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

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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. Nucleolus. - d. IE mRNA on the nuclear envelope (arrowheads) at relative short distance from the main spot. Nucleus. – nu Nucleolus. – c Cytoplasm. – Bars 5 µm (a, b), 1 µm (c, 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% CO₂ 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 NaHCO₃, 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 Na₂HPO₄, 0.9 mM KH₂P0₄, 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 x SSC (0.3 M NaCl, 0.03 M sodium citrate) overnight at 37°C. Specificity controls and immunodetection by anti-digoxigenin-peroxidase F(ab)₂ fragments (Boehringer-Mannheim) followed by diaminobenzidine (DAB)/H₂O₂ detection and preparation for transmission electron microscopy were as described [17].

**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

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**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.
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 (≈ 750 μm²), 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] confirmed 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.

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References


