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PROSPECTS

High Resolution Distribution of mRNA Within the Cytoskeleton

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Abstract It has been well documented that mRNA is associated with the cytoskeleton, and that this relationship is involved in translation and mRNA sorting. The molecular components involved in the attachment of mRNA to the cytoskeleton are only poorly understood. The objective of this research was to directly visualize the interaction of mRNA with the cytoskeleton, with sufficient resolution to identify the filament systems involved. This work required the development of novel in situ hybridization methods for use with electron microscopy.

Key words: cytoskeleton, in situ hybridization, mRNA localization, mRNA-cytoskeletal interaction

Most cell types are defined by their unique shape. Many have a polarity which belies a complex organization of internal structure. For example, a skeletal muscle fiber is characterized by the parallel and highly registered arrays of myofibrils. Neuronal cells have two morphologically and functionally distinct processes: axons and dendrites. A major challenge is to identify the organizational principles which make possible this cellular diversity, operationally defined as non-random distribution of cellular proteins.

To approach this question experimentally, considerable biochemical research has been devoted to the identification of mechanisms involved in protein sorting. Signal peptide sequences have been identified which allow the nascent chains of membrane and secretory proteins to be co-translationally translocated into the rough endoplasmic reticulum for further modification and transport. Similarly, other sequences have been identified which permit proteins to be targeted to mitochondria, lysosomes, and nuclei.

More recently evidence has been provided which indicates that nucleic acids are not simply diffuse throughout the cytoplasm. Specific mRNAs are localized to distinct subcellular regions. For example, β-actin mRNA was localized to fibroblast lamellae, yet vimentin mRNA was localized to the perinuclear cytoplasm [Lawrence and Singer, 1986]. Microtubule-associated protein mRNA (MAP-2) was found concentrated to neuronal dendrites, whereas tubulin mRNA was localized to cell bodies [Garner et al., 1988]. Myelin basic protein mRNA has been shown to be regionalized to oligodendrocyte processes [Colman et al., 1982; Trapp et al., 1987]. Acetylcholine receptor mRNA was shown to be localized to the neuromuscular junction [Fontaine et al., 1988]. Earlier studies with nontranslated maternal mRNAs have demonstrated nonrandom distributions in ascidian eggs [Jeffrey et al., 1983; Jeffrey, 1984], *Xenopus* oocytes [Rebagliati et al., 1985], and *Drosophila* embryos [Frigerio et al., 1986]. The sorting of mRNAs could play an important role in establishing localized synthesis of proteins at their sites of function or assembly, and hence may directly influence cellular organization (see also Fulton, this issue).

The intracellular distribution of mRNA has been studied using in situ hybridization followed by light microscopy, permitting a broad definition of cellular regions in which a specific mRNA is concentrated, i.e., peripheral, perinuclear. However, the definition of the compartments within a cell to which these mRNAs are targeted requires greater resolution in order to correlate their spatial distribution with specific cellular components. The spatial organization of mRNA appears to be defined by structures which are not resolved by light microscopy [Sundell and Singer, 1991; Taneja et al., 1992]. mRNA,

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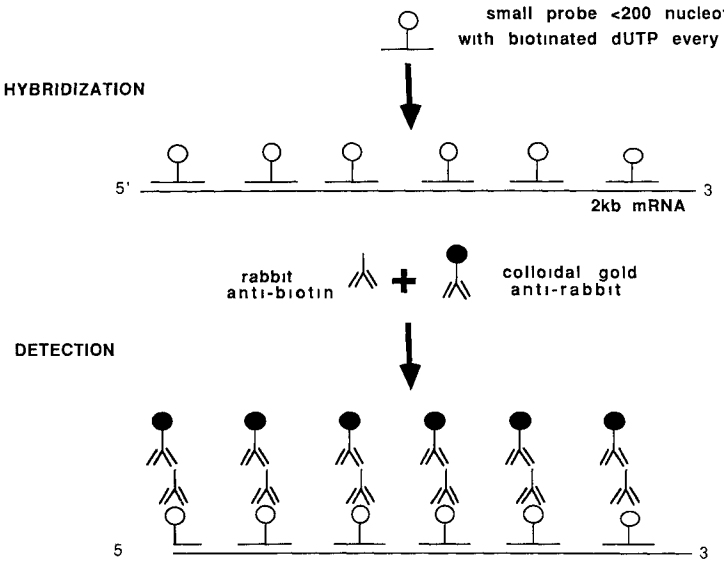

