Immunoelectron Microscope Visualization of Nuclear Ribonucleoprotein Antigens within Spread Transcription Complexes

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Abstract. The ultrastructural distribution of nuclear ribonucleoproteins (RNP) within spread active chromatin has been investigated using specific anti–RNP antibodies. Monoclonal antibodies directed against the core proteins of heterogeneous nuclear (hn)RNP or small nuclear (sn)RNP have been incubated directly with lysed mouse or Drosophila tissue culture cells and the bound antibodies visualized by means of a protein A–colloidal gold complex.

The hnRNP core proteins have been localized on growing RNP fibrils within non-nucleolar transcription complexes. Anti–snRNP antibodies, directed either against the Sm-antigen (common for nucleoplasmic snRNP species containing U1, U2, U4, U5, and U6 RNAs) or against U1–snRNP, were bound by two morphological types of RNP structures. Within areas of chromatin that do not completely disperse, labeling was observed on RNP-fibril gradient type structures or on groups of fibrogranular material. In the well-dispersed regions containing individual nonribosomal transcription complexes, snRNP antigens were associated with growing RNP fibrils.

Our results provide direct evidence for association of some U-snRNP species (including U1–snRNP) with extranucleolar RNA as early as during transcription elongation. In addition, the presence of core hnRNP proteins on the same type of nascent RNA transcripts has been confirmed.

The ultrastructural distribution of ribonucleoproteins (RNPs) within the cell nucleus in situ has recently been analyzed by means of electron microscope immunochemistry. Using polyclonal or monoclonal anti–heterogeneous (hn)RNP or anti–small nuclear (sn)RNP antibodies, and the protein A–colloidal gold complex as a marker, association of the two above types of RNP antigens with perichromatin fibrils has been demonstrated (7). The perichromatin fibrils have previously been shown by high resolution autoradiography of ultrathin sections contrasted with a staining technique differential for nuclear nucleoproteins (1), as being the in situ nuclear structure containing newly synthesized hnRNA (8, for review see reference 6). Therefore, the immunocytochemical studies have not only confirmed the hnRNP nature of the perichromatin fibrils, but also suggested an early association of at least some snRNPs with these nuclear structural components. Because some snRNPs have recently been proposed as factors playing an important role in the process of splicing of pre-mRNA (13, for review see reference 21), the question arises as to whether the association of snRNPs with pre-mRNA already occurs during the transcription process, or whether it is a posttranscriptional event. To examine this question, localization of specific snRNP antigens has been analyzed directly on preparations of spread chromatin after lysis of tissue culture cells and labeling with the specific antibodies.

Materials and Methods

Mouse P815 or Drosophila Schneider II cell lines were maintained under standard conditions.

Exponentially growing cells (2–3 × 10⁵ cells/ml, 5–10 ml for P815 cells, or 2–3 × 10⁶ cells/ml, 1–2 ml for Drosophila cells) were gently centrifuged and the pellet resuspended in 4 ml of slightly hypotonic ice-cold phosphate buffer (0.2 mM [pH 7.5]) containing 0.1 M sucrose (RNase free). The cell lysate was prepared as previously described (25). Briefly, after re-centrifugation, 1 ml of the same phosphate buffer was mixed with the pelleted cells, which were then thoroughly resuspended using a Vortex mixer at low speed. 1 ml of 0.5% Nonidet P-40 in 0.2 mM EDTA (pH 7.5) was then added dropwise under constant Vortex stirring. The clear lysate was subsequently diluted in 20 ml of 0.2 mM EDTA (pH 7.5) and kept on ice.

Two types of monoclonal antibodies have been used for immuno labeling. In one series of experiments, an anti–hnRNP antibody purified from ascitic fluid, eF5, raised against 30S hnRNP subcomplexes and specific for the hnRNP core group polypeptides of 32–40 kDa, reacting predominantly with the 35-kD species (15), was used. In another series of experiments, anti–snRNP antibodies were applied. These were either a monoclonal anti–Sm Y12 derived from an autoimmune mouse hybridoma and characterized as reacting with 25- and 26-kDa polypeptides of snRNP containing U1, U2, U4, U5, and U6 snRNAs (14), or a mouse hybridoma antibody specific for the 70-kD "RNP"-antigen of U1–snRNP (4).

The antibody has been added to the fresh cell lysate in EDTA to obtain specific snRNP antigens has been analyzed directly on preparations of spread chromatin after lysis of tissue culture cells and labeling with the specific antibodies.

Abbreviations used in this paper: hnRNP, heterogeneous ribonucleoprotein; snRNP, small nuclear RNP.

