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Subcellular Localization of mRNA in Neuronal Cells

Contributions of High-Resolution In Situ *Hybridization Techniques*

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Abstract

The development of technologies for high-resolution nucleic acid localization in cells and tissues has contributed significantly to our understanding of transcriptional and translational regulation in eukaryotic cells. These methods include nonisotopic *in situ* hybridization methods for light and electron microscopy, and fluorescent tagging for the study of nucleic acid behavior in living cells. *In situ* hybridization to detect messenger RNA has led to the discovery that individual transcripts may be selectively targeted to particular subcellular domains. In the nervous system, certain species of mRNA have been localized in distal processes in nerve cells and glia. Direct visualization of mRNA and its interactions with subcellular features, such as synaptic specializations, cytoskeletal elements, and nuclear pores, have been achieved. Of particular interest is the presence of mRNA and ribosomes in dendrites, beneath synaptic contacts, suggesting the possibility of synaptic regulation of protein synthesis. The following article will describe the application of high-resolution *in situ* hybridization and live imaging techniques to the study of mRNA targeting in neurons.

Index Entries: Dendrites; synaptic plasticity; electron microscopy; dendritic spines; review.

Introduction

The development of high-resolution techniques for studying the localization of nucleic acid sequences has led to an expansion in our thinking about ways in which cells regulate and achieve protein targeting. As *in situ* hybridization methods became widely used, researchers began to observe that mRNAs encoding certain proteins were not randomly distributed, but occupied specific sites within the cell. In some cases, the message was found concentrated in regions where there was a high concentration of the protein it encoded (Lawrence and Singer, 1986; Fontaine et al., 1988; Singer et al., 1989; Goldman and Staple,

1989). For example, actin mRNA is concentrated in the lamellopodia of developing cells (Lawrence and Singer, 1986), whereas the message for the acetylcholine receptor is clustered beneath synaptic sites in muscle cells (Merlie and Sanes, 1985; Fontaine et al., 1988; Goldman and Staple, 1989). Thus, although protein packaging and transport remain a dominant method for protein trafficking, the cellular repertoire also includes the means of directing mRNA molecules to specific locations. Subcellular targeting of mRNA may be a means not only of protein targeting, but also of setting up protein synthesis domains subject to local regulation (Steward, 1995a).

Several early reports detailed the subcellular localization of specific transcripts in large cells, for example Vg1 in frog oocyte, *bicoid* in the *Drosophila* oocyte, and the acetylcholine receptor in skeletal muscle cells (Melton, 1987; Fontaine et al., 1988; Berleth et al., 1988; Goldman and Staple, 1989). These early observations were achieved because the spatial dimensions of these large cells permitted the resolution of differential labeling within the cell, even when using relatively low-resolution autoradiographic methods. As high-resolution nonisotopic methods for *in situ* hybridization have been developed, the number of reported instances of subcellular targeting has increased. These methods have been particularly valuable for investigating dendritic localization of messages in neuronal cells, where the complex organization of the neuropil in tissue sections makes interpretation of low-resolution autoradiograms problematic. It has become possible not only to observe mRNA localization with high precision, but also to visualize the interaction and relationship of mRNA with subcellular features, such as the cytoskeleton and nuclear pores, and to study the behavior of mRNA in living cells. These types of studies have provided insight into the mechanisms and significance of mRNA targeting in neurons and other cells. Several excellent reviews on the subcellular targeting of mRNA have been published (Steward and Banker, 1992; Wilhelm and Vale, 1993; Steward, 1995a; St. Johnston, 1995; Steward et al., 1996). The current article will provide an overview of the contribution of high-resolution *in situ* hybridization techniques to our understanding of subcellular targeting in neuronal cells.

Subcellular Targeting of mRNA in Neuronal Cells

Neurons have traditionally been divided into dendritic, somatal and axonal compartments, each with unique functional specializations (reviewed in Peters et al., 1991). The majority of protein synthetic machinery, including most of the Golgi apparatus and the rough endoplasmic reticulum, is localized within the soma. There is no clear morphological transition between the somal and dendritic compartments, although the amounts of both the Golgi apparatus and the rough endoplasmic reticulum (RER) gradually decrease with increasing distance from the nucleus. However, polyribosomes do exist in distal dendrites, sometimes in close association with membranous cisterns (Steward and Levy, 1982; Steward and Reeves, 1989). In contrast, a distinct transition zone, the axon initial segment, distinguishes the axonal compartment from the somtato/dendritic compartment. This zone is characterized by tight bundling of microtubules, the exclusion of RER and Golgi apparatus, and the presence of a dense plasmalemmal protein coat. Polyribosomes are present in the initial segment, but have not been described in the axon proper in most mammalian neurons.

In situ hybridization has confirmed that the majority of mRNA molecules are localized to the cell soma in neuronal cells, consistent with its role as the major trophic center of the cell. However, in both neurons and glia, a small but growing number of mRNAs have been localized to distal processes. Both in culture and in tissue, mRNAs have been detected not only in proximal dendrites, where mRNA might be expected owing to the presence of many of the same organelles found in the soma, but also in

distal dendrites. In certain neurons, mRNA is also found in axons.

Dendritic mRNAs have been described in a variety of neurons, both in vivo and in vitro, suggesting that dendritic mRNAs are a general neuronal feature. Within each cell type, at least some mRNAs appear to be stablely expressed in the dendrites of adult animals. These include the microtubule-associated protein MAP2 (Garner et al., 1988; Tucker et al., 1989), the α-subunit of the calcium/calmodulin-dependent protein kinase II (αCAMKII) (Burgin et al., 1990; Steward and Wallace, 1994; Steward, 1995b), the Type 1 IP3 receptor (Furuichi et al., 1993); the RNA polymerase III untranslated transcript BC1 (Tiedge et al., 1991), the olfactory mucosal protein OMP (Krishna et al., 1995), dendrin (Neunar-Jehle et al., 1996; Herb et al., 1997), and the α-subunit of the glycine receptor (Racca et al., 1997). Other messages may be present only transiently during development, as has been reported for the mRNA encoding Type 1 calmodulin (Berry and Brown, 1996) and protein kinase C (γ) (Moriya and Tanaka, 1994). Still other mRNAs can be induced in dendrites following stimulation or pathological insult, including *arc,* an activity-related transcript encoding a protein with some homology to spectrin (Link et al., 1995; Lyford et al., 1995) and the mRNA encoding HSC70, a member of the hsp70 multigene family of heat-shock proteins, which was induced in dendrites following an episode of hyperthermia (Foster and Brown, 1996). Biochemical studies on isolated dendrites or synaptosomes have suggested that the population of dendritic mRNAs may be larger (Rao and Steward, 1993; Chicurel et al., 1993; Miyashiro et al., 1994; Crino and Eberwine, 1996).

