View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Radboud Repository

Radboud Repository

Radboud University Nijmegen

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/21773

Please be advised that this information was generated on 2017-12-05 and may be subject to change.

Short Communication

Detection of specific messenger RNA by electron microscopic in situ hybridization: implications for nucleocytoplasmic transport

Merryn V. E. Macville[1)a, b], Karien C. Wiesmeijer[a, b], Jack A. M. Fransen[2)b], Roeland W. Dirks[a], Anton K. Raap[a]

[a] Department of Cytochemistry and Cytometry, University of Leiden, Leiden/The Netherlands
[b] Department of Electron Microscopy, University of Leiden, Leiden/The Netherlands

Received June 2, 1995
Accepted July 24, 1995

Human cytomegalovirus – immediate early messenger RNA – electron microscopy – in situ hybridization – nucleocytoplasmic transport

Introduction

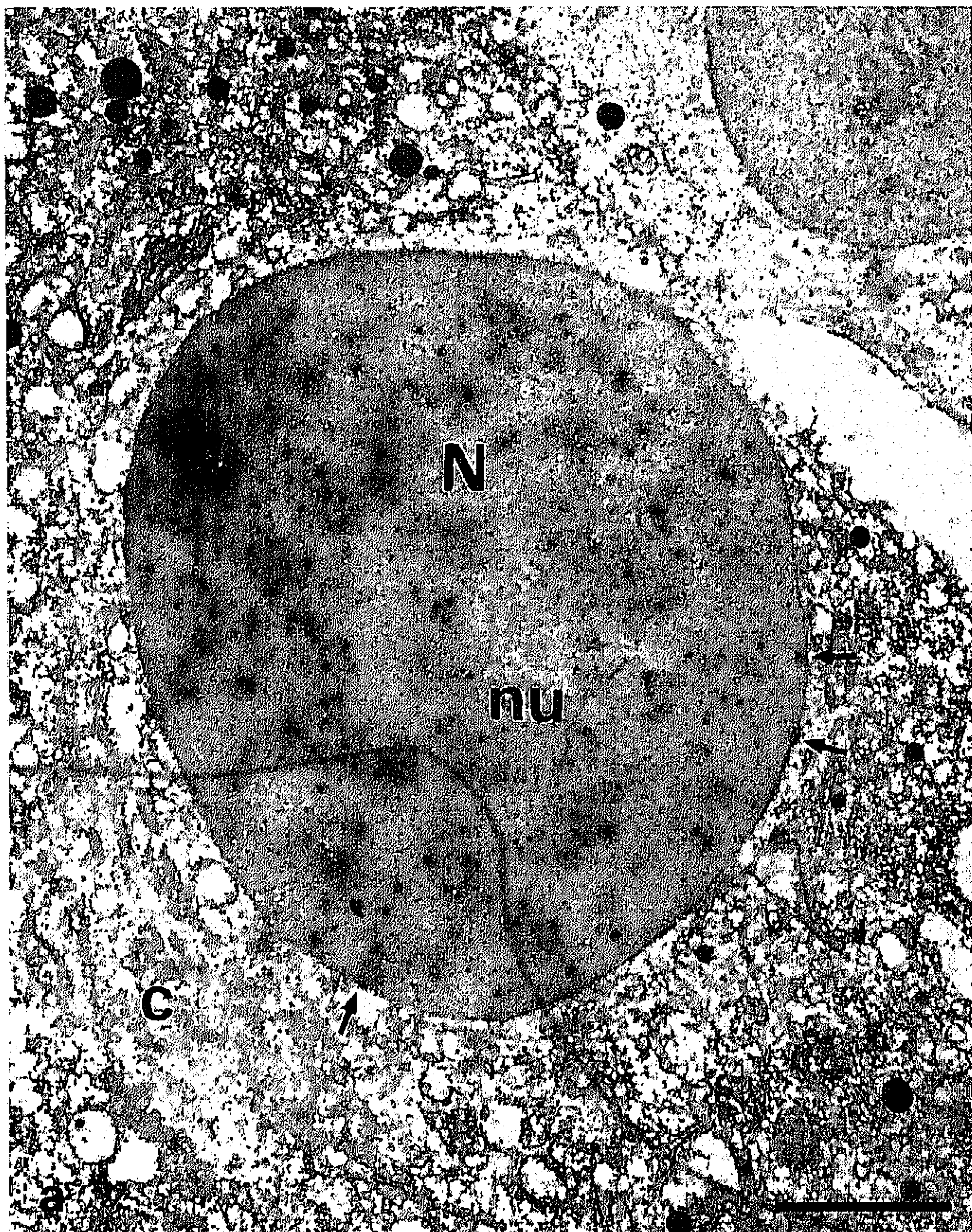
The past two decades have witnessed major advance in defining the basic molecular mechanisms of RNA synthesis, processing, and transport with most studies focusing on how interactions between protein factors and specific DNA or RNA sequences control these events. Messenger RNA (mRNA) is formed through a complex series of steps which includes transcription, 5' end capping, 3' end cleavage, addition of 3' poly(A) tail, and removal of introns followed by nucleocytoplasmic transport of mature mRNA. Despite powerful biochemical analysis in vitro, the full complexity of these processes in vivo is still poorly understood. One of the unanswered questions is along which pathway newly synthesized mRNA molecules are transported from the gene in the nucleus to the cytoplasm.