Fig. 1. Schematic showing the basis for detection of bona fide signal from mRNA-biotinated DNA hybrids Iterative detection of a mRNA molecule is possible using a biotinated DNA probe cut into small fragments so that each segment hybridizes independently The antibody detection of the biotin groups results in a string of colloidal gold particles, and this distinguishes the signal from the noise generated by non-specific sticking, either of probe or of antibody The schematic is not intended to represent the number of secondary antibody molecules adsorbed per gold particle or the number of secondary antibody complexes per primary antibody

and specifically translatable mRNA, is associated with the detergent-insoluble cytoskeletal framework [Lenk et al., 1977; Farmer et al., 1978; Lenk and Penman, 1979; Fulton et al., 1980; Cervera et al., 1981]. This association has now been studied in several laboratories using a biochemical approach to demonstrate that a variety of mRNA species are enriched in cytoskeletal fractions [reviewed in Hesketh and Pryme, 1991]. It is reasonable to suggest that the cytoskeleton is involved in the maintenance of nonuniform mRNA distributions [see Singer, 1992]. An understanding of the molecular mechanisms governing mRNA localization required elucidation of the physical association of mRNA with the cytoskeleton. Investigation of a relationship between mRNA attachment to the cytoskeleton and mRNA localization within the cell required a level of resolution not provided by light microscopy. The development of an electron microscopic in situ hybridization methodology was directly suited for this purpose, as the high resolution (0.3 nm) provided simultaneous visualization of mRNA molecules and individual cytoskeletal filaments. With this technology it has been possible to describe cellular components involved in attachment of mRNA to the cytoskeleton. These observations provided data essential for understanding the mechanisms involved in the spatial organization of mRNA.

The development of ultrastructural in situ hybridization methodology was directed toward visualizing single mRNA molecules on cytoskeletal filaments. Initial studies focused on the localization of mRNAs for the cytoskeletal proteins actin, vimentin, and tubulin [Singer et al., 1989]. Recent work on the localization of poly(A) mRNA has permitted a broader analysis of mechanisms involved in the attachment of mRNAs to the cytoskeleton [Taneja et al., 1992; Bassell and Singer, 1992] (Bassell et al., submitted). These data taken together with the previous work are used to develop a model to explain how specific mRNA molecules are localized within the cytoskeleton.

SITES OF mRNA BINDING REVEALED BY HIGH RESOLUTION

The ultrastructural localization of actin mRNA was studied in cultured fibroblasts grown on carbon and formvar coated EM grids and extracted in cytoskeletal buffer prior to fixation [Singer et al., 1989]. Full length β-actin cDNA was nick translated with biotinated dUTP and hybridized as probe. Figure 1 is a schematic of what one would expect to visualize if biotin labeled actin probe molecules were hybridized along the length of a 2 kb actin mRNA molecule. The biotin groups are detected by an antibiotin antibody followed by a colloidal gold conjugated