Although the presence of mRNAs within dendrites has been widely reported, a smaller number of studies have indicated that mRNA may be present in some axons as well (reviewed in (Van Minnen, 1994). In immature cultured neurons, poly $(A)^+$-containing mRNA is initally present within all developing neurites, but gradually disappears from the neurite destined to become an axon, as it begins to elongate and differentiate (Bassell et al., 1994). Focal concentrations of mRNAs may still be present within growth cones of differentiated axons (Bassell et al., 1994a; Olink-Coux and Hollenbeck, 1996) and at branch points (Olink-Coux and Hollenbeck, 1996). The mRNAfor at least one axonal protein, microtubule associated protein tau, has been observed to be concentrated at the axonal pole of cultured neurons and continues to be present in the axon initial segment in mature cultures (Litman et al., 1993; 1994; Behar et al., 1995). Whether the neurite destined to become an axon can be distinguished by a distinct complement of mRNAs prior to differentiation remains to be determined.

In the adult animal, mRNA has been described in axons in invertebrates and in certain specialized mammalian neurons in the hypothalamus/pituitary (Dirks et al., 1989; Guitteny and Bloch, 1989; Jirikowski et al., 1990; Mohr et al., 1991, Trembleau et al., 1994, 1995) and olfactory bulb (Krishna et al., 1995), but most *in situ* hybridization studies have not reported much axonal labeling in other areas of the brain. The absence of mRNA in most vertebrate axons is consistent with the lack of ribosomes reported in this compartment in ultrastructural studies (Peters et al., 1991) and the lack of protein synthesis reported for axons in culture systems (Torre and Steward, 1992). As the resolution and sensitivity of our detection systems improve, we may find additional instances of both ribosomal and mRNA localization in vertebrate axons. However, the considerably lower levels of mRNA and ribosomes in axons compared to somata/dendrites (Bruckenstein et al., 1990; Bassell et al., 1994a; Knowles et al., 1996) suggests that local protein synthesis in axons is minimal compared to the somatal and dendritic compartments. Consistent with the lack of demonstrated protein synthesis, a combined *in situ* hybridization–immunocytochemical study found little immunoreactivity for arginine vasopressin associated with focal concentrations of its mRNA in axonal swellings in the posterior pituitary (Trembleau et al., 1996). Some authors have suggested that perhaps mRNA is not translated in these axons, but

is temporarily stored in the axon away from protein synthetic components until it is needed (Mohr et al., 1995; Trembleau et al., 1996). Evidence that translational delay can occur has been provided in a study of the transcription factor GHF-1 in the pituitary where GHF-1 protein was not detected until 3 d after the mRNA was present, suggesting that posttranscriptional regulation of protein levels occurs (Dolle et al., 1990).

Glial cells, like neurons, must develop and maintain an extensive array of specialized processes at some distance from the perikarya. Not surprisingly then, targeting of mRNA has also been observed in glial cells. As is the case with neurons, the majority of protein synthesis in glial cells occurs in the perinuclear region (Gould and Mattingly, 1990), but protein synthetic components have been described in the fine processes of astrocytes, oligodendrocytes, and Schwann cells (Trapp et al., 1987; Gould and Mattingly, 1990; Peters et al., 1991; Ainger et al., 1993; Landry et al., 1994; Barbarese et al., 1995). In Schwann cells, both newly synthesized protein and mRNA were found within the cytoplasmic channel network stretching between the perinuclear region and distant paranode (Gould and Mattingly, 1990). The mRNA for myelin basic protein has been localized in fine, distal processes in oligodendrocytes, both in culture and in vivo (Trapp et al., 1987; Ainger et al., 1993; Landry et al., 1994; Barbarese et al., 1995). Similarly, the message for the astrocytic protein GFAP has been described in astrocytic processes (Sarthy et al., 1989; Erickson et al., 1992; Landry et al., 1994). Poly $(A)^+$-containing mRNA was localized at the ultrastructural level to astrocytic profiles surrounding synaptic structures in the hippocampal neuropil (Martone et al., 1996).

High-Resolution *In Situ* Hybridization Detection

Most early studies on mRNA targeting in neuronal cells used autoradiographic methods of detection. Although the sensitivity of isotopic methods has yet to be surpassed, the relatively poor spatial resolution restricted these studies to the use of cultured neurons, where the processes of individual neurons are well separated from the cell bodies or to brain regions with a distinct laminar organization. In laminar regions like the cerebellar cortex and hippocampus, the dendrites and somata of the principal cells occupy different strata, and silver grains of the exposed emulsion overlying dendritic regions can be distinguished from those over somata. Although high-resolution detection is possible with autoradiographic methods, particularly when ^3H is used as the label, these methods are time-consuming (they may takes months to develop) and the level of resolution is still not equal to nonradioactive methods. The lack of resolution stems from the nature of the autoradiogram: the radioactive particle can interact with the film overlying a certain radius surrounding the site of decay, depending on the energy of the particle and the thickness of the emulsion. Fine-level, detailed analyses of the neuropil in these preparations require statistical methods to correlate silver grains with particular subcellular structures (Williams, 1977).