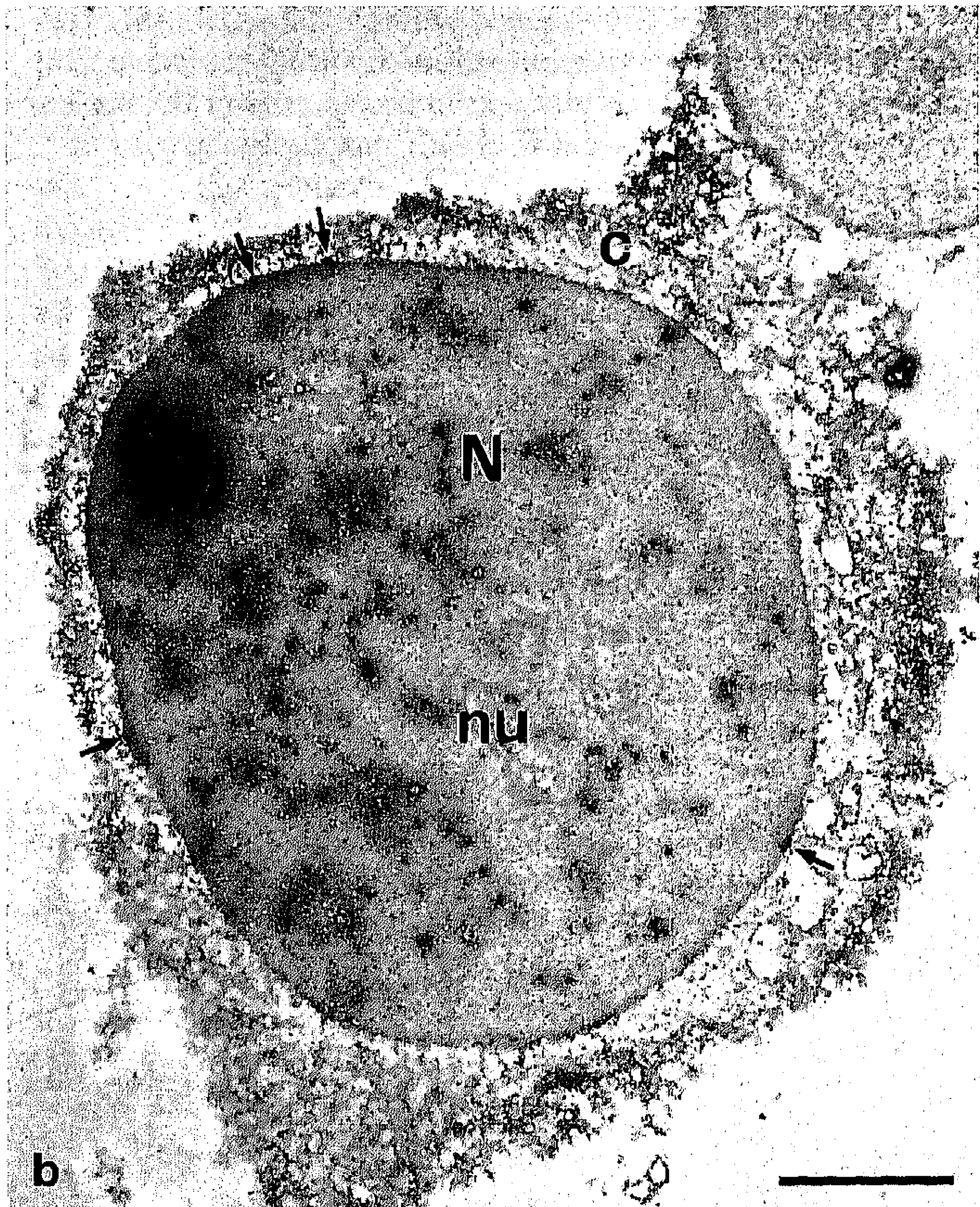
Possible mRNA transport mechanisms have been proposed based on in situ hybridization studies (ISH). By fluorescence ISH for viral mRNAs as well as for some endogenous gene transcripts, nuclear signals were observed in foci or "tracks" [12, 13, 15, 16, 20, 24]. The discussion about the nature of the tracks focuses on the question whether they represent nascent mRNA associated with the DNA template ("christmas-tree"), accumulation during pre-mRNA processing, a defined nucleocytoplasmic transport route, or combinations of the above (see [14, 25] and references herein). Evidence for "channeled diffusion" was obtained from ISH studies on Drosophila larvae [26] and mammalian cells [6, 27]. These studies conclude that tracks exclusively represent nascent mRNAs, which are cotranscriptionally spliced before they diffuse through an interchromosomal channel network to the nuclear surface.