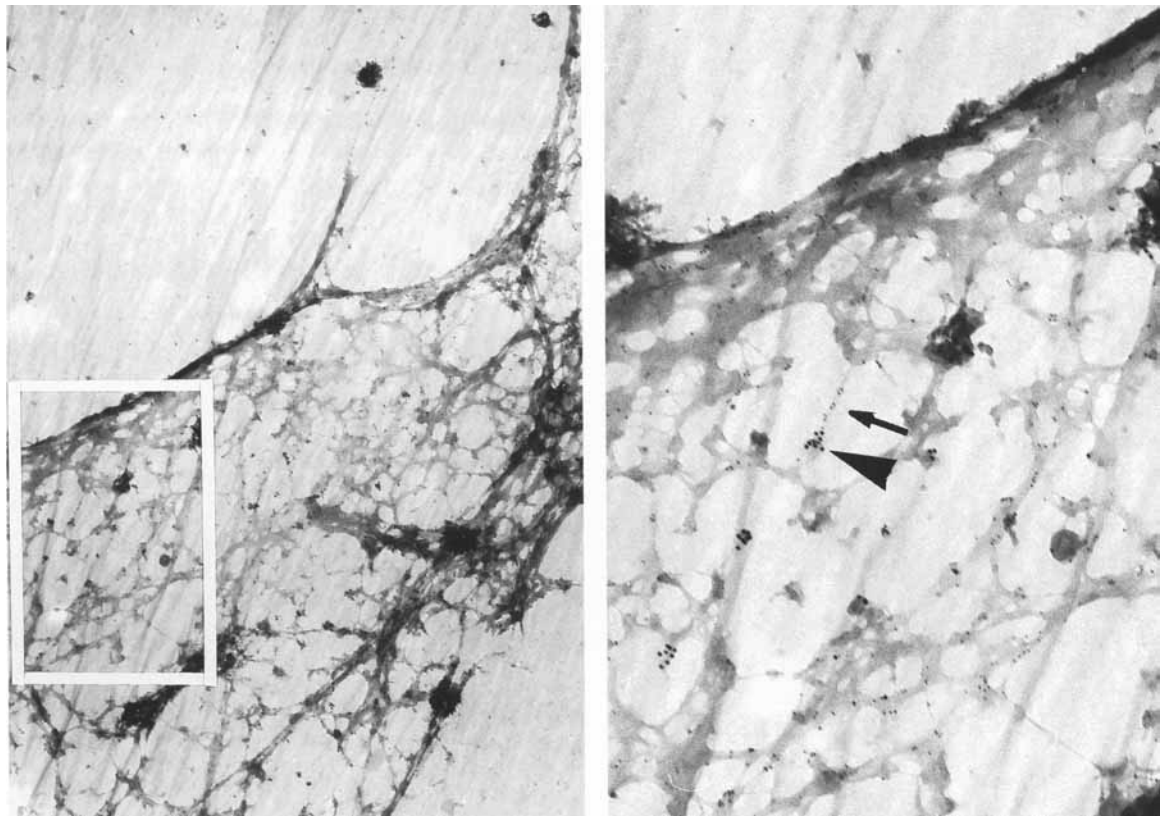

Fig. 2. Ultrastructural localization of actin mRNA A Triton-extracted fibroblast was hybridized with a biotinated β-actin cDNA probe and detected by 10 nm gold after a primary rabbit antibiotin antibody After hybridization, actin protein was detected by 5 nm gold anti-mouse after a monoclonal antibody to actin was used The leading edge of the cell is shown on the left and a magnified area (boxed) is shown on the right The arrow points to an actin filament approximately the thickness of 5 nm gold and the arrowhead points to an actin mRNA indicated by the cluster of eight 10 nm gold particles There are three other actin mRNAs nearby, illustrating the density of actin mRNA found within the leading edge They average a distance of 0 4 μm apart **Left,** ×10,000, **right,** ×35,000

secondary antibody, resulting in a tightly linked array of gold particles. By this approach, sequence specific hybridization could be both qualitatively and quantitatively distinguished from background noise, since nonspecific binding of probe or antibodies would be independent events and not expected to produce an iterative pattern of gold particles. In fact, this is what was observed experimentally. Extensive quantitative data were obtained to show that the actin mRNA hybridization signal in the form of these gold particle clusters was significantly above background noise. Gold particles were counted from samples hybridized with biotinated actin DNA probe or control biotinated pBR322 vector probe. As the number of gold particles in a cluster increased, progressively fewer clusters were observed with the control probe compared to the actin probe. This resulted in a proportional increase in signal to noise ratios (i.e., frequency for each size of gold particle cluster observed for the actin probe divided by the control probe), which began at 3:1 for single gold particles and increased to 30:1 for clusters of eight or more gold particles. When as many as eight particles are seen within a cluster, the probability was 97% that the hybridization signal represented actin mRNA. Figure 2 is illustrative of the clustered appearance of colloidal gold particles when actin mRNA molecules are visualized ultrastructurally. In addition, clusters of gold particles were observed not simply present in an aggregate, but often in a characteristic array with relatively constant spacing. The clusters were frequently present in spiral or circular conformations. This appearance was consistent with biotinated actin probe molecules hybridizing to a template, most likely a single mRNA molecule,