The development of nonradioactive techniques employing biotinylated or digoxygenin-labeled probes has greatly advanced in the past few years. A variety of detection methods are available for these types of probes, imparting flexibity to labeling schemes and the ability to achieve double-labeling *in situ* hybridization (Bassell et al., 1994b; Trembleau et al., 1995) or combined *in situ* hybridization and immunocytochemistry with relative ease (Singer et al., 1989; Bassell, 1993; Trembleau et al., 1996; Racca et al., 1997). Although the sensitivity of these techniques has not reached the level of the isotopic methods, they are sufficiently sensitive and reliable to be able to detect moderately abundant messages. One drawback of nonisotopic methods, particularly those based on enzymatic detection, is that they are not as amenable to quantitative analysis as are the isotopic methods. However, if accessibility of tar-

get sequences is ensured, e.g., by using highly extracted preparations, some quantitation is possible with colloidal gold-based methods (Singer et al., 1989; Bassell et al., 1994b).

Using nonradioactive detection, a variety of protocols have now been developed for high-resolution light and electron microscopic detection. Electron microscopic studies have been particularly valuable in determining the patterns of mRNA in complex tissues like neuropil, and for examining the relationship of mRNA with other subcellular features, such as synaptic contacts. Detailed protocols can be found in Morel (1993), and a survey of some recent methods used for light and electron microscopic study of mRNA is provided in Table 1. Most protocols generally employ oligonucleotide or cDNA probes, because the high-stringency conditions necessary for the use of riboprobes result in excessive degradation of ultrastructure. However, riboprobes have been used in postembedding methods where tissues are first embedded in epoxy resin, thin-sectioned, and then subjected to hybridization conditions (Wenderoth and Eisenberg, 1991; Dorries et al., 1993). Detection methods include enzymatic reactions using either peroxidase or alkaline phosphatase, fluorescence detection, or colloidal gold, with or without silver intensification. Each protocol has its advantages and disadvantages, in terms of sensitivity and resolution. Of these methods, the digoxygenin-alkaline phosphatase detection system is reported to be the most sensitive for light microscopy (Trembleau and Bloom, 1995; personal observations), although most substrates for alkaline phosphatase are not suitable for electron microscopic analysis. Under favorable conditions, single mRNA molecule visualization is possible as concluded from statistical analyses of colloidal gold labeling patterns in electron micrographs of highly extracted cells (Singer et al., 1989; Bassell et al., 1994b). The greater amount of noise present in intact cells or tissue sections has precluded similar resolution in these preparations.

In our own laboratory, we have developed several methods for electron microscopic and correlated light and electron microscopic detection of nucleic acid sequences (Pollock et al., 1990; Deerinck et al., 1994; Martone et al., 1996). We and others have found that the use of ultracryosections combined with colloidal gold detection provides a fairly sensitive method for localizing individual messages ultrastructurally (Wenderoth and Eisenberg, 1991; Le Guellec et al., 1992). This method is particularly useful when combined with high-voltage electron microscopy (HVEM). By using HVEM, it is possible to examine 5–25 times thicker sections than is possible with a conventional electron microscope (1–5 μm for HVEM vs 0.05–0.2 μm for conventional microscopes). The thicker specimens offer two advantages: first, they are easier to prepare and survive the often harsh pretreatments better than do thinner sections, and second, they allow for a greater number of mRNA molecules to be contained within a single section. This latter point is important for lower-abundance messages where only a few molecules might be contained within a single thin section. Using this method, Pollock and coworkers (1990) successfully localized the mutant transcript *chaoptic* in the photoreceptors of the fly eye and were able to demonstrate that abberant localization correlated with a defect in rhabdomere organization. We were also able to gain some information on dendritic localization of CAMKII isoforms using this approach (Martone et al., 1996).

Although the use of ultracryosections has proven to be advantageous in terms of sensitivity, there are some problems associated with this method (Wenderoth and Eisenberg, 1991; Le Guellec et al., 1992). First, adequate ultrastructural morphology can be difficult to achieve, especially in delicate tissue, such as neuropil. Ultracryosections also suffer from an inherent lack of contrast compared to conventionally prepared sections. This lack of contrast is a particular problem when attempting to interpret staining patterns in the neuropil with high-voltage electron microscopy. Thus, in our study of CAMKII in hippocampal dendrites, we found that although preservation and interpretation of staining patterns in the perikarya

Table 1
Some Recent Protocols for High-Resolution Nonradioactive Nucleic Acid Detection[a]

Study	Target message	Tissue	Method	Probe	LM	EM	IC-ISH	Resolution
Erickson et al. (1992)	GFAP	Retina	Postembedding gold	cDNA-b	No	Yes	No	Good
Bassell et al. (1994a,b; Bassell, 1993)	Poly (A)$^+$	Cultured cells	Postembedding gold	Oligo-b	No	Yes	Yes	Excellent
Huang et al. (1994), Deerinck et al. (1994)	Poly (A)$^+$ mRNA	Cultured cells	Photo-oxidation	Oligo-b	Yes	Yes	No	Excellent
Knowles et al. (1996)	RNA	Cultured neurons	SYTO vital dye	n/a	Yes	Yes	Yes	Good
Martone et al. (1996)	Poly (A)$^+$ mRNA, CAMKII isoforms	Hippocampus	Pre-embedding peroxidase, fluorescence, ultracryosection/ gold	Oligo-bd	Yes	Yes	No	Fair–good
Pollock et al. (1990)	Chaoptic	Fly eye	Ultracryosection/ gold	cDNA-b	No	Yes	No	Excellent
Racca et al. (1997)	Glycine receptor, α-subunit	Spinal cord	Peroxidase, fluorescence, SIG	Oligo-d	Yes	Yes	Yes	Fair-good
Trembleau et al. (1994, 1996), Trembleau and Bloom (1995)	Vasopressin, tyrosine hydroxylase, galanin, others	Hypothalamus	Pre-embedding perocidase	Multiple oligos-b	Yes	Yes	Yes	Fair-good