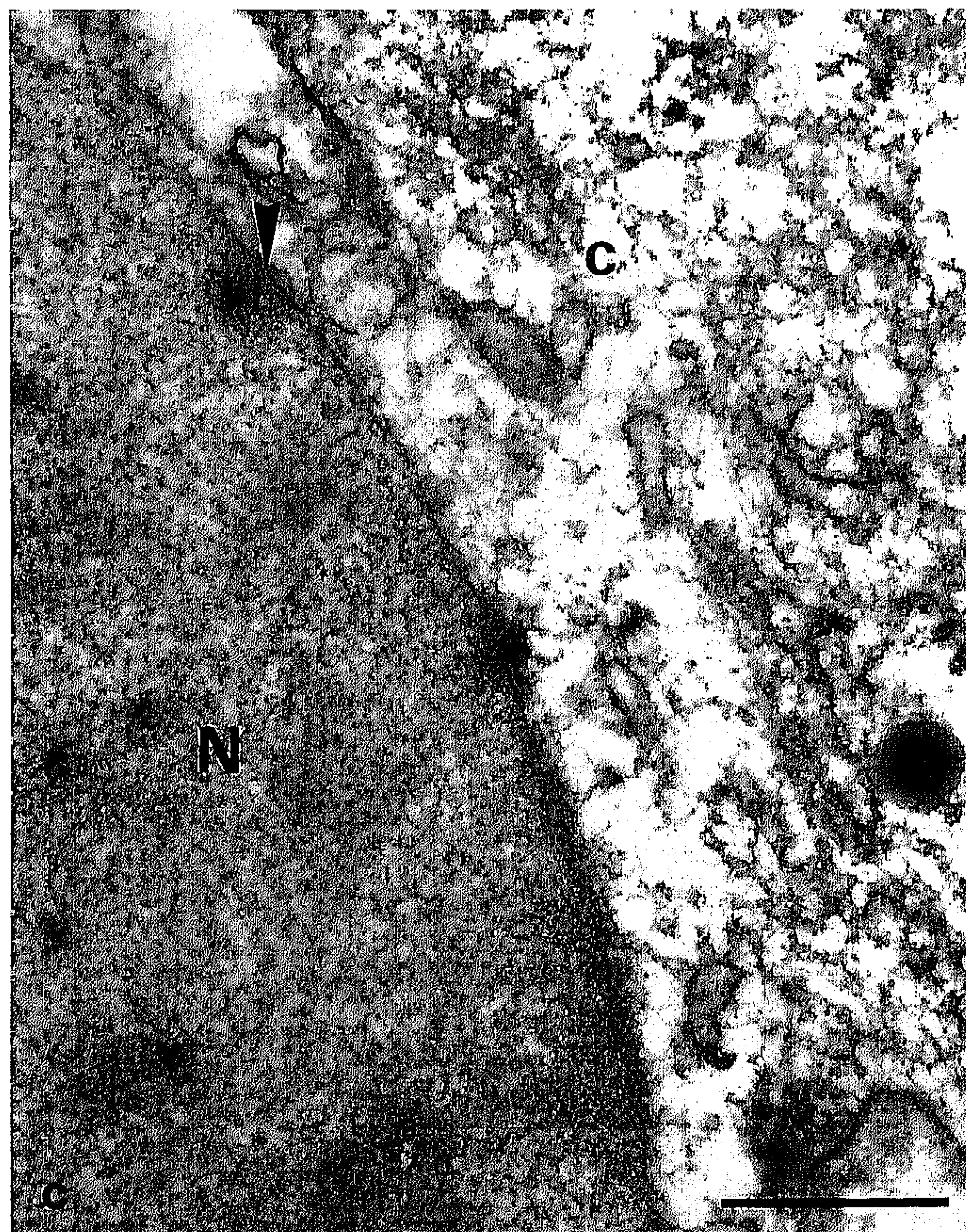
In previous fluorescence ISH studies, we reported the presence of nuclear tracks of human cytomegalovirus (HCMV) immediate early (IE) mRNA in rat 9G cells, as well as many small nuclear IE mRNA signals [7, 20]. We also analyzed the nuclear distribution of mRNAs from two other genes with different mRNA-splicing and mRNA-export characteristics [7]. On basis of these results, we hypothesized that the tracks represent sites of transcription, processing, and accumulation of pre-mRNAs, and that the small nuclear ISH signals radiating from these tracks represent IE mRNA in transport towards the nuclear envelope. To support the latter, visual proof would be provided by detection of mRNAs in transit over the nuclear envelope. This requires electron microscopic (EM) resolution.

