Histochemistry (1994) 101:223–232

ORIGINAL PAPER

Ody C. M. Sibon · Bruno M. Humbel · Arjan De Graaf
Arie J. Verkleij · Fons F. M. Cremers

Ultrastructural localization of epidermal growth factor (EGF)-receptor transcripts in the cell nucleus using pre-embedding in situ hybridization in combination with ultra-small gold probes and silver enhancement

Accepted: 2 January 1994

Abstract A high-resolution in situ hybridization method is described for localizing epidermal growth factor (EGF)-receptor transcripts in nuclei of A431 epidermoid carcinoma cells. The method is based upon the use of ultra-small gold particles in combination with silver enhancement. The RNA of the EGF-receptor was detected mainly around the nucleoli. After removal of the DNA using nucleases and high salt extraction, the RNA of the EGF-receptor appears to be associated with the nuclear matrix. The RNA of the EGF-receptor was observed in close contact with the SC-35 splicing protein, but no exact colocalization was observed. These results demonstrate that high resolution pre-embedding in situ hybridization in combination with immunocytochemistry, both using ultra-small gold as a detection method, provides a powerful tool to unravel the organization of nuclear processes.

Introduction

The cell nucleus contains several essential functions, such as replication of DNA, transcription, processing and transport of RNA from the nucleus to the cytoplasm. Many biochemical studies have already been done on these nuclear processes but relatively little is known how the nuclear processes are spatially integrated into the nuclear architecture.

DNA replication sites have been identified in different spatial and temporal patterns during S-phase (O'Keefe et al. 1992; Raska et al. 1989). Recently DNA replication sites were visualized as discrete ovoid bodies (Hozak et al. 1993). Transcription sites have been localized using UTP-analoque-5-bromouridine 5'triphosphate incorporation in vivo; 300 to 500 focal sites of RNA synthesis have been detected (Jackson et al. 1993; Wansink et al. 1993). Other localization studies have been done using specific antibodies to factors involved in the splicing process. These studies revealed that proteins involved in splicing are concentrated in a speckled pattern (Habets et al. 1989; Huang and Spector 1992; Spector 1990; Spector et al. 1991). Specific small nuclear (sn) RNAs are also concentrated in speckles (Carmo-Fonseca et al. 1991a, b). However, it is still unclear whether these speckles represent storage and/or assembly of splicing factors or if splicing itself takes place at those regions. Localization of specific RNA sequences in the nucleus would give more clues about the organization of nuclear processes. Some reports already demonstrate that transcripts are not homogeneously distributed in the nucleus (Bond and Wold 1993; Lawrence et al. 1989; Raap et al. 1991; Xing et al. 1993).

Recently an endogenous RNA, representing transcripts of the EGF-receptor, was localized in the nuclei of A431 cells by light microscopy. This specific RNA was mainly present at discrete sites around the nucleoli (Sibon et al. 1993). The studies mentioned above support the hypothesis that nuclear processes are not randomly distributed throughout the nuclear plasma but occur at discrete sites, most likely organized by the nuclear matrix. The nuclear matrix is a reticular structure remaining after extraction of nucleic acids by nucleases and high salt treatments and is thought to be the solid scaffold to support most of the nuclear processes (reviewed in Berezney 1991; Verheijen et al. 1988).

In this study, we have used an electron microscopical approach in order to obtain detailed information on the localization of particular components in relation to nuclear structures. Sub-nuclear structures that can be visualized by electron microscopy include e.g. interchromatin granules, nucleolar substructures and the nuclear matrix. Studies on the application of electron microscopical localization may include pre- or post-embedment labelling.

O. C. M. Sibon (✉) · B. M. Humbel · A. De Graaf
A. J. Verkleij · F. F. M. Cremers
Department of Molecular Cell Biology,
University of Utrecht,
Padualaan 8, 3584 CH Utrecht, The Netherlands
Tel. +31-30-533449; Fax +31-30-513655

Pre-embedding techniques employ labelling and detection on permeabilized cells before embedment, whereas post-embedding techniques involve labelling and detection directly on thin sections. Several reports already exist about post-embedding in situ hybridization studies (e.g. Dirks et al. 1993; Puvion-Dutilleul 1993; Puvion-Dutilleul et al. 1991; Thiry and Thiry-Blaise 1989). However during post-embedment labelling, only those targets which are exposed at the surface of the section can be reached by the reporter molecules (Kellenberger et al. 1987; Stierhof et al. 1986, 1991). Therefore the post-embedding method could result in rather a low level of labelling. The latter may cause a problem when the protein or nucleic acid sequence of interest is present in a small quantity. In contrast, application of the pre-embedment labelling method allows labelling of the target molecules throughout the whole cell. Therefore pre-embedment labelling in principle, provides the most sensitive method to detect proteins or nucleic acid sequences at the electron microscopical level.

Using immunocytochemistry, the application of ultra-small gold in combination with silver enhancement was demonstrated to provide the most sensitive detection technique (De Graaf et al. 1991; Humbel and Biegelmann 1992). Therefore we have applied the pre-embedment labelling method in combination with in situ hybridization to localize at the ultrastructural level a specific endogenous RNA sequence in the nucleus. In this paper we show that the RNA of the EGF-receptor is mainly located around the nucleolus. Hardly any label was observed at interchromatin granules, inside the nucleolus or at the periphery of the nucleus. In nuclear matrix preparations, the RNA of the EGF-receptor is associated with the filamentous structure.

Furthermore we show that the pre-treatment needed for in situ hybridization does not interfere with subsequent immunolabelling. For this purpose, the essential splicing protein SC-35 (Fu and Maniatis 1990) was immunolabelled and found to be located mainly at the interchromatin granules. Double labelling experiments, i.e. combining in situ hybridization with the EGF-receptor transcripts and immunolabelling of SC-35, were performed to reveal their structural relationship. It was found that the EGF-receptor RNA does not exactly colocalize with the SC-35 protein. Nevertheless there were several sites of close contact of the EGF-receptor RNA and the SC-35 protein. These results show that double labelling at the electron microscopical level using in situ hybridization and immunocytochemistry has the potential to become an important technique to correlate RNA processing with the structures and enzymes involved.

Materials and methods

Cell culture

A431 and NIH-3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Flow Labs, Irvine, Scotland, UK) in a 7% CO_2 humidified atmosphere. Cells were routinely grown to about 50% confluency on glass coverslips for light microscopy or on Thermanox coverslips (LUX, Naperville, Ill., USA) for electron microscopy, the coverslips being placed in culture dishes (Costar, Cambridge, Mass., USA).