although an aggregate of more than one actin mRNA molecule could not be ruled out.

We then developed a double-label technology which could evaluate the spatial relationship of specific nucleic acids and their corresponding proteins using electron microscopy. This methodology enabled us to address the role of the specific cytoskeletal filaments in mRNA attachment. In this method, a monoclonal antibody to a cytoskeletal protein is incubated with the rabbit polyclonal antibiotin primary antibody. After washing the unbound primary antibodies, 5 nm gold labeled goat anti-mouse and 10 nm gold labeled goat anti-rabbit secondary antibodies were incubated simultaneously to complete the detection of mRNA and its corresponding protein.

Figure 2 provides an example of the codistribution of actin mRNA with microfilaments using a monoclonal antibody to actin. From this micrograph it is evident that actin protein (5 nm gold) is distributed throughout the area occupied by actin mRNA (10 nm gold). In order to evaluate the association of actin mRNA molecules with the three major filament systems, parallel samples were evaluated with antibodies to either tubulin or vimentin. The percentage of actin mRNA signals (detected by cluster of 10 nm gold particles) was quantitated which localized within 5 nm of a series of 5 nm gold particles (antibody labeled cytoskeletal filament). Using these criteria, the majority (88%) of all actin mRNAs examined were observed along microfilaments. Further evidence for interactions of actin mRNA with microfilaments has recently been obtained using drugs which perturb either microfilaments or microtubules. Low doses of the actin severing drug, cytochalasin-D, resulted in a dose dependent decrease in the number of cells which exhibit lamellipodial localization of β-actin mRNA [Sundell and Singer, 1991].

Is the actin cytoskeleton involved in the localization of other mRNAs to specific intracellular locations? Using probes for vimentin and tubulin mRNA in combination with antibodies for their cognate proteins revealed that these messages were not colocalized with their own corresponding proteins and also exhibited a tendency to be localized near actin filaments [Singer et al., 1989]. This research provided a high resolution approach to the investigation of mRNA-filament interactions which indicated involvement of microfilaments in the attachment of these mRNAs to the cytoskeleton. But how can microfilaments be involved in localizing different mRNAs to distinct intracellular locations, i.e., β-actin mRNA to the lamella and vimentin mRNA over the nucleus (see also Fulton, this volume)? One possibility is that there are proteins unique to actin in the lamella which anchor actin mRNAs and other microfilament specific proteins in the perinuclear cytoplasm which are selective for vimentin mRNA. The hypothesis to be tested is that mRNAs are not bound directly to the core microfilament, but rather to specific filament binding proteins which act as mRNP receptors within the actin cytoskeleton. Numerous actin binding proteins have been identified which assemble cytoplasmic actin into several structurally and functionally distinct arrangements [reviewed in Hartwig and Kwiatkowski, 1991]. Evidence for an involvement of actin binding proteins in interactions of mRNP with microfilaments has been observed in *Dictyostelium,* where ABP50, which crosslinks microfilaments, was shown to be elongation factor 1A [Yang et al., 1990] (see also Edmonds, this volume). However, it is not yet known whether distinct actin binding proteins are involved in localizing mRNAs to different sites within the actin cytoskeleton. Recent ultrastructural studies indicate that mRNA does not associate with the actin filament itself, but instead is distributed on subcompartments of the actin cytoskeleton, mainly where the filaments intersect (Bassell et al., submitted). Therefore, the different subcompartments of the filament may provide regional specificity for the localization of specific mRNAs.

