a chance event due to relocalization of the molecules when the nuclear sap spread on the slides. However, the distribution of the protein was always the same. If it were due to chance, there would be no reason for the protein to be associated with one structure rather than another; in addition, when the nucleus was gelified with phalloidin (i.e., when all the molecules and
The ultrastructural data provide information on the distribution of the protein on the different elements of the nucleus: nucleoli, RNP particles on the loops and in the nucleoplasm. We do not presently know the nature of the association nor the percent of nucleoplasmin involved in these associations. Nucleoplasmin, which is known to be a readily diffusible molecule in situ and in vivo (18, 19), is also known to be a molecule which can associate in complexes containing four major histone components (16); in this case, only a minor quantity of the protein is concerned. The nucleoplasmin we described may be included in the diffusible category and, in this case, the nature of the association with RNP particles would be reversible, or might constitute a third state of the protein.

Some nuclear proteins, such as histones, appear to be associated with DNA whatever the level of transcriptional activity. On the contrary, other nuclear proteins such as nucleoplasmin and HMG1 protein (our unpublished data) are associated only with active chromatin. When we compare the two proteins, they show similarities in amino acid composition, and both are rich in potentially charged amino acids (9).

Nucleoplasmin is known as a histone-binding protein (9, 19). In vitro, this protein binds all four histones which constitute the nucleosomal core. In the amphibian nucleus, histones are stored in a soluble form (33). A certain quantity of histones H3 and H4 is associated with one 110,000-mol-wt polypeptide of the karyophilic proteins, called N1/N2 (15). Nucleoplasmin is found associated in complexes containing four major histone components (16). These complexes could explain the nuclear accumulation of histones of the oocyte. Earnshaw et al. (9) have presented nucleoplasmin as a factor which maintains an ionic environment conducive to chromatin assembly in vitro. They noted that polyanions can also remove histones from DNA and therefore cause decondensation of chromatin, followed by its activation in transcription. Along these lines, it should be pointed out that nucleoplasmin is not detectable in inactive cell types (19).

We have detected nucleoplasmin at the site of transcription, where it is associated with transcription products. When transcription decreased, nucleoplasmin was associated with the residual loops that retained RNP particles, and when transcription stopped, nucleoplasmin was not associated with the chromosomes but with the large RNP particles that formed.

The non-association of nucleoplasmin with the chromosome axis might have been due to a problem of accessibility, since treatment with the antibody was carried out before sectioning of material; however, the results described with an antihistone (see above) show that no such problem existed. Thus, it appears that nucleoplasmin is involved in the formation of RNPs and perhaps with transcriptional activity.

However, the role(s) of nucleoplasmin remains unclear: this protein interacts with histones, is associated with active chromatin and transcription products, and is absent from transcriptionally inactive cells. Nucleoplasmin could be involved with transcription, perhaps by modifying the structure of DNA or by intervening in the transport of transcription products.

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