Permeabilization and fixation of the cells

After removal of the medium and washing of the cells with phosphate-buffered saline (PBS; 140 m*M* NaCl, 26 m*M* KCl, 6.4 m*M* Na_2HPO_2, 1.4 m*M* KH_2PO_4, pH 7.4), the cells were extracted for 3 min on ice with a detergent containing cytoskeleton stabilizing buffer [CSK-buffer; 100 m*M* NaCl, 2 m*M* $MgCl_2$, 1% Triton X-100 (Sigma, St. Louis, Mo., USA), 0.5 m*M* $CaCl_2$, 10 m*M* PIPES, pH 6.8, 300 m*M* sucrose, 4 m*M* vanadyl ribonucleoside complex (Gibco, Breda, Netherlands)]. The extracted cells were immediately fixed with 0.25% glutaraldehyde in CSK-buffer for 10 min. After fixation the extracted cells were washed with PBS for 10 min and subsequently for 10 min in PBS supplemented with 50 m*M* glycine to inactivate free aldehyde groups.

Nuclear matrix preparations

Preparation of nuclear matrices for in situ hybridization was performed essentially as earlier described (Fey et al. 1986). A431 cells grown on coverslips were washed in RNase-free PBS, pH 7.4, and permeabilized as described above in a CSK-buffer. To isolate the nuclear matrix, chromatin was removed by digestion with 1000 U/ml RNase-free DNase I (Boehringer Mannheim, Germany) in CSK-buffer for 30 min at room temperature. Ammonium sulphate was added to a final concentration of 250 m*M*. After 3 min incubation in 250 m*M* ammonium sulphate the extracted components were removed by washing in CSK-buffer. Removal of the DNA after the matrix isolation was monitored by staining the cells with 5 µg/ml of 4′, 6 diamidino-2-phenylindol-dihydrochloride (DAPI; Boehringer, Mannheim, Germany) for 5 min in PBS.

Probes

Labelling of EGF-receptor antisense RNA probe

A 3.8 kb EGF-receptor cDNA (Den Hertog et al. 1991) was inserted in a PGEM-3ZF(−)vector (Promega, Madison, Wisc., USA) containing an SP6 promoter and a T7 promoter. The plasmid was linearized with *Xba*I at the 5′ end of the EGF-receptor cDNA insert, for making the antisense RNA probe (positive RNA probe) with SP6 polymerase. The plasmid was linearized with *Hin*dIII at the 3′ end of the EGF-receptor cDNA to generate the sense RNA probe (control RNA probe).

The RNA probes were made by adding 16 µl transcription buffer of the following final concentrations to 1 µg linearized DNA; 1 µg DNA, 25 m*M* TRIS/HCl (pH 7.5), 4 m*M* $MgCl_2$, 1.3 m*M* spermidine, 12.5 µ*M* 1,4-dithiothreitol, 30 µ*M* EDTA, 125 µg/ml bovine serum albumin (BSA). Finally, 2 µl digoxigenin-RNA labelling mixture (Boehringer Mannheim) and 2 µl SP6 (40 U) or T7 (40 U) RNA polymerase (Boehringer Mannheim) were added. After incubation for 2 h at 40° C, the reaction was stopped by adding 2 µl 0.2 *M* EDTA (pH 8.0). The RNA was precipitated with 2.5 µl 4 *M* LiCl and 75 µl prechilled (−20° C) ethanol. The RNA was hydrolysed in 0.1 *M* sodium carbonate buffer (pH 10.2) at 60° C for 40 min. After hydrolysis the amount of detectable probe was estimated by spotting the labelled probe in a series of dilutions onto a nylon membrane (Kessler et al. 1990). The length of the RNA probe, determined by agarose gel electrophoresis, varied between 100 and 150 bases.

In situ hybridization

In situ hybridization with an EGF-receptor RNA probe

After permeabilization and fixation the cells were hybridized for 3 h in: 50% formamide, 2× SSC (1× SSC is 150 m*M* NaCl, 15 m*M* sodium citrate, pH 7.4), 0.3% Ficoll (Pharmacia, Uppsala, Sweden), 0.3% polyvinyl pyrrolidone (Sigma), 0.3% BSA (Sigma), herring sperm DNA (0.2 mg/ml) and yeast tRNA (0.2 mg/ml), 5% dextran sulphate (Sigma) and 1 ng/µl digoxigenin labelled RNA probe (RNA sense probe or the RNA antisense probe) at 55° C. The hybridization mix was boiled for 5 min prior to use; 20 µl hybridization mixture was added per coverslip. After hybridization the cells were washed for 30 min with 50% formamide, 2× SSC at 55° C, 20 min with 2× SSC, 20 min with 1× SSC and 20 min with 0.1× SSC at room temperature. All buffers used before the fixation step were RNase-free, buffers used after the fixation step were sterilized only.

As one of the controls an RNase A treatment was done after the permeabilization step and before the fixation step as follows: cells were incubated for 30 min in CSK-buffer with 100 µg/ml RNase A (Boehringer Mannheim) at 37° C. After the RNase step the cells were fixed and hybridization with the RNA probe was performed under the same conditions as mentioned above.

Detection of the hybrids with alkaline phosphatase

After the stringent washing steps, the hybridized cells were blocked in 100 m*M* TRIS/HCl (pH 7.5), 150 m*M* NaCl, 1% BSA, 0.3% Tween 20 (Sigma) for 30 min. Then the cells were incubated for 2 h with anti-digoxigenin Fab-fragments, directly coupled to alkaline phosphatase (sheep anti-digoxigenin-AP Fab-fragments from Boehringer Mannheim) diluted 1:500 in the blocking buffer. After extensive washing, the alkaline phosphatase staining reaction was performed: the cells were incubated in a substrate solution containing 0.33 mg/ml nitro-blue tetrazolium (NBT) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100 m*M* TRIS/HCl, pH 9.5 in the presence of 100 m*M* NaCl and 100 m*M* $MgCl_2$ for 15 min. The preparations were subsequently mounted in Mowiol (Hoechst, Frankfurt/Main, Germany) and examined with a bright field microscope (photomicroscope III, Zeiss, Oberkochen, Germany).