Another possibility is that other filament systems are involved in transport, or anchoring. Evidence indicates that the actin cytoskeleton is not the only filament system which is involved in mRNA localization. In *Xenopus* oocytes, the localization of veg-1 RNA to the posterior pole was sensitive to perturbation of both microfilaments and microtubules [Yisraeli et al., 1990]. In *Drosophila* embryos, the localization of bicoid mRNA to the anterior pole appeared to require intact microtubules [Pokrywka and Stephenson, 1991]. We have recently applied high resolution fluorescence and ultrastructural in situ hybridization methods to test the hypothesis that multiple filament systems are involved in the attachment of mRNAs to the cytoskeleton. In our previous ultrastructural study, microfilaments appeared to be solely involved in the attachment of three different mRNAs to the cytoskeleton. However, it may be that other

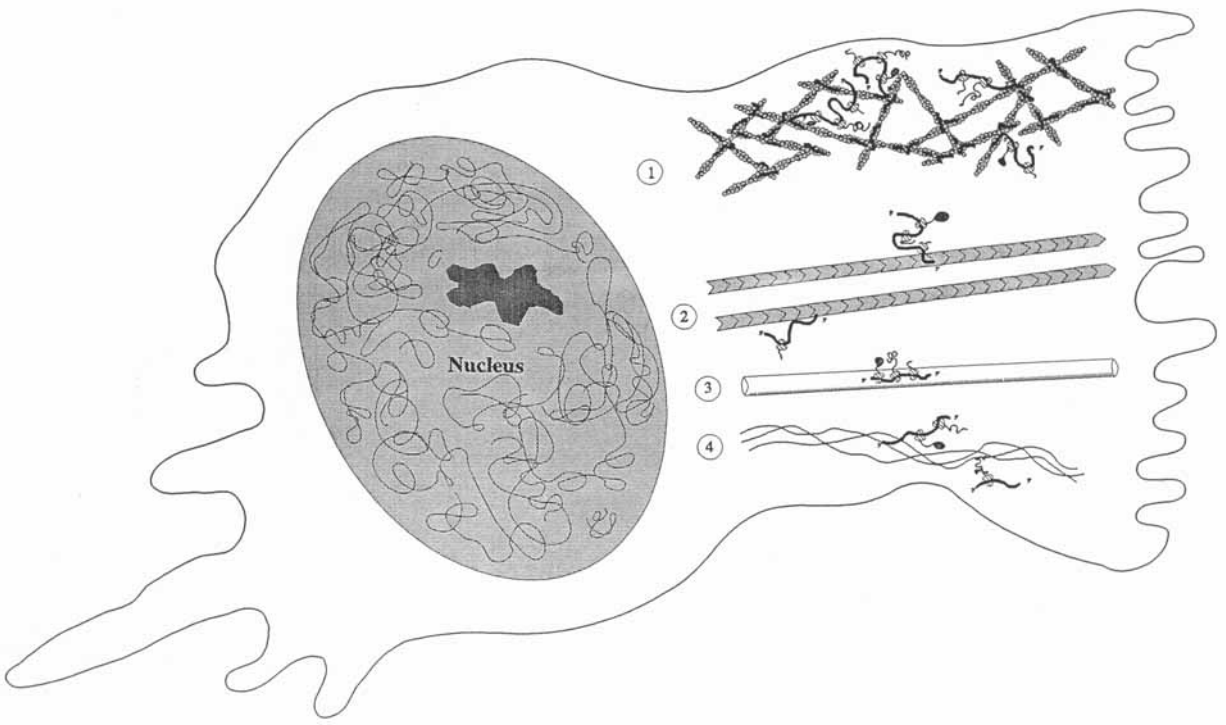

Fig. 3. Proposed mechanisms for intracellular compartmentalization of mRNA **1:** Localization of mRNA to microfilament subdomains Shown here are mRNAs at intersections of orthogonal actin networks **2:** Possible localization or treatment of mRNA on parallel microfilament bundles, e g , stress fibers **3:** Localization or transport of mRNAs on microtubules **4:** Localization of mRNAs to intermediate filaments

mRNAs utilize other filament systems for anchoring. Since most eukaryotic mRNAs are polyadenylated, this served as a convenient sequence to probe, permitting observations of a more general significance to cellular mRNA and allowing the relative involvement of each filament system to be evaluated.