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a final dilution of 50 or 25 µg/ml (occasionally 10 µg/ml). This mixture was then incubated for 30 min at room temperature, with occasional gentle stirring. Afterwards, 20 µl of protein A—colloidal gold complex solution (mean particle diameter 15 nm; prepared as in reference 22) were added to 180 µl of incubation mixture (occasionally the protein A—gold complex was added to make a final dilution of 1:20) and incubated for another 30 min at room temperature. In some experiments the lysate was incubated with the antibody for 2 h followed by protein A—gold labeling for 30 min, both on ice.

Alliquots of the final incubation mixture were then layered onto formaldehyde (4 %)–sucrose cushions (pH 7.5–7.9) in special plastic centrifugation chambers (18), on the bottom of which were placed previously glow-discharged (11), formvar- and carbon-coated electron microscope gold grids. Spreading was performed by centrifugation at 2,200 g, for 10 min at 4°C. The grids were then removed and, without drying, immediately immersed in 0.4 % Photo-fluo 600 (Eastman Kodak Co., Rochester, NY) solution (pH 7.5–7.9) for 30–45 s, blotted on filter paper to remove excess liquid, and air dried.

Two types of control experiments have been performed. In one, the antibody was omitted and the lysate was incubated only with the protein A—colloidal gold complex to assess a possible nonspecific association of protein A with nuclear material. In competition controls, the antibody was first preincubated for 1–5 h with the antigen-containing nuclear RNP fraction (8S sucrose density gradient fraction for anti-snRNP antibodies, 30S fraction for the anti—hnRNP antibody). Then the antigen—antibody preincubation mixture was briefly centrifuged (microfuge B, 10, 4°C, Beckman Instruments Inc., Fullerton, CA) to sediment possible protein aggregates, and the supernatant was used for the incubation with the cell lysate as described above.

All grids were stained for 1 min with 1 % phosphotungstic acid in 70 % ethanol, rinsed for 20 s in 95 % ethanol, and air dried. Preparations were observed with a Philips EM 400 electron microscope at 40 kV or a Zeiss EM 10 electron microscope at 40 or 60 kV, both equipped with a 40-µm objective aperture.

Results

The anti—hnRNP and the anti—snRNP antibodies have never been observed to bind to the ribosomal RNP type of transcription complexes. These complexes are easily recognizable; they always appear in the form of regular and closely spaced gradients and their morphological features have been well characterized (19; for review see reference 23).

As expected from previous studies (15), the anti—hnRNP antibody used in this work reacts only with the vertebrate cell antigens, but not with those of Drosophila cells. After incubation of the mouse cell lysate with the antibody, relatively strong labeling is observed associated with aggregates of RNP fibrils occurring within regions of rather poorly dispersed chromatin (Fig. 1 a). In the areas showing well dispersed chromatin fibers the growing RNP chains on individual transcription complexes are discernible; it is to these nascent RNP fibrils that the label is restricted (Fig. 1 b).

Anti—snRNP antibodies are, for both mouse and Drosophila cell lysates, bound by RNP constituents in two morphologically different zones of the spread material. In one type of organization, either RNP—fibrill gradient structures distinct from ribosomal transcription units (Figs. 2, a and b) or groups of fibro-granular material (Figs. 2, a and c) are sites of antibody localization. These structures are often found within the areas where chromatin fibers are not completely dispersed, and are surrounded by stretches of inactive chromatin (Fig. 2, a and b). Sometimes the groups of labeled RNP material can be identified in a form of small individual aggregates, separated from the main chromatin masses (Fig. 2 c). The second class of the snRNP antibody distribution is represented by individual transcription complexes, where label is associated with RNP fibrils on nonribosomal transcription units at lower frequency but qualitatively similar to those labeled by anti—hnRNP antibodies (Fig. 2 d).

The results of competition experiments, in which the antibodies have first been preincubated with the corresponding antigen and then incubated with cell lysate, reveal a very marked reduction of RNP labeling. The control incubations with the protein A—gold complex in the absence of antibodies revealed only background levels of labeling. The background within, as well as outside, the spread material is usually very low.

Discussion

The application of the techniques of ultrastructural immunocytochemistry using monoclonal anti—RNP antibodies, combined with the Miller type spreading for visualization of active chromatin (a) confirms the presence of hnRNP proteins within the growing RNP chains in the transcription complexes of non-nucleolar type; (b) indicates an early association of U1—snRNP and possibly also of other snRNP species, with transcripts occurring in the extranucleolar RNA transcription complexes.