Detection of the hybrids with ultra-small gold particles

After stringent washing subsequent to in situ hybridization, the coverslips were washed with PBS supplemented with 50 m*M* glycine for 10 min and then blocked for 30 min in PBG [PBS supplemented with 0.5% BSA (Sigma) and 0.1% cold water fish skin gelatin (Sigma)]. The preparations were incubated overnight in PBG containing anti-digoxigenin Fab-fragments directly coupled to ultra-small gold particles. The ultra-small gold particles used are smaller than 0.8 nm in diameter (Aurion, Wageningen, The Netherlands). After overnight incubation the coverslips were washed in PBG for 2 h and in PBS for 10 min. The samples were post-fixed with 1% glutaralydehyde in PBS for 10 min. The ultra-small gold particles were enlarged by silver enhancement (Danscher 1981) during 40 min for light microscopy and 20 min for electron microscopy respectively. Light microscopical preparations were mounted in Mowiol as described above and observed by bright field microscopy. The electron microscopical preparations were stained with 0.5% uranyl acetate in H_2O, dehydrated and embedded in Epon. Sections of 0.2 µm in thickness were cut parallel to the substratum, using an Ultracut E (Reichert-Jung, Nussloch, Germany). Sections were examined in a Philips EM 420 electron microscope operated at 120 kV.

In situ hybridization combined with immunolabelling

Initial in situ hybridization was done as described above. The hybrids were detected first with an anti-digoxigenin Fab-fragment coupled to ultra-small gold and the ultra-small gold particles were silver enhanced for 20 min as described above. After extensive washing the SC-35 protein was localized. The SC-35 protein is an essential non-sn ribonucleoprotein (RNP) splicing factor (Fu and Maniatis 1990) with a molecular weight of 35 kDa. To localize the SC-35 protein the samples were first incubated with the primary antibodies for 1 h and subsequently incubated overnight with a secondary antibody, conjugated to ultra-small gold particles; a second silver enhancement step was performed for 20 min. The gold particles which represent the EGF-receptor RNA were silver enhanced twice and were therefore visible as large particles. The gold particles representing the SC-35 protein were silver enhanced once and therefore visible as small particles.

Results

Localization of the EGF-receptor RNA at the light microscopical level

The EGF-receptor RNA was localized in nuclei of A431 cells using a digoxigenin-labelled RNA probe with in situ hybridization detected at the light microscopical level. The optimal hybridization conditions of the antisense RNA probe were first tested using the alkaline phosphatase detection method. An optimal signal was obtained when hybridization was performed for 3 h at a temperature of 55° C. Hybridization at a lower temperature led to aspecific binding of the probes and longer hybridization times did not increase the hybridization signal. Under these conditions, a 15-min colour reaction with alkaline phosphatase was sufficient to produce a pronounced signal (Fig. 1A).

The EGF-receptor RNA was mainly found at sites around the nucleolus. If the cells were treated with RNase before the hybridization (data not shown) or if they were hybridized with the control probe i.e. the RNA sense probe (Fig. 1 B), no signal could be detected. In addition when NIH-3T3 cells, which do not contain the EGF-receptor mRNA, were hybridized with the EGF-receptor RNA probe, again no hybridization signal was visible (Fig. 1 C). Therefore we concluded that the hybridization signal, as shown in Fig. 1 A, represents the EGF-receptor transcripts in the nucleus.

In order to locate the EGF-receptor RNA more precisely and to identify the underlying substructures, the in situ hybridization and detection protocol had to be adjusted for electron microscopy. As a detection system we chose gold-tagged antibodies. First the detection of the hybrids, using ultra-small gold and silver enhancement, was performed at the light microscopical level. A431 cells were permeabilized, fixed and hybridized with the digoxigenin-labelled EGF-receptor RNA probe, as described in the 'Materials and methods'. Then the detection of the hybrids was performed with a gold conjugated antibody. After 40 min of silver enhancement a specific hybridization signal was observed, using bright field light microscopy. The signal, visible as a black precipitate, is mainly present around the nucleoli (Fig. 1 D).