Using quantitative double-label fluorescence microscopy, most poly(A) mRNA codistributed with the diffuse actin network. Two thirds of poly(A) mRNA was released from the cytoskeleton after cytochalasin treatment [Taneja et al., 1992]. Complete release of mRNA from the cytoskeleton has not been observed, even at high cytochalasin doses which dramatically altered the morphology of the cell, i.e., 50% at 10 μg/ml [Ornelles et al., 1986]. A fundamental question concerning these observations is whether the mRNA which is still retained on the cytoskeleton is bound to microfilaments which were not perturbed by cytochalasin treatment, or instead to another filament system. This issue can be resolved by ultrastructural examination of poly(A) mRNA-cytoskeletal associations using the double-label colloidal gold method. Our recent data indicate that a significant fraction of poly(A) is associated with filaments other than actin. Although microfilaments still contain the majority of poly(A) mRNA, significant interactions with intermediate filaments were observed (Bassel et al., submitted). Microtubules also had mRNA associated, albeit in minor amounts. We propose that multiple filament systems are utilized by a cell to sort mRNAs to distinct intracellular locations. Moreover, it is likely that filament-associated proteins and their subdomains within the cytoskeleton are the actual molecules involved in mRNA localization, rather than the filaments themselves. This hypothesis (see Fig. 3) suggests that at least a part of the addressing system for mRNA can be acheived through multiple interactions of mRNA with the cytoskeleton.

PERSPECTIVE

Investigation of the interaction of mRNA with the cytoskeleton by direct visualization has recently become an important analytical tool. The original observations of the cytoskeleton which illustrated mRNA attached to the cytoskeleton [Lenk et al., 1977] led to numerous biochemical studies showing that the majority of protein

synthesis is associated with the cytoskeleton [reviewed in Hesketh and Pryme, 1991]. However, the interaction of mRNA with the cytoskeleton has spatial implications impossible to understand through the exclusive use of biochemical manipulation. Our work continues efforts to characterize the mRNA-cytoskeletal interaction within a spatial context. This work has required the development of ultrastructural in situ hybridization methodology.

The identification of cytoskeletal mechanisms involved in mRNA localization may ultimately lead to a better understanding of the functional significance of mRNA sorting. One hypothesis is that the targeting of mRNAs to specific locations will allow proteins to be synthesized at or near their sites of function. For example, in neurons, MAP-2 is localized to dendritic microtubules and plays and important role in the establishment of dendritic architecture and polarity. The mechanism of MAP-2 localization involves the targeting of its mRNA [Garner et al., 1988]. To test whether the dendritic localization of MAP-2 mRNA is the essential determinant of protein localization will require identification of the molecular components involved in mRNA localization, their perturbation or mutation, and assessment of MAP-2 protein distribution. Similar approaches will need to be performed for other localized mRNAs.

A major challenge of future research on mRNA localization will be to determine whether this phenomenon functions to influence where the majority of proteins are synthesized within a cell, not just structural proteins. Since mRNAs which code for structural proteins are abundant, they are more amenable to in situ hybridization analysis. To resolve between these fundamentally distinct classes of proteins, it will be essential to evaluate whether mRNAs coding for non-cytoskeletal proteins are also localized to specific intracellular domains. For example, rough endoplasmic reticulum bound mRNAs have been shown to be associated with the cytoskeleton [Zambetti et al., 1990]. It will be interesting to learn whether mRNAs which code for integral membrane, secretory proteins, or mitochondrial proteins are localized to specific locations close to their final disposition and whether interactions of mRNP complexes with filament systems somehow mediate this organization. Nonetheless, the fact that poly(A) interacts with various filaments indicates that localization of mRNA can create cellular microenvironments of specific protein synthesis.

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