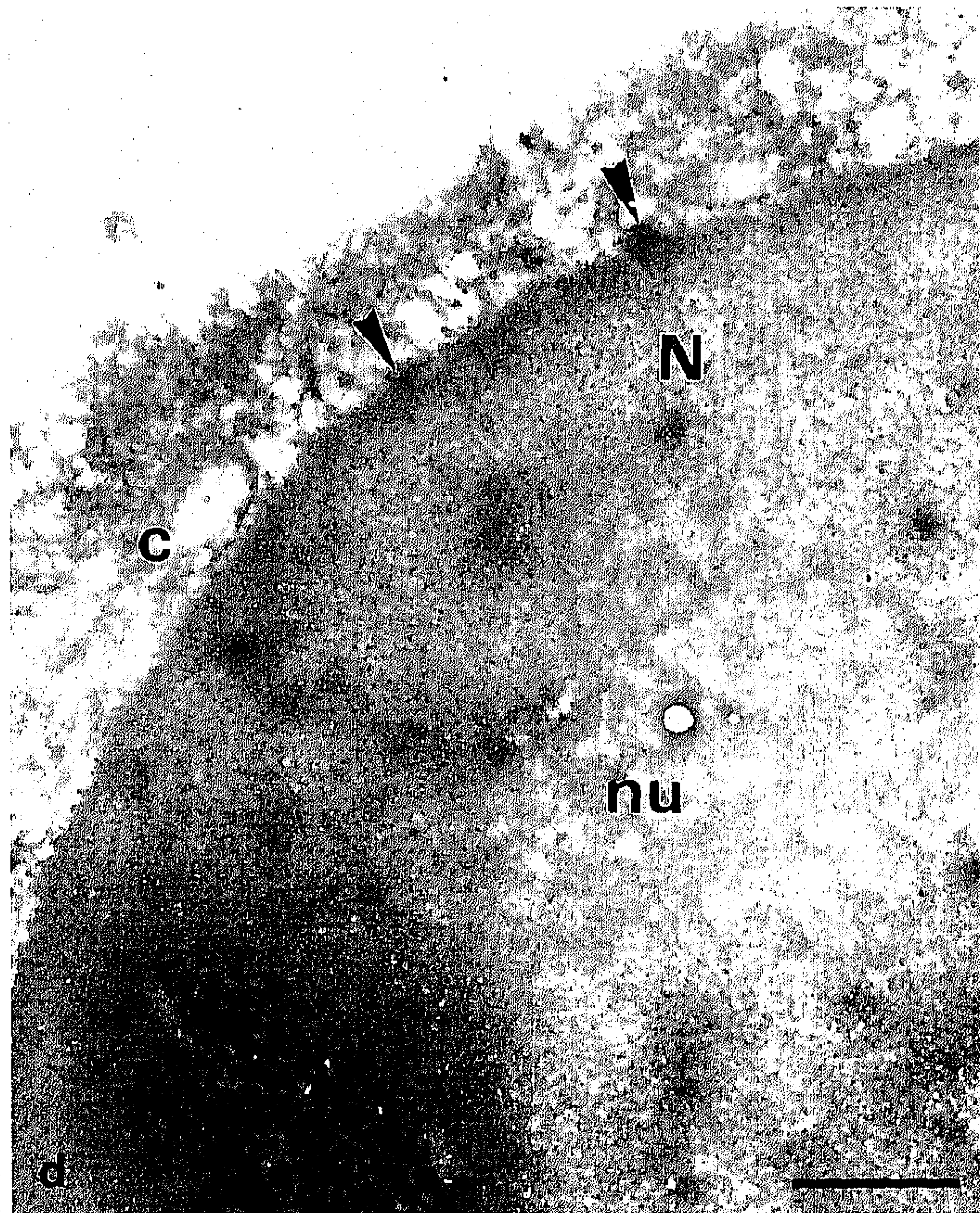
Fig. 1a. Ultrastructural IE mRNA distribution over the nucleus as observed in a horizontal section through the lower part (3rd section) of a 1% FA-fixed rat 9G cell. Many small nucleoplasmic DAB spots represent IE mRNA presumably radiating from the main spot towards the nuclear envelope. IE mRNA non-expressing cells do not reveal ISH signals in the cytoplasm and nucleus (*top right corner*). – **b.** Horizontal section through a higher part of the cell in (**a**), at approximately 0.8 μm distance (12th section), showing a similar distribution pattern. *Arrows* in (**a**) and (**b**) point at IE mRNA on the nuclear envelope, shown in detail in (**c**) and (**d**), respectively. – **c.** IE mRNA on the nuclear envelope at relative long distance from the main spot. Nuclear IE mRNA close to the inner nuclear membrane shows a tapering ISH signal (*arrowhead*). – **d.** IE mRNA on the nuclear envelope (*arrowheads*) at relative short distance from the main spot. – N Nucleus. – nu Nucleolus. – c Cytoplasm. – Bars 5 μm (**a, b**), 1 μm (**c, d**).