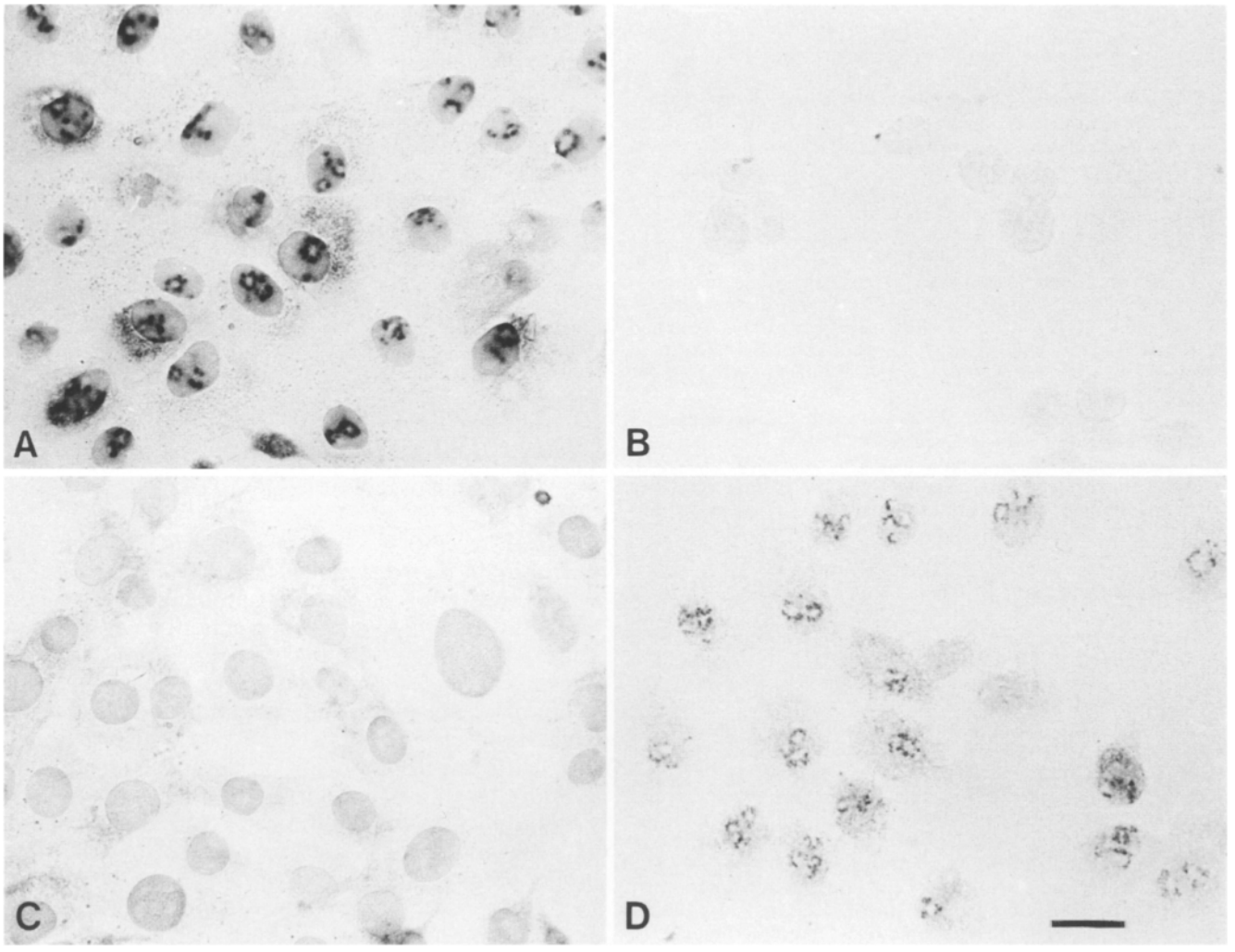

Fig. 1 A–D In situ hybridization with digoxigenin labelled probes at the light microscopical level. **A** In situ hybridization was performed on permeabilized A431 nuclei with a digoxigenin-labelled RNA probe; the hybrids were detected with alkaline phosphatase. **B** In situ hybridization was performed on permeabilized A431 nuclei with the digoxygenin-labelled control RNA probe i.e. the sense RNA probe; the hybrids were detected with alkaline phosphatase. **C** In situ hybridization was performed with a digoxigenin-labelled RNA probe on permeabilized NIH-3T3 cells, which do not contain the epidermal growth factor (EGF)-receptor mRNA; the detection was performed with alkaline phosphatase. **D** In situ hybridization was performed with a digoxigenin-labelled RNA probe; the detection was performed with ultra-small gold particles and silver enhancement. *Bar*, 25 μm

Localization of the EGF-receptor RNA at the electron microscopical level

The antisense RNA probe was also used to perform in situ hybridization at the ultrastructural level. To prepare samples for electron microscopy, cells were grown on Thermanox coverslips, then permeabilization, hybridization and detection of the hybrids were performed exactly as for the light microscopical analyses. The only modification was that the time of silver enhancement was reduced to 20 min. An electron microscopical image is shown in Fig. 2. Most of the label is present around the nucleolus and a few particles are directly associated with the nucleolus. Hardly any label was observed inside the nucleolus, at the interchromatin granules, at the lamina pore complex or at other places in the nucleus.

To investigate whether the RNA of the EGF-receptor is associated with the nuclear matrix, the in situ hybridization was repeated on nuclear matrices. Therefore cells were treated with nuclease and high salt before the hybridization in order to remove the DNA from the nuclei (see the 'Materials and methods'). In the matrix preparations, no DAPI signal was observed by light microscopy thus confirming the extraction of the chromatin. In this matrix preparation the RNA of the EGF-receptor is still present in areas around the nucleolus. Furthermore, EGF-receptor transcripts are attached to the fibril-like structure of the nuclear matrix (Fig. 3).

Immunolabelling of nuclear proteins after the hybridization procedure

In order to correlate the localization of a specific endogenous RNA with nuclear proteins, immunolabelling

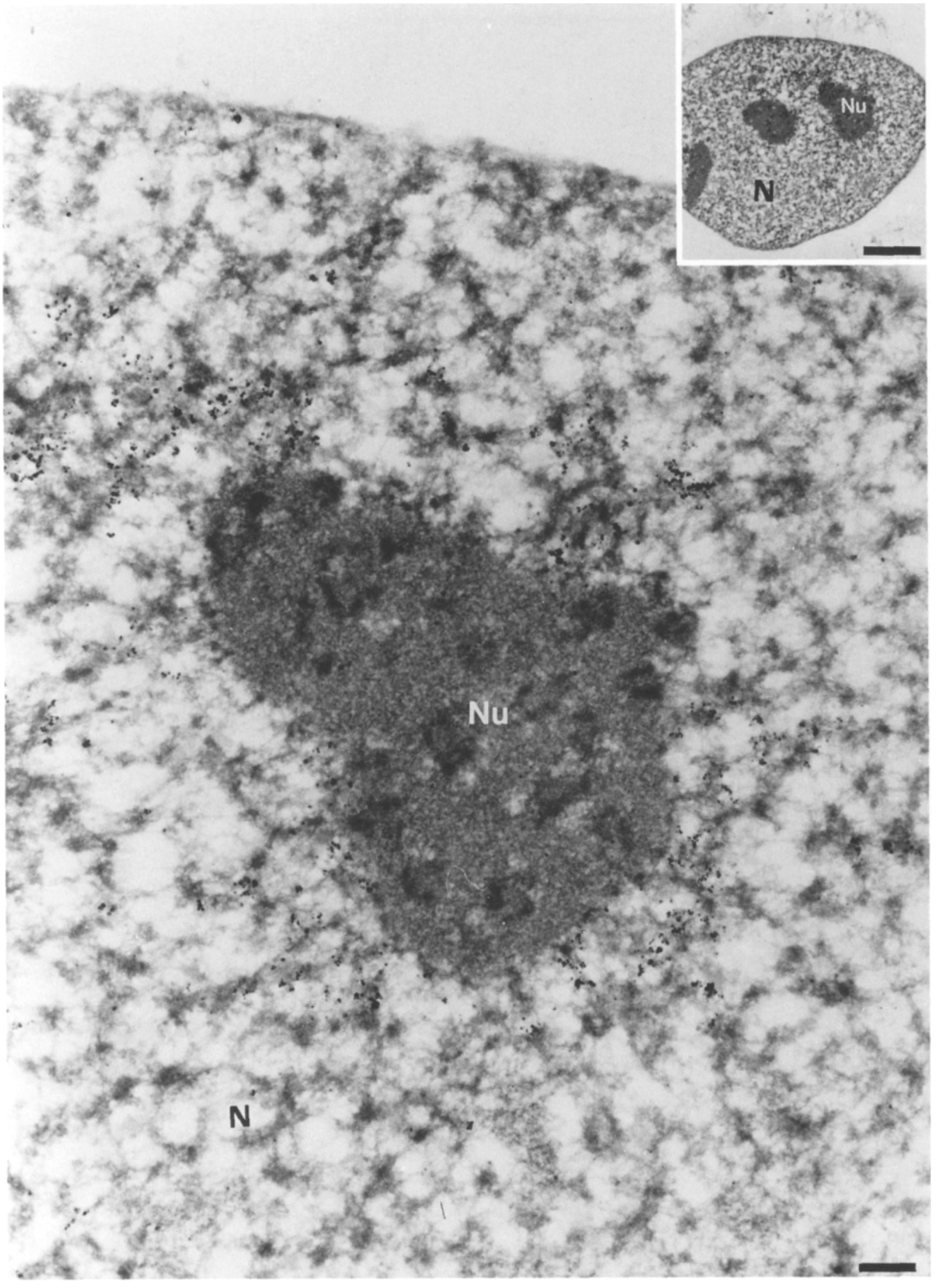

Fig. 2 Electron microscopical visualization of the EGF-receptor RNA after in situ hybridization and detection of the hybrids with ultra-small gold particles and silver enhancement. (*N* Nucleus, *Nu* nucleolus.) ×8800; *bar*, 0.4 μm. Insert ×3800; *bar*, 3 μm