[a] A sampling of protocols developed recently for high-resolution *in situ* hybridization, mostly in neuronal tissue. All of these protocols involve electron microscopic (EM) detection and some are suitable for light microscopic (LM) visualization as well. Three types of protocols are distinguished: pre-embedding where labeling is done prior to embedding in epoxy resin for electron microscopy; ultracryosection where labeling is performed on ultrathin cryosections prior to embedding for electron microscopy; and postembedding, where sections are first embedded in resin, thin-sectioned, and then labeling is performed. The type of probe (oligo = synthetic oligonucleotide probe) as well as the type of tag (b = biotin, d = digoxygenin) is identified and whether or not immunocytochemistry (IC) was performed along with the *in situ* hybridization (IC-ISH). The determination of resolution is subjective, but takes into account the diffusibility of the detection label and the preservation of ultrastructure.

and large dendrites were possible, distinguishing fine dendritic processes and other structures in the neuropil was very difficult. Fortunately, pre-embedding protocols have been developed that overcome some of the difficulties of the ultracryosection approach. By using a three-step detection protocol developed by McQuaid and Allan (1992), involving successive layering of biotinylated reagents and streptavidin–peroxidase, we were able to obtain a sufficiently sensitive signal to localize poly $(A)^+$-containing mRNA in hippocampal dendrites using both fluorescence detection for confocal microscopy and enzymatic detection with peroxidase-3, 3'-diaminobenzidine (DAB) for electron micoscopy (Fig. 1). More recently, Racca et al. (1997) described the ultrastructural localization of the message for the α glycine receptor in spinal motor neurons, using both enzymatic and silver-intensified colloidal gold detection. Thus, although not yet routine, non-radioactive electron microscopic detection is becoming feasible for localizing dendritic messages at the ultrastructural level.

A third promising experimental approach developed in our laboratory involves fluorescence *in situ* hybridization using detection with the fluorophore eosin, a brominated derivative of fluorescein, followed by photo-oxidation for electron microscopic analysis (Deerinck et al., 1994). When a fluorophore is excited in the presence of DAB, the DAB is oxidized into an insoluble polymer, which can be rendered electron-dense using osmium tetroxide (Maranto, 1982). The fluorescence output of eosin is 20% lower than that of fluorescein, making it less desirable as a fluorophore for most applications, but its quantum yield of singlet oxygen is 20× greater than fluorescein, making it an excellent oxidizer of DAB. Like the pre-embedding peroxidase technique, this method is suitable for correlated light and electron microscopy, but unlike the peroxidase-based methods, it does not appear to be as subject to diffusion artifacts, resulting in excellent resolution. Using biotinylated oligonucleotide probes, followed by a streptavidin–eosin conjugate, Huang et al. (1994) were able to visualize mRNA tracking

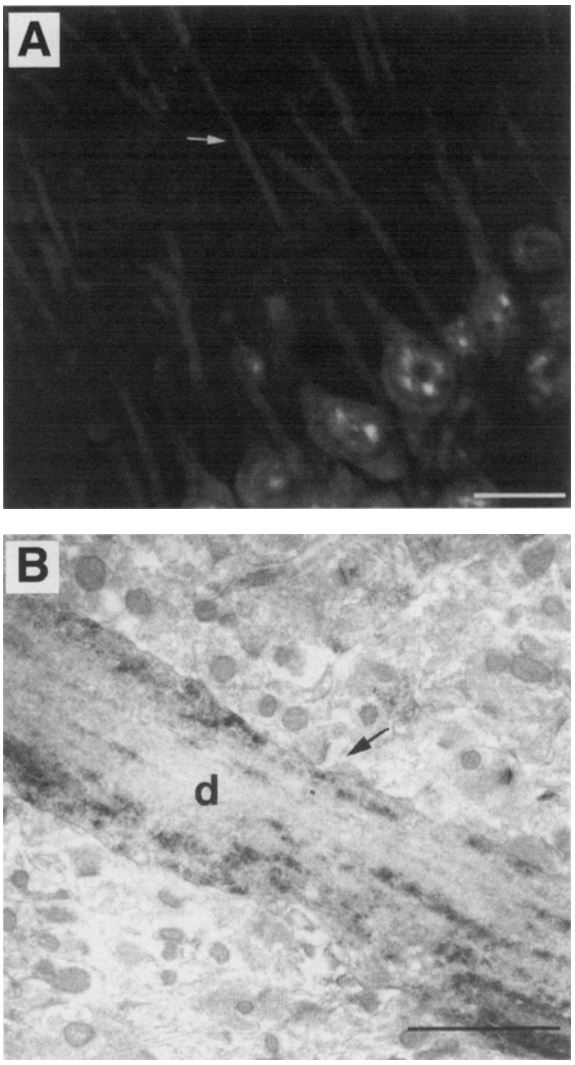

Fig. 1. Distribution of poly $(A)^+$ mRNA in area CA1 of adult rat hippocampus at the light **(A)** and electron microscopic **(B)** levels. (A) Fluorescence *in situ* hybridization for Poly $(A)^+$ mRNA localized in pyramidal neurons using a biotinylated oligonucleotide probe and FITC. Messenger RNA extended into apical dendrites (arrow) for 100–200 μm. (B) Electron micrograph of apical dendrite (d) of a CA1 pyramidal cell labeled using a pre-embedding peroxidase technique. The arrow points to a small, unlabeled dendritic spine. The DAB reaction product was distributed in a longitudinal pattern in proximal dendrites. Additional details can be found in (Martone et al., 1996). Scale bar in A = 20 μm; in B = 2 μm.