[1)] Dr. Merryn V. E. Macville, Department of Cytochemistry and Cytometry, Sylvius Laboratories, University of Leiden, Wassenaarseweg 72, 2333 AL Leiden/The Netherlands.
[2)] Present address: Department of Cell Biology and Histology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen/The Netherlands.


N
nu
c
a


c
N
nu
b


c
N
c


N
c
nu
d

We employed a newly developed preembedding EM ISH technique for non-radioactive detection of specific mRNAs [18]. We show specific gene transcripts in transport over the nuclear envelope in spatial relation to their transcription site.

Materials and methods

Rat 9G cells were cultured in Dulbecco's modified Eagle medium supplemented with 5 % fetal calf serum at 37 °C in a 5 % CO_2 atmosphere. HCMV-IE mRNA expression was induced by incubating the cells for 5 h with 50 μg/ml cycloheximide [3]. Cells were fixed in 1 % formaldehyde (FA) or 1 % FA with 0.05 % glutaraldehyde (GA; EM grade, Fluka Chemie, Buchs/Switzerland) dissolved in 0.15 M $NaHCO_3$, pH 8.6 [2], followed by permeabilization with 0.1 % saponin (Fluka Chemie) in phosphate-buffered saline (136 mM NaCl, 2.7 mM KCl, 8.4 mM Na_2HPO_4, 0.9 mM KH_2PO_4, pH 7.4) for 30 min at room temperature [18]. For IE mRNA specific ISH, the digoxigenin-labeled (Boehringer-Mannheim, Mannheim/Germany) plasmid probe pSS [3] was used at a concentration of 5 ng/μl in 60 % formamide/2 × SSC (0.3 M NaCl, 0.03 mM sodium citrate) overnight at 37 °C. Specificity controls and immunodetection by anti-digoxigenin-peroxidase $F(ab)_2$ fragments (Boehringer-Mannheim) followed by diaminobenzidine (DAB)/H_2O_2 detection and preparation for transmission electron microscopy were as described [17].

Transmission electron microscopy

Ribbons of ultrathin horizontal sections (60–90 nm) were cut on a Reichert Ultracut E microtome and collected on copper slotgrids (1 × 2 mm^2) essentially according to Wells [23]. Each slotgrid with its droplet of water was placed on a 0.8 % parlodion-filmed plastic ring (5 mm in diameter, 1 mm thick, 1 mm high), and air-dried. Serial sections were examined without carbon coating or contrast staining using a Philips EM 410LS operating at 60 kV.

Results

In rat 9G fibroblasts, nuclear and cytoplasmic IE mRNA ISH signals were examined at the ultrastructural level. Hybridization results were obtained without in situ denaturation of DNA and were absent in RNase A-treated cells, proving that the hybridization signals were specific for RNA. No ISH signals were obtained in hybridizations without probe or with IE nonspecific control probes. A maximum of 30 % of a rat 9G cell population will express IE mRNA upon induction [3], providing an internal specificity control (Fig. 1a).