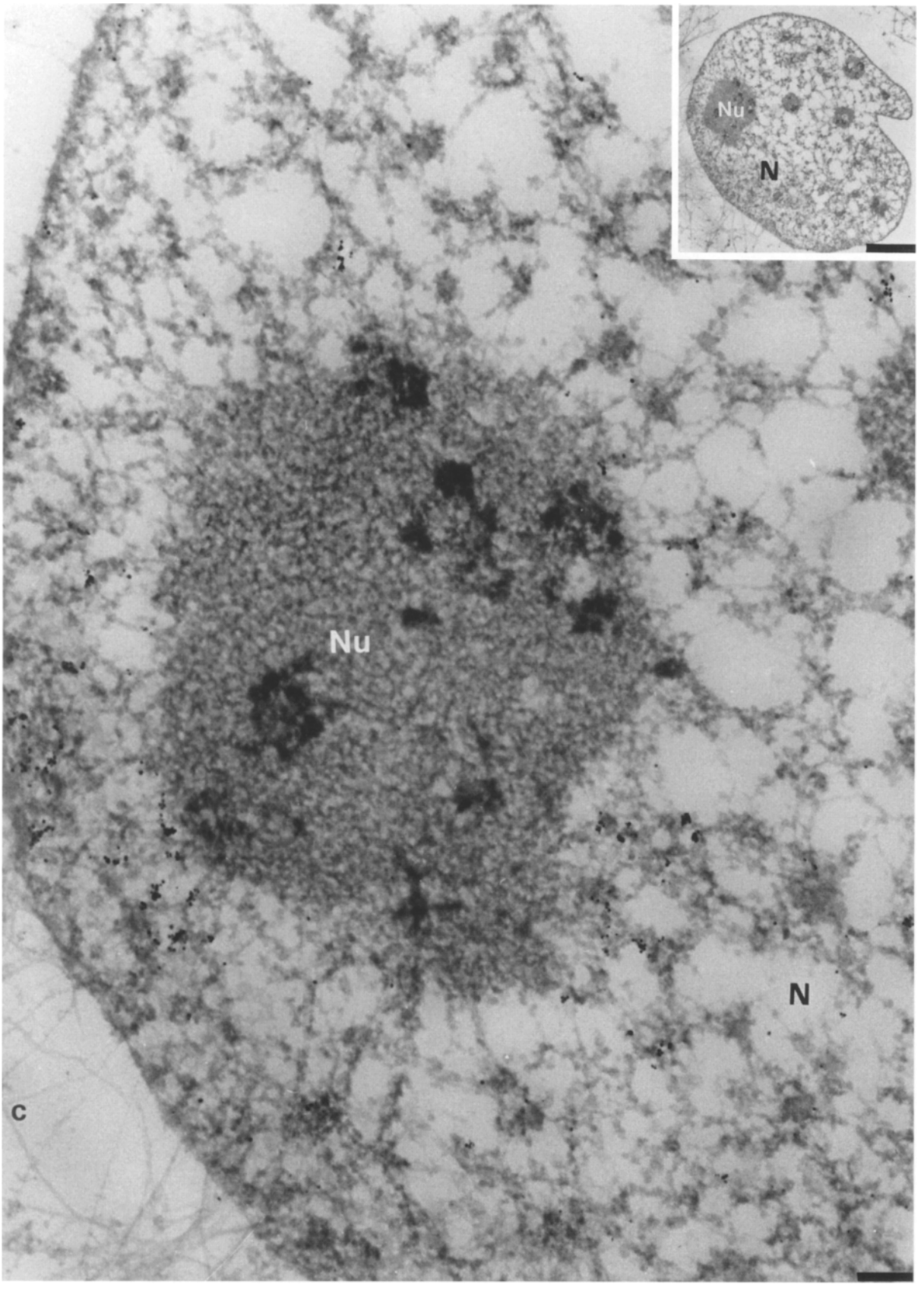

Fig. 3 Visualization of the EGF-receptor RNA in the nuclear matrix by pre-embedding in situ hybridization and detection with ultra-small gold and silver enhancement. (*N* Nucleus, *Nu* nucleolus, *C* cytoskeletal element) ×10500; *bar*, 0.3 μm. Insert ×3800; *bar*, 3 μm