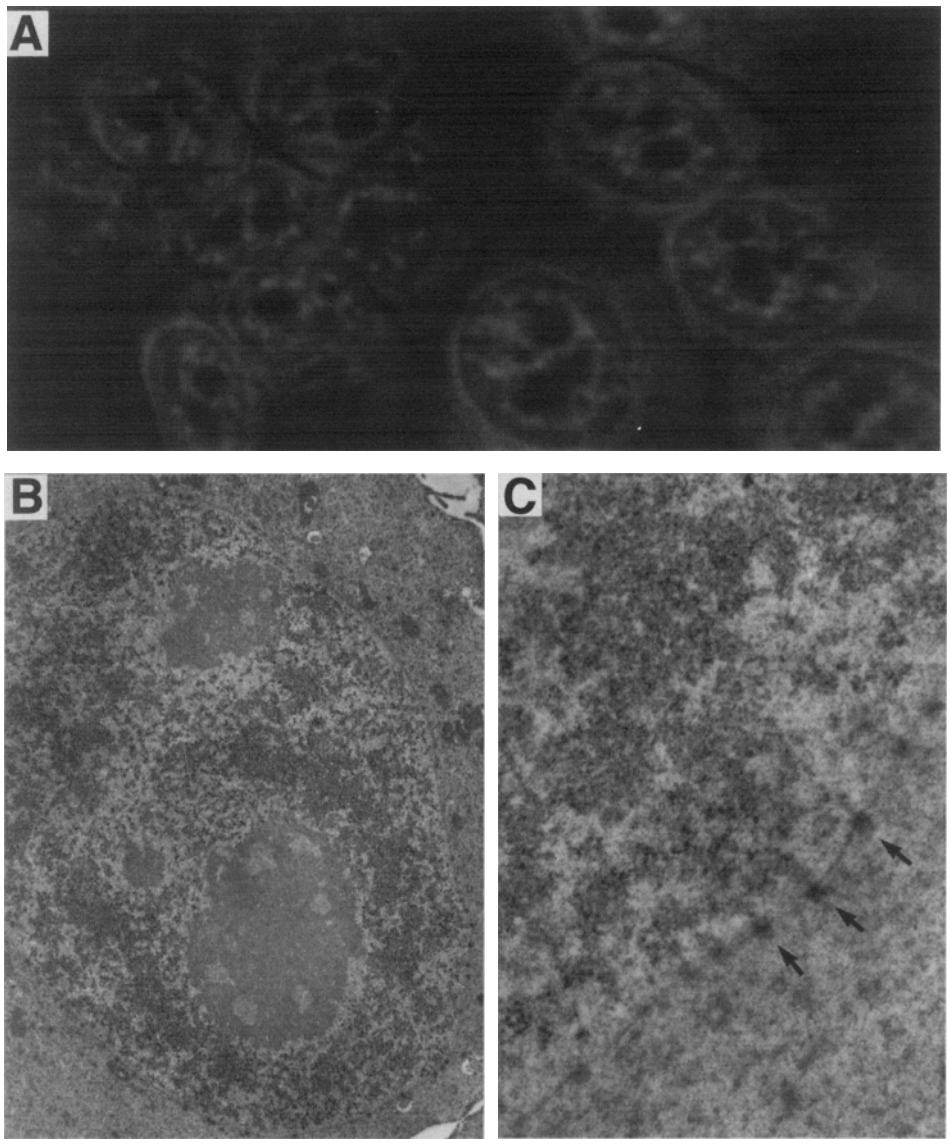

Fig. 2. *In situ* hybridization for poly $(A)^+$ mRNA in cultured HeLa cells using fluorescence detection with eosin-conjugated reagents **(A)** followed by photo-oxidation and subsequence electron microscopic analysis (**B** and **C**). (A) A light microscopic image derived from a series of optical sections showing the prominent speckled pattern of mRNA in the nucleus. (B) Electron micrograph of poly $(A)^+$ localization in the nucleus. (C) Higher-magnification electron micrograph showing the association of mRNA with the nuclear pores (arrows). Streams of mRNA can be seen contacting the nuclear envelope and concentrating on the cytoplasmic side of the nuclear pore. Additional details can be found in (Deerinck et al., 1994; Huang et al., 1994).

from the nucleus out through the nuclear pores in cultured HeLa cells (Fig. 2). Unfortunately, this technique is not particularly sensitive. Although we have used this protocol on neural tissue, we were only able to photoconvert the highly fluorescent poly $(A)^+$-containing focal domains within the nucleus of hippocampal cells (unpublished observations). Because of its

superior resolution and utility for correlated light and electron microscopy, we are working to improve the sensitivity, both at the chemical level by seeking more potent producers of singlet oxygen and at the detection level by the incorporation of additional amplification schemes (van Gijlswijk et al., 1997).

Localization of mRNA Within Dendrites

Early studies indicated that transport of mRNA into dendrites is an active process (Davis et al., 1987). Direct visualization of mRNA transport has been achieved using live imaging of fluorescently labeled mRNA, both in glia (Ainger et al., 1993; Barbarese et al., 1995) and neurons (Knowles et al., 1996). This has been accomplished both by fluorescent tagging of individual mRNA molecules that are then microinjected into cultured cells (Ainger et al., 1993; Barbarese et al., 1995) and by the use of RNA-specific dyes, such as SYTO (Molecular Probes, Inc.), which are compatible with live imaging (Knowles et al., 1996). All of these studies reported that mRNA is transported in the form of granules, ~0.3–0.8 μm in diameter, and that the transport of these granules could be disrupted by microtubule depolymerizing agents. In the case of neurons, RNA granules were initially present in all developing neurites, but were excluded from axons once they began to elongate (Knowles et al., 1996). Ribosomal RNA, elongation factors, tRNAs, and aminoactyl tRNA synthetase were shown to be present within a subset of granules as well (Barbarese et al., 1995; Knowles et al., 1996). In collaboration with Roger Knowles and Ken Kosik, we were able to show that some of the granules corresponded to discrete clusters of ribosomes that were not membrane-bound (Knowles et al., 1996). These results indicate that mRNA may be shipped to distal locations as part of a macromolecular protein synthetic complex, containing all of the machinery required for translation. Whether or not mRNAs are transported as parts of macromolecular complexes in intact tissue is unknown, but *in situ* hybridization studies have consistently reported spotty labeling of mRNAs (Olink-Coux and Hollenbeck, 1996; Martone et al., 1996; Racca et al., 1997). Kobayashi et al. (1991) also reported that the dendritically localized RNA BC1 was extracted from tissue as part of a 10S ribonucleoprotein particle.