In a recent study [18], we showed that nucleoplasmic mRNA distribution of rat 9G cells was best studied when they were fixed in 1 % FA, whereas cytoplasmic distribution was

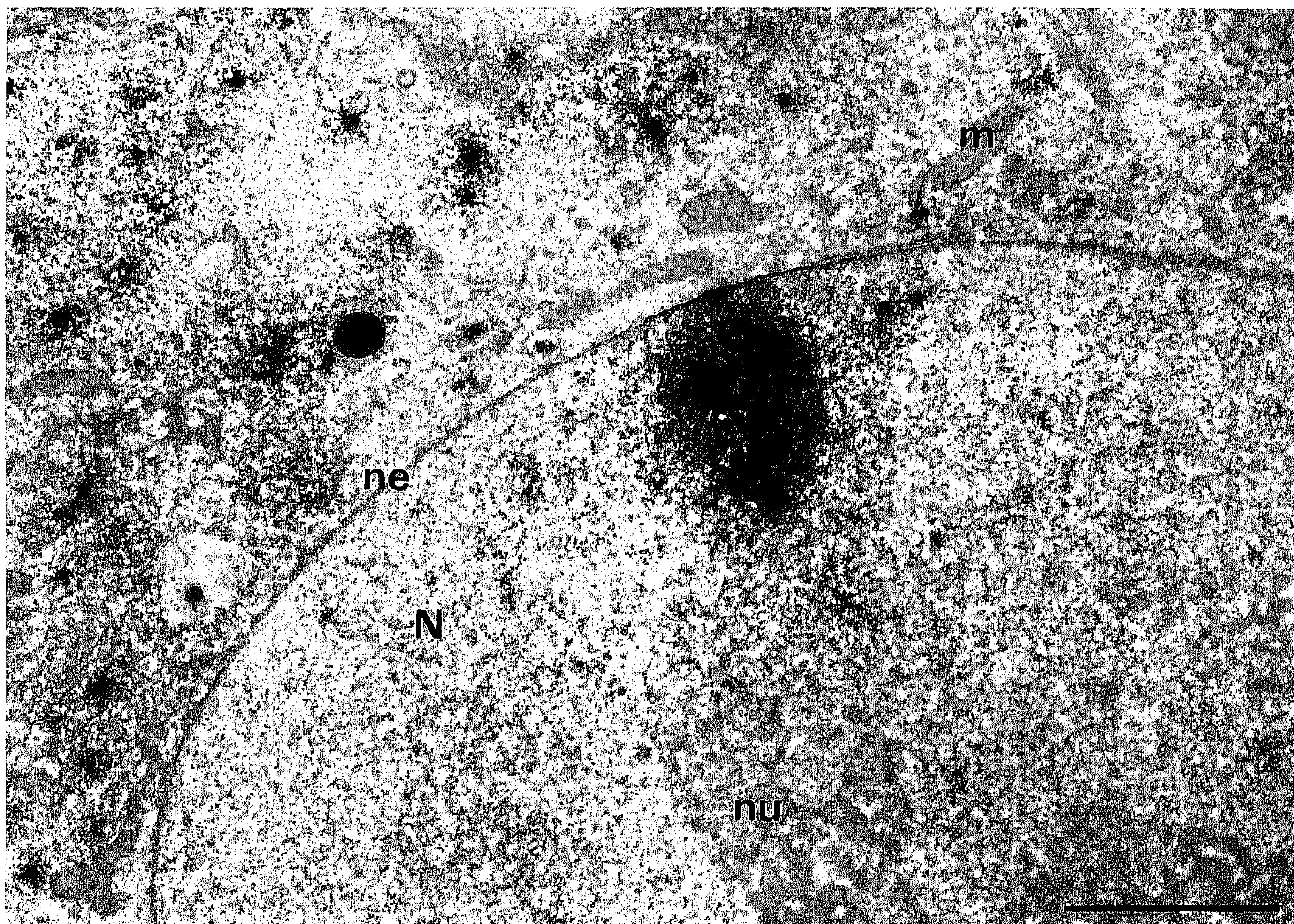


Fig. 2. Electron microscopic in situ hybridization for IE mRNA followed by immunoperoxidase/DAB detection, as shown in a 1 % FA/0.05 % GA-fixed rat 9G cell. The main nuclear ISH signal is in contact with the nuclear envelope. No cytoplasmic IE mRNA ISH signals are observed directly in contact with the main spot. – N Nucleus. – nu Nucleolus. – ne Nuclear envelope. – m Mitochondrion. – Bar 2 μm.

best studied when they were fixed in 1 % FA/0.05 % GA. Figure 1a shows IE mRNA detection in 1 % FA fixed-cells. Nuclei showed one main DAB spot with many small spots throughout the nucleoplasm, but no DAB spots over the nucleoli. The small nuclear spots formed a radiating pattern around the main spot: many spots were present close to the main spot, gradually decreasing in number away from the spot (see also [7]). In 1% FA/0.05% GA-fixed cells the main spot was clearly visible, but the number of small nuclear spots was reduced as a consequence of reduced accessibility. In approximately 90 % of the IE mRNA positive cells, the main spot was present in the periphery of the nucleus close to the nuclear envelope. In a few cases the main spot was in contact with the nuclear envelope (Fig. 2), but we never observed cytoplasmic IE mRNA ISH signals in direct contact with the main nuclear spot.