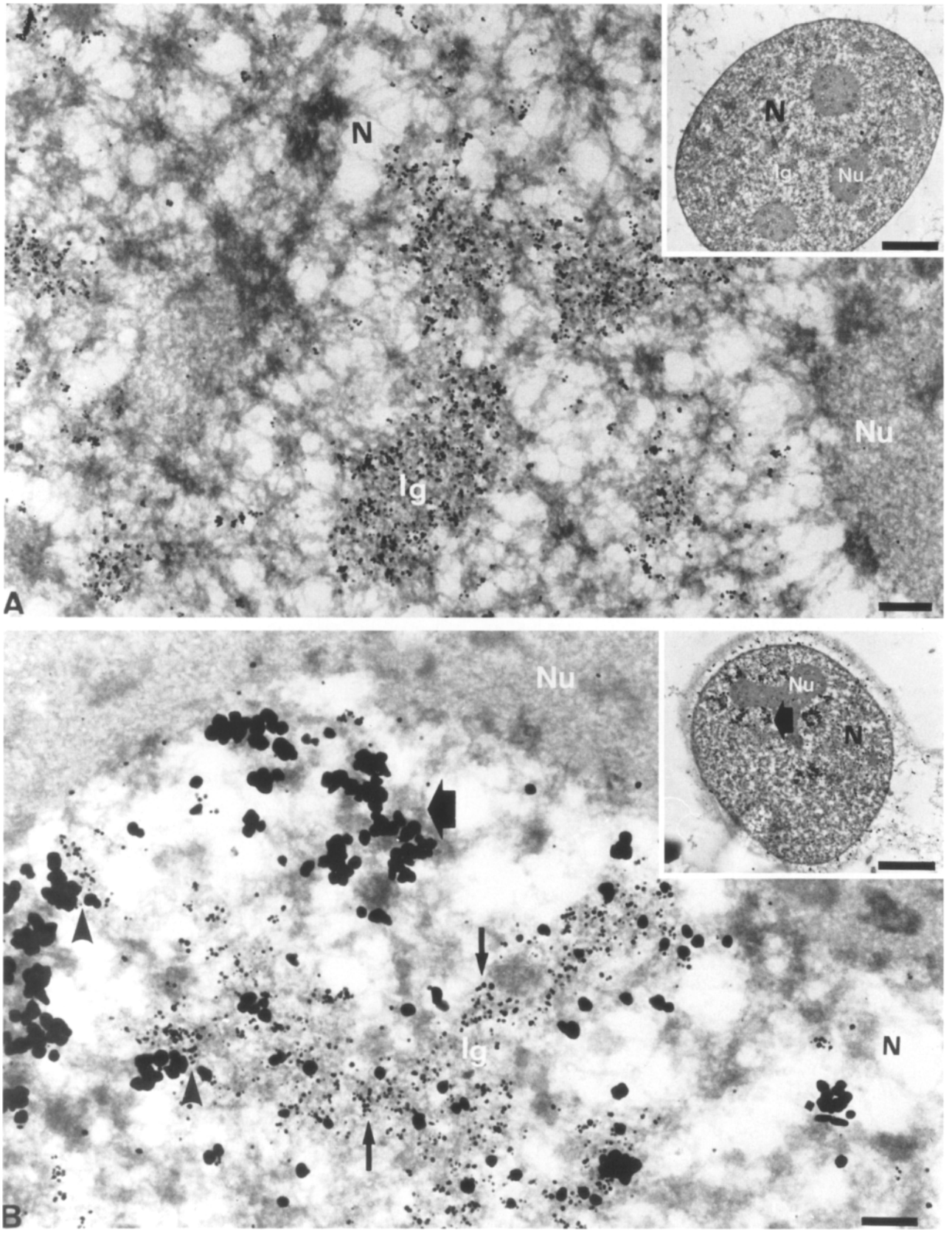

Fig. 4 **A** Localization of SC-35 protein at the electron microscopical level using pre-embedment labelling with ultra-small gold particles and silver enhancement. (*N* Nucleus, *Nu* nucleolus, *Ig* interchromatin granula cluster) × 10500; *bar*, 0.3 μm. Insert × 3800; *bar*, 3 μm. **B** Double labelling of the EGF-receptor RNA and the SC-35 protein by pre-embedding in situ hybridization in combination with immunocytochemistry. (*N* Nucleus, *Nu* nucleolus) *Large arrows* indicate EGF-receptor RNA; *small arrows* indicate SC-35 protein and *arrowheads* the close contact sites between the EGF-receptor RNA and the SC-35 protein. × 13500; *bar*, 0.3 μm. Insert × 3300; *bar*, 3 μm

was performed to localize the essential splicing protein SC-35 (Fu and Maniatis 1990). After the hybridization procedure, which was carried out without a probe, the cells were incubated with an anti-SC-35 antibody, followed by detection with ultra-small gold and silver enhancement. The SC-35 protein was located mainly over clusters of interchromatin granules (Fig. 4A), a localization previously observed by other workers by labelling of sections (Spector et al. 1991). These findings indicate that relocation does not occur during the permeabilization and hybridization procedure and that antigenicity is maintained under the hybridization conditions. Furthermore the ultra-small gold particles were demonstrated to penetrate into the clusters of interchromatin granules.

A double-labelling experiment was also performed to localize the EGF-receptor RNA and the SC-35 protein. First a hybridization was done with the EGF-receptor antisense RNA probe, detecting the hybrids with ultra-small gold followed by silver enhancement for 20 min. Then the nuclei were incubated with the SC-35 antibody and detection of this antibody was performed with a secondary antibody, coupled to ultra-small gold; silver enhancement was carried out for a second time. The large particles represent the EGF-receptor RNA (also visible at a lower magnification, insert in Fig. 4B) and the small particles represent the SC-35. SC-35 is mainly present in the interchromatin granules (indicated by small arrows, Fig. 4B). The EGF-receptor RNA is present in patches around the nucleolus (large arrows in Fig. 4B). Both labels at some sites are close to each other (indicated as arrowheads in Fig. 4B), but no exact colocalization is observed.

The image obtained in the double labelling experiment, shows background labelling scattered over the interchromatin granules and over the nucleoplasm. This background was also observed in cells which were hybridized with either a control probe or without a probe, followed by detection with the gold marker system and 40 min silver enhancement. Therefore it was concluded that the background is probably a result of aspecific binding of the probes and anti-digoxigenin antibodies, which become clearly visible after a silver enhancement step of 40 min. The background label was not a result of autoprecipitation during the silver enhancement steps, because when only silver enhancement was performed on cells without prior incubation with a probe or antibodies, no particles could be observed. These results demonstrate that the combination of in situ hybridization and immunocytochemistry, as described here, provides a powerful method to detect specific RNA sequences and specific proteins simultaneously, at the electron microscopical level.

Discussion

We have described a non-radioactive in situ hybridization method at the electron micoscopical level in combination with immunocytochemistry. The EGF-receptor transcript was localized in A431 nuclei using pre-embedding in situ hybridization, and was found mainly located around nucleoli. The pre-embedding in situ hybridization technique was also used to label the RNA of the EGF-receptor in nuclear matrix preparations. In an electron microscopical double labelling study, the EGF-receptor RNA was localized together with the essential splicing protein SC-35 using both pre-embedding in situ hybridization and pre-embedding immunocytochemistry.

During the in situ hybridization experiments, ultra-small gold particles in combination with silver enhancement have been used to detect the hybrids. These small gold particles have been used because several studies demonstrate convincingly that labelling efficiency increases with decreasing diameters of the gold particles (De Graaf et al. 1991; Humbel and Biegelmann 1992). After silver enhancement, the particles can be visualized by light microscopy, which provides the advantage that the specificity of the labelling can be monitored by light microscopy, before the application of electron microscopy.