A body of evidence has accumulated that at least one signal determining whether a given mRNA is targeted is present within the mRNA molecule itself, in the 3′ untranslated region (3′UTR). Translation of the targeted mRNA does not appear to be necessary for correct targeting to occur in the majority of cases (Yisraeli and Melton, 1988). Deletion of sequences in the 3′UTR in targeted mRNAs led to the lack of subcellular localization (MacDonald and Struhl, 1988; Davis and Ish-Horowicz, 1991; Mowry and Melton, 1992; Behar et al., 1995; Veyrune et al., 1996; Mayford et al., 1996). This work has been carried out primarily in embryonic cells but the 3′UTR has also been implicated in targeting in neuronal cells as well (Behar et al., 1995; Mayford et al., 1996). Mayford and coworkers (1996) showed that the 3′UTR from αCAMKII was sufficient to cause the dendritic localization of a nonlocalized transcript in transfected cultured neurons and transgenic animals. Analyses of the sequences of 3′UTRs of targeted mRNAs have failed to find a common sequence among these molecules (MacDonald, 1990; Mayford et al., 1996). However, in a detailed analysis of 3′UTRs from *bicoid* homologs from different species of *Drosophila*, MacDonald (1990) determined that despite the lack of extensive sequence homology, all of the 3′UTRs were capable of forming similar secondary stem-loop structures. Thus, the three-dimensional structure of the UTR rather than a specific sequence may determine whether or not a given transcript is transported. Recent evidence suggests that specific RNA binding proteins may also be involved in mediating the association of mRNA with microtubules (Elisha et al., 1995), which are involved in localization of mRNA (*see* the following).

Numerous studies have concluded that microtubules are particularly important for

subcellular localization of mRNA. These studies have consistently shown that disruption of microtubules using depolymerizing drugs results in a diffuse distribution of mRNAs that were normally selectively localized in both neural and nonneuronal cells (Pokrywka and Stephenson, 1991; Mowry and Melton, 1992; Ainger et al., 1993; Bassell et al., 1994a; Litman et al., 1994; Knowles et al., 1996). In support of these studies, high-resolution studies in neural cells have shown a direct association between mRNA molecules and microtubules in cultured cells with the electron microscope (Bassell et al., 1994a) and with tubulin-labeled structures in the confocal microscope (Ainger et al., 1993). In tissue sections, we observed that labeling for poly $(A)^+$ mRNA was distributed longitudinally within proximal dendrites (Fig. 1B), a pattern consistent with an association between mRNA and microtubules, which also run longitidually within dendrites (Peters et al., 1991). The role of microfilaments in mRNA transport and localization is less clear, but an association of individual mRNAs with actin filaments has been reported in nonneuronal cells using EM level *in situ* hybridization (Singer et al., 1989; Bassell et al., 1994b).

Although a critical role of the 3'UTR and the cytoskeleton in subcellular mRNA targeting appears to be well established, the complex patterns of mRNA localization in neuronal cells indicates that additional cellular factors likely come into play in determining the ultimate localization of a given mRNA. Steward and Wallace (1994) have shown that different mRNAs can occupy distinct sites within dendrites: MAP2 mRNA is most concentrated in the inner third of the molecular layer of the dentate gyrus, whereas CAMKII mRNA is more evenly distributed throughout the dendrite. Because the localization of MAP2 mRNA corresponded to the afferent input from the commisural/hilar fiber input, these authors proposed that perhaps afferent influences were modulating the localization of mRNA, although lesioning experiments of hippocampal pathways did not indicate that dennervation disrupts mRNA localization (Steward, 1995b). Third, both *in situ* hybridization studies of cultured neurons (Kleiman et al., 1994) and analysis of amplified mRNAs from single, aspirated dendrites (Miyashiro et al., 1994) indicate that not all neurites from the same cell may have the same mRNA profile, suggesting that differential stabilization or transport of mRNA may occur in different dendrites. Finally, the same mRNA can have a different subcellular distribution depending on the cell type. This observation was first made by Landry et al. (1994), who localized the mRNA for MAP2 in dendrites in many forebrain neurons, but not in some brainstem neurons. A more recent study by Paradies and Steward (1997) showed that even some mRNAs that were thought to be strictly localized in the cell body, e.g., neurofilament protein 68kd subunit, can exhibit extensive dendritic localization in specific neuronal types, in this case, neurons of the lateral vestibular nucleus. These authors concluded that "there are no general rules for mRNA localization that apply to all neuron types."

Although the above studies suggest that multiple cellular influences on mRNA localization exist, their nature is unknown. Behar and coworkers (1995) have purified RNA binding proteins that showed specific binding to sequences in the 3'UTR of the tau mRNA that contained the localization signal. In *Drosophila*, St. Johnston and colleagues (1995) have identified at least three stages involved in the localization of bicoid mRNA, each requiring additional genes. Thus, trans-acting signals may exist that differ across cell types or subcellular regions to impart specificity in the localization of a particular mRNA. In addition, interaction with the cytoskeleton through the 3'UTR may not be the only mechanism of localization. Mohr et al. (1995) reported that a point mutation in the coding region was sufficient to eliminate the axonal localization of the arginine-vasopressin mRNA in the Brattleboro rat, suggesting that this mRNA may be transported along with its nascent protein. Several authors have also raised the possibility that mRNA may be selectively transported through

a subset of nuclear pores, implying that directed transport from the nucleus could determine some aspects of mRNA localization (Pollock et al., 1990; Davis and Ish-Horowicz, 1991). Consistent with the idea of vectorial transport, patchy labeling of RER domains has been noted in *Drosophila* (Pollock et al., 1990) and rat hypothalamic neurons (Trembleau et al., 1994; Trembleau and Bloom, 1996). Trembleau and Bloom (1996) observed that the mRNAs for a G-protein subunit and vasopressin each occupied distinct domains of RER in the same cell.

In summary, neurons likely utilize a variety of mechanisms to orchestrate the distribution of mRNA molecules within neuronal compartments. These mechanisms may involve both *cis*- and *trans*-acting factors, and combine ways to direct transport to and/or restrict diffusion away from certain cellular regions (Paradies, and Steward, 1997). Additional local signals may be present, which "fine-tune" the localization of different mRNAs in a given dendrite. Detailed electron microscopic analysis of mRNAs, which are differentially distributed in dendites, e.g., MAP2 and CAMKII, may reveal unique association of these mRNAs that may provide clues to structural solutions utilized by neurons to ensure proper targeting of mRNA species.

Why Target mRNA?