In serial sections throughout the Z-direction of the nucleus similar distribution patterns were observed (Figs. 1a, b). The average long axis of the main spot was approximately 2 μm.

Many DAB ISH signals were found on the nuclear envelope, most likely representing IE mRNA in transport from the nucleus to the cytoplasm (Figs. 1c, d). Transport sites were not preferentially located close to the main spot (Fig. 1d). They were even found at a relative long distance from the main spot (Fig. 1c). On basis of the number of transport sites observed in transverse sections through the nuclear envelope and the dimensions of the nuclear surface ($\approx$ 750 μm^2), we estimate approximately 200 transport sites in the cell shown in Figure 1.

Figure 1c shows an IE mRNA signal tapering towards the inner nuclear membrane, which could indicate association with a nuclear pore complex (NPC) [5, 11, 22].

Discussion

In a recent fluorescence ISH study, we provided indirect evidence that the elongated dot or track-like signals for HCMV-IE mRNA in rat 9G cells represent sites of mRNA transcription, processing, and accumulation, whereas the small radiating signals represent processed mRNAs in transport through the nucleoplasm [7]. Even though at the light microscopic level, the main nuclear mRNA tracks are often in close association with the nuclear border, it was concluded that the track is not involved in nucleocytoplasmic transport of mRNAs. Due to its limited spatial resolution, however, direct visual proof of mRNA transport over the nuclear envelope could not be provided by fluorescence ISH. With a recently developed EM ISH technique [18], not only we confirmed the light microscopic data, but also showed IE mRNA located on the nuclear envelope. This localization strongly implies nucleocytoplasmic translocation through nuclear pores [1, 4, 9, 19, 21].

The distribution of DAB spots at the nuclear envelope did not indicate preferential transport sites. This raises evidence that IE mRNA translocation occurs randomly over the nuclear envelope. Feldherr and Akin [8] already showed that all nuclear pores are capable of RNA export. Huang et al. [11] conformed this by showing poly(A)$^+$ RNA ISH signals associated with all nuclear pores. In addition, Huang et al. [11] described tapering of poly(A)$^+$ mRNA ISH signals as they reach the inner nuclear membrane. A similar observation was made for IE mRNA ISH signals. Tapering was explained by the hypothesis that mRNAs might associate with intranuclear filaments of the NPC before being exported [5, 11, 22]. Moreover, evidence for attachment of these filaments to the nuclear matrix [10] could provide a basis for solid-state transport.

In our interpretation, newly synthesized mRNAs accumulate near the transcription site, but no transport over the nuclear envelope is observed directly emanating herefrom. Specific mRNA molecules presumably radiate to potentially all nuclear pores, even at long distance from the transcription site.

Acknowledgements. The authors are grateful to Dr. R. Boom (Department of Virology, University of Amsterdam/The Netherlands) for providing the rat 9G cell line, L.D.C. Verschragen and J. Van der Meulen for superb electron microscopic service, and to Dr. Ch. Schöfer (Histology and Embryology Institute, University of Vienna/Austria) for technical instruction. – This work was funded by The Netherlands Organization of Scientific Research, Department of Medical Sciences, grant no. NWO-MW 901-34-079.

References

[1] Agutter, P. S.: Nucleoplasmic RNA transport. Subcell. Biochem. **10,** 281–357 (1984).

[2] Artvinli, S.: Biochemical aspects of aldehyde fixation and a new formaldehyde fixative. Histochem. J. **7,** 435 (1975).

[3] Boom, R., J. L. Geelen, C. J. Sol, A. K. Raap, R. P. Minnaar, B. P. Klaver, J. Van der Noordaa: Establishment of a rat cell line inducible for the expression of human cytomegalovirus immediate early gene products by protein synthesis inhibition. J. Virol. **58,** 851–859 (1986).

[4] Clawson, G. A., C. M. Feldherr, E. A. Smuckler: Nucleocytoplasmic RNA transport. Mol. Cell. Biochem. **67,** 87–99 (1985).

[5] Cordes, V. C., S. Reidenbach, A. Köhler, N. Stuurman, R. Van Driel, W. W. Franke: Intranuclear filaments containing a nuclear pore complex protein. J. Cell Biol. **123,** 1333–1344 (1993).

[6] Cremer, T., A. Kurz, R. Zirbel, S. Dietzel, B. Rinke, E. Schroeck, M. R. Speicher, U. Mathieu, A. Jauch, P. Emmerich, H. Scherthan, T. Ried, C. Cremer, P. Lichter: Role of chromosome territories in the functional compartimentalization of the cell nucleus. Cold Spring Harbor Symp. Quant. Biol. **58,** 777–792 (1993).