Electron microscopical pre-embedding studies have the advantage that labelling is achieved throughout the whole section. Therefore pre-embedment labelling is more sensitive compared to post-embedment labelling, where only those targets can be reached that are present at the surface of the section. A disadvantage of pre-embedment labeling is the requirement for an initial permeabilization step e.g. with Triton X-100, as described here, to make the cells accessible for the probes and the antibodies. The use of this detergent prior to fixation may induce artefacts since it causes extraction of lipids and soluble proteins. However, we show here that after the permeabilization and hybridization procedures the SC-35 protein is localized mainly over the interchromatin granules, as was previously observed by other workers using labelling on ultra-thin sections (Spector et al. 1991). Therefore relocation of the SC-35 protein appears not to occur during the extraction procedure.

We have also done in situ hybridization studies to localize 28S rRNA with both pre-embedding and post-embedding in situ hybridization. Pre-embedding in situ hybridizations were performed as described in the Materials and methods; post-embedding in situ hybridizations were carried out on Lowicryl K4M sections. For both hybridization techniques the same labelling pattern of 28S rRNA was observed (Sibon et al., manuscript in preparation). The pre-embedment labelling method has been previously used successfully to localize some nuclear proteins with good preservation of the ultrastructure (de Graaf et al. 1991, 1992a, b). Moreover the usefulness of the pre-embedding method for in situ hybridization studies has been proven by in situ hybridization studies at the ultrastructural level for detecting cytoskeletal mRNAs (Bassell 1993; Cremers et al. 1990; Singer et al. 1989). The use of Triton X-100 in the cell permeabilization procedure is justified as it has already been shown that Triton X-100 treatment

does not alter localization nor cause reduction of viral RNA sequences in the nucleus compared to intact cells (Xing and Lawrence 1991). Pre-embedding in situ hybridization is a sensitive tool to study the localization of specific RNA sequences in the nucleus and especially in nuclear matrix preparations because of the open structure of the latter. This pre-embedding in situ hybridization can be used at the level of both the light and the electron microscope and can be combined with immunocytochemistry.

The RNA of the EGF-receptor was found to occur in close proximity to the nucleolus. Hardly any label was found over the interchromatin granules, inside the nucleolus or at the periphery of the nucleus. The apparent association of the EGF-receptor RNA and the nuclear matrix (Fig. 3) provides some evidence that the nuclear matrix may play a role in pre-mRNA processing, as previously shown by biochemical studies (Jackson and Cook 1985; Krainer 1988; Smith et al. 1989).

Some earlier reports deal with the localization of specific RNA sequences in the nucleus. Generally two patterns can be distinguished, i.e. track-like appearances of RNA (Huang and Spector 1991; Lawrence et al. 1989; Raap et al. 1991; Xing et al. 1993) and foci-like appearances (Sibon et al. 1993; Xing et al. 1993). The reason why some transcripts, rather than others, are located in a track remains unclear. From the double labelling experiments with the SC-35 splicing protein, it was concluded that the only place were the SC-35 and the EGF-receptor RNA are close to each other is at the border of the interchromatin granules. These results support the hypothesis that splicing takes place at the edges of the interchromatin granules (Huang and Spector 1991; Spector et al. 1990).

It is still not known why the RNA of the EGF-receptor is located around nucleoli; however, this specific localization was also observed by other workers. Recently, c-myc transcripts were localized to the nucleoli by radioactive in situ hybridization (Bond and Wold 1993). However, the resolution that is reached after radioactive in situ hybridization is too low to conclude whether the c-myc transcripts are located inside the nucleolus or just at the outside of the nucleoli. In addition, poly(A) RNA has been localized in several cell types and was also found mainly around the nucleoli (Carter et al. 1991). Further studies will focus on the rationale behind the specific location of the EGF-receptor transcripts around the nucleolus. The pre-embedding in situ hybridization technique in combination with immunocytochemistry, as described here, will provide a powerful tool to perform such studies. This technique enables the localization of specific RNA sequences and nuclear proteins together with subnuclear structures.

Acknowledgements The authors are very indepted to J. Boonstra for critical reading of the manuscript. We acknowledge M. Kasperaitis for giving helpful instructions for synthesis of the RNA probes and L. Verspui for her excellent photographical work. We thank Drs. X.-D. Fu and T. Maniatis for the generous gift of the SC-35 antibody.

References

Bassell GJ (1993) High resolution distribution of mRNA within the cytoskeleton. J Cell Biochem 52:127–133

Berezney R (1991) The nuclear matrix: a heuristic model for investigating genomic organization and function in the cell nucleus. J Cell Biochem 47:109–123

Bond VC, Wold B (1993) Nucleolar localization of myc transcripts. Mol Cell Biol 13:3221–3230

Carmo-Fonseca M, Pepperkok R, Sproat BS, Ansorge W, Swanson MS, Lamond AI (1991a) In vivo detection of snRNP-rich organelles in the nuclei of mammalian cells. EMBO J 10:1863–1873

Carmo-Fonseca M, Tollervey D, Pepperkok R, Barabino SML, Merdes A, Brunner C, Zamore PD, Green MR, Hurt E, Lamond AI (1991b) Mammalian nuclei contain foci which are highly enriched in components of the pre-mRNA splicing machinery. EMBO J 10:195–206

Carter KC, Taneja KL, Lawrence JB (1991) Discrete nuclear domains of poly(A) RNA and their relationship to the functional organization of the nucleus. J Cell Biol 115:1191–1202

Cremers AFM, De Graaf A, Van Bergen en Henegouwen PMP, Humbel BM, Verkleij AJ (1990) The use of ultra small gold particles at the light and electron microscopical level. In: Elder HY (ed) Transaction of the Royal Microscopical Society. Adam Hilger, Bristol, pp 609–614

Danscher G (1981) Localization of gold in biological tissue. A photochemical method for light and electron microscopy. Histochemistry 71:81–88

De Graaf A, Van Bergen en Henegouwen PMP, Meijne AML, Van Driel R, Verkleij AJ (1991) Ultrastructural localization of nuclear matrix proteins in HeLa cells using silver enhanced ultra small gold probes. J Histochem Cytochem 39:1035–1045

De Graaf A, Humbel BM, Stuurman N, Van Bergen en Henegouwen PMP, Verkleij AJ (1992a) Three-dimensional immunogold labeling of nuclear matrix proteins in permeabilized cells. Cell Biol Int Rep 16:827–836

De Graaf A, Meijne AML, Van Renswoude J, Humbel BM, Van Bergen en Henegouwen PMP, De Jong L, Van Driel R, Verkleij AJ (1992b) Heat shock induced redistribution of a 160 kDa nuclear matrix protein. Exp Cell Res 202:243–251