The subcellular targeting of mRNA molecules offers several potential advantages to a cell (*see* Wilhelm and Vale, 1993; St. Johnston, 1995 for more extensive discussion). Obviously, the targeting of mRNA to subcellular domains is an efficient way to obtain a high protein concentration at a particular site. The transport of relatively few mRNA molecules that can be translated again and again is more energy efficient than the transport of large amounts of protein (Wilhelm and Vale, 1993). Although this mode of protein targeting would appear to be advantageous to a cell, it is curious that in the case of neuronal mRNAs, only a small proportion of mRNAs show specific targeting to the dendrite. For example, several dendritic proteins, including, most strikingly, those encoding neurotransmitter receptors and various ion channels, are more concentrated in the cell body than in the dendrites, indicating that protein targeting is sufficient in most cases for maintaining established cell domains. At least one reason for restricting mRNAs to the cell body is the need for further processing by the Golgi apparatus and rough endoplasmic reticulum. Because these organelles are by and large restricted to cell bodies and proximal dendrites, proteins that require posttranslational modification in these structures, e.g., integral membrane proteins, will be by necessity restricted to the cell body (but *see below*). The relatively small number of mRNAs that show targeting suggests that their localization may confer some other advantage to the cell beside transport efficiency.

A specific role for restricted mRNA localization has been demonstrated most conclusively in embryogenesis, where the targeting of mRNA to particular regions of the cell appears to be an important mechanism for establishing cellular polarity. In oocytes, focal concentrations of mRNA at opposite poles act as point sources for setting up protein gradients important for the specification of the anterior–posterior axis of the animal (reviewed in St. Johnston, 1993). These protein gradients then activate the transcription of subordinate genes in a concentration-dependent fashion (MacDonald, 1990). Thus, in *Xenopus* oocytes, the selective localization to the vegetal pole of the mRNA Vg1, a maternal mRNA encoding for a member of the transforming growth factor-β family, and localization of bicoid mRNA to the anterior pole and nanos mRNA to the posterior pole in *Drosophila* are required for proper axis specification in both these animals (Melton, 1987; Berleth et al., 1988; MacDonald and Struhl, 1988).

The contribution of mRNA localization to the development and differentiation of somatic cells like neurons has been less well studied. The distributions of several mRNAs in neurons indicate that restriction of mRNAs to

axonal and dendritic poles may contribute to the development and/or maintenance of neuronal polarity. Dendrites and axons can be distinguished by several criteria, including the differential distribution of certain microtubule-associated proteins: MAP2 is found predominantly in dendrites, whereas tau is localized to axons (reviewed in Matus, 1988; Ginzburg, 1991). As described previously, their mRNAs show a similar segregation: tau mRNA is concentrated in the axonal pole, whereas MAP2 mRNA is present in dendrites (Garner et al., 1988; Tucker et al., 1989; Bruckenstein et al., 1990; Litman et al., 1993, 1994, Kleiman et al., 1994, Behar et al., 1995). Further evidence for a role for local protein synthesis in neuronal development comes from the observation that polyribosomes are particularly numerous during times when active synaptogenesis is occuring, e.g., during development and following deafferentation (Steward and Fass, 1983; Steward and Falk, 1986).

In mature cells, the functional significance of selective mRNA targeting is more conjectural, although several hypotheses have been advanced. Several authors have noted that subcellular mRNA targeting allows for the assembly of proteins at the site of synthesis (cotranslational assembly). Such site-selective synthesis would ensure that proteins that spontaneously self-assemble, such as certain cytoskeletal proteins, are utilized in the correct location (reviewed in Wilhelm and Vale, 1993). Both MAP2 and αCAMKII, two mRNAs that are selectively targeted in neurons, are multifunctional proteins that can interact with a large number of cellular constituents. Their local synthesis may ensure that they do not interact with other proteins in inappropriate regions of the cell. Similarly, myelin basic protein is a highly charged, reactive protein that is very rapidly inserted into membranes, causing their compaction. Translation at the specific site of incorporation may be necessary to ensure that the protein does not incorporate into membranes enroute to its ultimate destination in the myelin sheath (St. Johnston, 1995).

Perhaps the most interesting aspect of subcellular targeting of mRNA in differentiated cells, at least from the point of view of neuroscientists, is the possibility for local regulation of protein synthesis in response to extracellular signals. Structural and biochemical changes at postsynaptic sites are hypothesized to be major mechanisms by which synaptic strength is modified as a result of synaptic transmission (reviewed in Weiler et al., 1994, 1995). The presence of protein synthetic machinery within dendrites leads naturally to the hypothesis that local protein synthesis may occur in response to synaptic transmission, allowing for the modification or growth of individual synapses (Steward and Banker, 1992; Weiler et al., 1994; Steward, 1995a). That dendritic mRNAs are influenced by stimulation has been shown in several studies. In the dentate gyrus, dendritic levels of MAP2 mRNAs have been reported to be increased in dendrites following application of NMDA or nitric oxide-releasing agents (Johnston and Morris, 1994). Stimulation of the perforant pathway also induced the dendritic expression of an mRNA termed *arc* that is normally not localized within dendrites (Link et al., 1995; Lyford et al., 1995). A study by Thomas et al. (1994) found persistent increases in mRNA for αCAMKII following induction of long-term potentiation (LTP) (however, *see*, Steward and Wallace, 1994). A recent study by Knowles and Kosik (1997) showed increased translocation of SYTO 14-labeled granules into distal dendrites of cultured cortical neurons in response to neurotrophin-3 application. This effect was blocked by the application of a tyrosine kinase inhibitor K252a.