[7] Dirks, R. W., K. C. Daniel, A. K. Raap: RNAs radiate from gene to cytoplasm are revealed by fluorescence in situ hybridization. J. Cell Sci. **108,** 2565–2572 (1995).

[8] Feldherr, C. M., D. Akin: EM visualization of nucleocytoplasmic transport processes. Electron Microsc. Rev. **3,** 73–86 (1990).

[9] Franke, W. W., U. Scheer, G. Krohne, E. D. Jarasch: The nuclear envelope and the architecture of the nuclear periphery. J. Cell Biol. **91,** 39–50 (1981).

[10] He, D., J. A. Nickerson, S. Penman: Core filaments of the nuclear matrix. J. Cell Biol. **110,** 569–580 (1990).

[11] Huang, S., T. J. Deerinck, M. H. Ellisman, D. L. Spector: In vivo analysis of the stability and transport of nuclear poly(A)$^+$ RNA. J. Cell Biol. **126,** 877–899 (1994).

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

[13] Kopczynski, C. C., M. A. T. Muskavitch: Introns excised from the delta primary transcripts are localized near sites of Delta transcription. J. Cell Biol. **119,** 503–512 (1992).

[14] Kramer, J., Z. Zachar, P. M. Bingham: Nuclear pre-mRNA metabolism: channels and tracks. Trends Cell Biol. **4,** 35–37 (1994).

[15] Lawrence, J. B., L. M. Marselle, K. S. Byron, C. V. Johnson, J. L. Sullivan, R. H. Singer: Subcellular localization of low-abundance human immunodeficiency virus nucleic acid sequences visualized by fluorescence in situ hybridization. Proc. Natl. Acad. Sci. USA **87,** 5420–5424 (1990).

[16] Lawrence, J. B., R. H. Singer, L. M. Marselle: Highly localized tracks of specific transcripts within interphase nuclei visualized by in situ hybridization. Cell **57**, 493–502 (1989).
[17] Macville, M. V. E., A. G. M. Van Dorp, K. C. Wiesmeijer, R. W. Dirks, J. A. M. Fransen, A. K. Raap: Monitoring morphology and signal during non-radioactive in situ hybridization procedures by reflection-contrast microscopy and transmission electron microscopy. J. Histochem. Cytochem. **43**, 665–674 (1995).
[18] Macville, M. V. E., K. C. Wiesmeijer, R. W. Dirks, J. A. M. Fransen, A. K. Raap: Saponin pre-treatment in pre-embedding electron microscopic in situ hybridization for the detection of specific RNA sequences in cultured cells: a methodological study. J. Histochem. Cytochem. in press (1995).
[19] Mehlin, H., B. Daneholt, U. Skoglund: Translocation of a specific premessenger ribonucleoprotein particle through the nuclear pore studied by electron microscope tomography. Cell **69**, 605–613 (1992).
[20] Raap, A. K., F. M. Van de Rijke, R. W. Dirks, C. J. Sol, R. Boom, M. Van der Ploeg: Bicolor fluorescence in situ hybridization to intron and exon mRNA sequences. Exp. Cell Res. **197**, 319–322 (1991).
[21] Scheer, U., M. C. Dabauvalle, H. Merkert, R. Benavente: The nuclear envelope and the organization of the pore complexes. Cell Biol. Int. Rep. **12**, 669–689 (1988).
[22] Sukegawa, J., G. Blobel: A nuclear pore complex protein contains zinc fingers motifs, binds DNA, and faces the nucleoplasm. Cell **72**, 29–38 (1993).
[23] Wells, B.: A convenient technique for the collection of ultrathin serial sections. Micron **5**, 79 (1975).
[24] Xing, Y., C. V. Johnson, P. R. Dobner, J. B. Lawrence: Higher level organization of individual gene transcription and RNA splicing. Science **259**, 1326–1330 (1993).
[25] Xing, Y., J. B. Lawrence: Nuclear RNA tracks: structural basis for transcription and splicing. Trends Cell Biol. **3**, 346–353 (1993).
[26] Zachar, Z., J. Kramer, I. P. Mims, P. M. Bingham: Evidence for channeled diffusion of pre-mRNAs during nuclear RNA transport in metazoans. J. Cell Biol. **121**, 729–742 (1993).
[27] Zirbel, R. M., U. R. Mathieu, A. Kurz, T. Cremer, P. Lichter: Evidence for a nuclear compartment of transcription and splicing located at chromosome domain boundaries. Chromosome Res. **1**, 92 (1993).