Den Hertog JD, De Laat SW, Schlessinger J, Kruijer W (1991) Neuronal differentiation in response to epidermal growth factor of transfected murine P19 embryonal carcinoma cells expressing human epidermal growth factor receptors. Cell Growth Differ 2:155–164

Dirks RW, Van Dorp AGM, Van Minnen J, Fransen JAM, Van Der Ploeg M, Raap AK (1993) Ultrastructural evidence for the axonal localization of caudodorsal cell hormone mRNA in the central nervous system of the mollusc *Lymneae stagnalis*. Microsc Res Tech 25:12–18

Fey EG, Krochmalnic G, Penman S (1986) The non-chromatin substructures of the nucleus: the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionating and resinless section electron microscopy. J Cell Biol 102:1654–1665

Fu X-D, Maniatis T (1990) Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. Nature 343:437–441

Habets WJ, Hoet MH, De Jong BAW, Van der Kemp A, Van Venrooij WJ (1989) Mapping of B cell epitopes on small nuclear ribonucleoproteins that react with human autoantibodies as well as with experimentally induced mouse monoclonal antibodies. J Immunol 143:2560–2566

Hozak P, Hassan AB, Jackson DA, Cook PR (1993) Visualization of replication factories attached to a nucleoskeleton. Cell 73:361–373

Huang S, Spector DL (1991) Nascent pre-mRNA transcripts are associated with nuclear regions enriched in splicing factors. Genes Dev 5:2288–2302

Huang S, Spector DL (1992) U_1 and U_2 snRNAs are present in nuclear speckles. Proc Natl Acad Sci USA 89:305–308

Humbel BM, Biegelmann E (1992) A preparation protocol for postembedding immunoelectron microscopy of *Dictyostelium discoideum* cells with monoclonal antibodies. Scanning Microsc 6:817–825

Jackson DA, Cook PR (1985) Replication occurs at a nucleoskeleton. EMBO J 4:919–925

Jackson DA, Hassan AB, Errington RJ, Cook PR (1993) Visualization of focal sites of transcription within human nuclei. EMBO J 12:1059–1065

Kellenberger E, Dürrenberger M, Villinger W, Carlemalm E, Wurtz M (1987) The efficiency of immunolabel on Lowicryl sections compared to theoretical predictions. J Histochem Cytochem 35:959–969

Kessler C, Höltke HJ, Seibl R, Burg J, Mühlegger K (1990) Nonradioactive labeling and detection of nuclei acids. A novel DNA labeling and detection system based on digoxigenin: anti-digoxigenin ELISA principle (digoxigenin system). Biol Chem Hoppe-Seyler 371:917–927

Krainer AR (1988) Pre-mRNA splicing complementation with purified human U_1, U_2, U_4/U_6, U_5 snRNPs. Nucleic Acids Res 16:9415–9429

Lawrence JB, Singer RH, Marselle LM (1989) Highly localized tracks of specific transcripts within the interphase nuclei visualized by in situ hybridization. Cell 57:493–502

O'Keefe RT, Henderson SC, Spector DL (1992) Dynamic organization of DNA replication in mammalian cell nuclei: spatial and temporally defined replication of chromosome-specific α-satellite. DNA sequences. J Cell Biol 116:1095–1110

Puvion-Dutilleul F (1993) Protocol of electron microscope in situ nucleic acid hybridization for the exclusive detection of double-stranded DNA sequences in cells containing large amounts of homologous stranded DNA and RNA sequences. Applications to adenovirus type 5 infected HeLa cells. Microsc Res Tech 25:2–11

Puvion-Dutilleul F, Bachellerie JP, Puvion E (1991) Nucleolar organization of HeLe cells as studied by in situ hybridization. Chromosoma 100:395–409

Raap AK, Van de Rijke FM, Dirks RW, Sol CJ, Boom R, Van der Ploeg M (1991) Bicolor fluorescence in situ hybridization to intron and exon mRNA sequences. Exp Cell Res 197:319–322

Raska I, Koberna K, Jarnik M, Petrasovicova V, Bednar J, Raska K, Bravo R (1989) Ultrastructural immunolocalization of cyclin/PCNA in synchronized 3T3 cells. Exp Cell Res 184:81–89

Sibon OCM, Cremers AFM, Boonstra J, Humbel BM, Verkleij AJ (1993) Localization of EGF-receptor mRNA in the nucleus of A431 cells by light microscopy. Cell Biol Int 17:1–11

Singer RH, Langevin GL, Lawrence JB (1989) Ultrastructural visualization of cytoskeletal mRNAs and their associated proteins using double-label in situ hybridization. J Cell Biol 108:2343–2353

Smith HC, Harris SG, Zillmann M, Berget SM (1989) Evidence that a nuclear matrix protein participates in pre-messenger RNA splicing. Exp Cell Res 182:521–533

Spector DL (1990) Higher order nucleolar organization: three-dimensional distribution of small nuclear ribonucleoprotein particles. Proc Natl Acad Sci USA 87:147–151

Spector DL, Fu X-D, Maniatis T (1991) Associations between distinct pre-mRNA splicing components and the cell nucleus. EMBO J 10:3467–3481

Stierhof YD, Schwarz H, Frank H (1986) Transverse sectioning of plastic embedded immunolabeled cryosections: morphology and permeability to protein A-colloidal gold complexes. J Ultrastruct Mol Struct Res 97:187–196

Stierhof YD, Schwarz H, Dürrenberger M, Villiger W, Kellenberger E (1991) Yield of immunolabel compared to resin sections and thawed cryosections. In: Hayat MA (ed) Colloidal gold: principles, methods, and applications, vol 3. Academic Press, San Diego, Calif., USA, pp 88–113

Thiry M, Thiry-Blaise L (1989) In situ hybridization at the electron microscope level: an improved method for precise localization of ribosomal DNA and RNA. Eur J Cell Biol 50:235–243

Verheijen R, Venrooij W, Ramaekers F (1988) Review. The nuclear matrix: structure and composition. J Cell Sci 90:11–36

Wansink DG, Schul W, Van der Kraan I, Van Steensel B, Van Driel R, De Jong L (1993) Fluorescent labeling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. J Cell Biol 122:283–293

Xing Y, Lawrence JB (1991) Preservation of specific RNA distribution within the chromatin-depleted nuclear substructure demonstrated by in situ hybridization coupled with biochemical fractionation. J Cell Biol 112:1055–1063

Xing Y, Johnson CV, Dobner PR, Lawrence JB (1993) Higher level organization of individual gene transcription and RNA splicing. Science 259:1326–1330