Association of certain proteins with pre-mRNA molecules during synthesis has been shown to occur very early after the initiation of transcription (for review see references 16 and 24). Because the monoclonal anti—hnRNP antibody used in this work is specific for the major hnRNP (pre—mRNP) core group polypeptides, our studies provide by use of novel ultrastructural analysis a direct visualization of the association of the hnRNP core proteins with nascent RNA, a result that could only be inferred from previous biochemical and immunofluorescence investigations (16, 17).

Perhaps more surprising are our findings on the binding of anti—snRNP antibodies within active chromatin, which reveal a very early association of at least some snRNP species with growing hnRNP chains of transcription complexes. Because the antibody probes are specific for protein epitopes, our data cannot provide completely unambiguous proof for the association of snRNAs per se with the RNP transcript; there is, however, no evidence that snRNP proteins exist free in the nucleus. Although it has been speculated that snRNPs might associate in such a way with pre—mRNA, no direct evidence has previously been provided. The snRNAs U1, U2, and U3 have been shown to be involved in splicing of pre—mRNA and our data demonstrate that the association of snRNPs occurs as early as during transcription elongation. This, of course, does not mean that splicing starts during this period, but it may suggest that relevant processing sites are being identified co-transcriptionally, thus permitting rapid processing after release of the completed transcript.

The localization sites of the two types of RNP antigens correspond to the same type of fibrillar structures shown to be radioactively labeled within chromatin spreads from cultured cells previously submitted to a short [3H]uridine pulse (25). These fibrillar RNP structures, as well as the radioactive label associated with them, can be specifically removed from such spread specimens by means of direct RNase treatment (Fakan, S., and Hughes, M. E., manuscript submitted for publication). In addition, the morphology of RNP structures visualized in the present work is identical with those visualized on specimens prepared by direct spreading of
freshly prepared lysates of the same cells. This argues against possible degradation artifacts that might occur during labeling with the antibodies. All these data together confirm that the lateral fibrils, which we observe on the spread chromatin preparations, are indeed RNP transcripts.

Another striking feature of the immunolocalization of anti–snRNP antigens observed in our work is the labeling of the gradient-like structures and the fibro-granular aggregates of RNP material (Fig. 2 a–c). Two hypotheses can be formulated concerning this point. Since the lateral fibrils within the gradient-like structures appear to be quite short (Figs. 2, a and b), the question arises whether they could represent transcription complexes for snRNA synthesis and snRNP assembly. Some evidence exists, however, that the assembly of snRNP is cytoplasmic (5, 9). Furthermore, we do not have good evidence at present that these are transcriptionally ac-
Figure 2. Spread chromatin containing RNP structures labeled with monoclonal anti-snRNP antibodies. Bars, 0.5 μm. (a-c) Chromatin from mouse cell lysate incubated with the anti-Sm antibody. (a) Two groups of labeled fibrillar material, one exhibiting a gradient-like appearance (left). (b) A gradient-type arrangement of labeled fibrillar material, surrounded by inactive chromatin fibers. (c) Aggregate of labeled fibro-granular material. (d) An individual transcription complex spread from Drosophila cell chromatin incubated with the anti-U1-snRNP antibody. Label is associated with the growing RNP fibril.
tive sites. Alternatively, one could speculate that some of these structures are possible morphological counterparts of the clusters of interchromatin granules observed in cell sections. These have been shown as an in situ nucleoplasmic the clusters of interchromatin granules observed in cell sec-
tions. Alternatively, one could speculate that some of these structures are possible morphological counterparts of the clusters of interchromatin granules observed in cell sections. These have been shown as an in situ nucleoplasmic structural component containing high amounts of snRNPs (7); the function of the structures is not known. However, these two ideas remain conjectural in the absence of more direct experimental evidence.

Because the intensity of immunolabeling depends on the degree of chromatin dispersion in each experiment and consequently on the spatial arrangement of the more or less dispersed active chromatin regions making the specific epitopes accessible to the antibodies, a systematic quantitative evaluation of the distribution of label appears, at least for the moment, difficult. We are trying, however, to use this experimental system to evaluate a possible order of arrangement of the RNP proteins along the growing RNP chain. In that regard it is most important to determine if snRNPs are located at distinct sites along transcripts in a manner suggesting sequence specificity. We are now attempting this assay, using protein A-gold markers of smaller size. We are encouraged to attempt such an analysis by the observation of a consistent pattern of RNP morphology for some transcription units (2, 3). Recently it has been suggested that some structures observed in chromatin spreads might represent the association of hnRNP and snRNP as spliceosomes at processing sites on nascent RNA transcripts (26). Our results indicate that these components are indeed present on transcription fibrils, and refinement of the techniques of immuno-
labeling may make the precise mapping of proteins on nascent transcript arrays possible in the future.

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