Of course, a consideration of the functional significance of dendritic mRNA hinges on the question of whether these mRNAs are translated into protein. Unlike the case for axons (*see* earlier discussion), a growing number of studies support the idea that local dendritic protein synthesis occurs, including the demonstration that new proteins are produced in dendrites isolated from their cell bodies in culture (Torre and Steward, 1992, 1996; Crino and Eberwine, 1996), in synaptosomal preparations

(Rao and Steward, 1991; Weiler and Greenough, 1991, 1993) and in dendritic fields in slice preparations (Phillips et al., 1987; Feig and Lipton, 1993). In a clever study by Mayford et al. (1996), a transgenic mouse was created in which the 3'UTR from CAMKII was used to drive the *lac*Z transcript. The transgene also contained a nuclear localization signal, so that protein transcribed within the cell body would be rapidly transported to the nucleus. In these animals, spotty βgal activity was observed within distal dendrites, suggesting that some protein was transcribed within the dendrites themselves. Thus, although not conclusive, converging lines of evidence support the idea that translation does occur in distal processes in intact tissue.

mRNA and Synaptic Sites

The hypothesis that dendritically targeted mRNAs are involved in activity regulated protein synthesis has been bolstered by morphological studies showing that both ribosomes (Steward and Levy, 1982; Steward and Falk, 1986; Steward and Ribak, 1986) and mRNA (Martone et al., 1996; Racca et al., 1997) are present beneath synaptic sites. Extensive studies on the distribution of polyribosomes in neurons by Steward and colleagues indicated that they were selectively localized to the base of dendritic spines and beneath synaptic sites on the dendrite and axon initial segment (Steward and Levy, 1982; Steward and Falk, 1986; Steward and Ribak, 1986). More recently, we investigated the localization of poly $(A)^+$ mRNA relative to synaptic sites and dendritic spines in the adult rat hippocampus. Although the number of dendritic spines examined was relatively small, we found an elevated concentration of mRNA in the dendritic shaft near to the base of almost every dendritic spine observed emanating from the dendritic shaft (Fig. 3A). Labeling was also observed consistently beneath slight bulges in the plasmalemma. Steward and Levy (1982) estimated that approx 70% of ribosomes detected were similarly found beneath these "mounds" in the dentate gyrus. Their analysis of serial sections confirmed that many of these mounds marked bases of spines out of the plane of section. Because spine numbers can change rapidly even in the adult animal (Woolley et al., 1990), some of the labeled "mounds" could represent sites of developing spines.

An interesting finding in our study was the presence of labeling for poly$(A)^+$ mRNA within the dendritic spines themselves. Labeled spines were observed in the neuropil and also in continuity with the dendritic shaft, although not all spines with reaction product at their base were labeled. Three-dimensional analyses of dendritic spines in various brain regions indicated that polyribosomes are also present within a sizable portion of spines (Steward and Reeves, 1988; Spacek, 1985; Spacek and Harris, 1997). In addition, Chicurel et al. (1993) suggested that some mRNAs, such as GAP43 and BC1, may be specifically localized within spines based on an analysis of synaptosomal preparations.

Labeling for poly $(A)^+$ mRNA was not restricted to neuronal structures within the neuropil, but was also present in glial processes, some in close association with synaptic structures (Fig. 3B). Glial processes have been shown to undergo dynamic regulation in synaptic plasticity paradigms (Sirevaag and Greenough, 1991; Wenzel et al., 1991) and Steward and colleagues have found that GFAP mRNA levels are upregulated by neuronal activity (Steward and Wallace, 1994). Thus, the presence of glial mRNA surrounding synaptic sites suggests that local protein synthetic events in astrocytes could also influence synaptic plasticity. At the very least, the presence of mRNA in glial processes surrounding synaptic complexes points to the need for caution in interpreting biochemical results based on isolated synaptosomes and the interpretation of autoradiograms detecting protein synthetic events in the neuropil. These biochemical or low-resolution techniques may allow for confusion of postsynaptic and glial components of the signal.

An intriguing observation in both our study (Martone et al., 1996) and that of Racca and

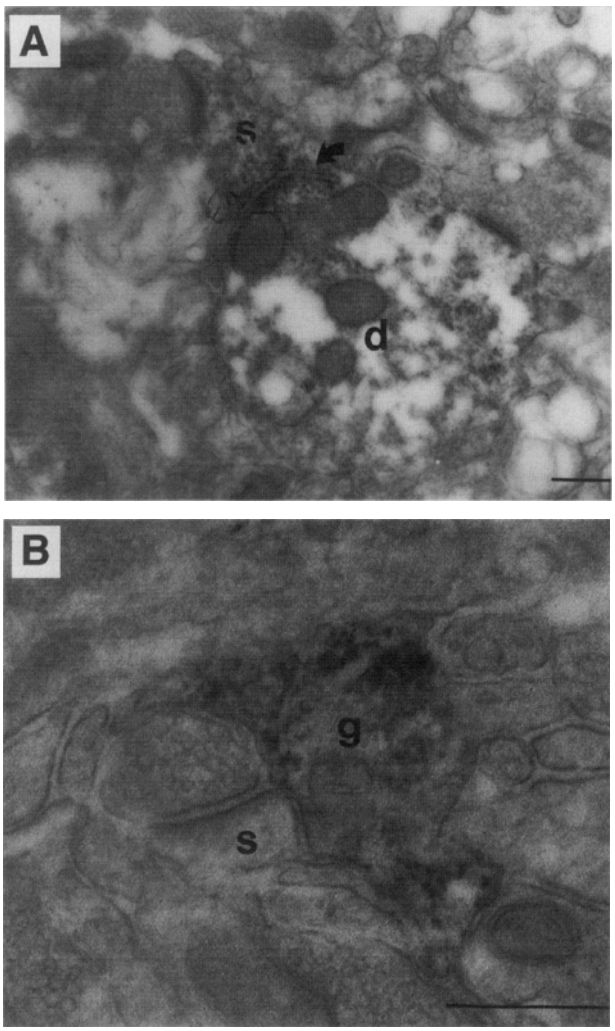

Fig. 3. The localization of mRNA in the hippocampal neuropil at the electron microscopic level. (A) Cross-section of a dendrite (d) with a large spine(s) attached. Note the intesely labeled cistern of endoplasmic reticulum at the base of the spine (curved arrow). A synapse onto the dendritic shaft does not show an accumulation of label at its base. (B) mRNA localization in glial process (g) surrounding a synaptic contact onto an isolated spine(s). Additional details can be found in (Martone, et al., 1996). Scale bars = 500 nm.

coworkers (1997) was the presence of intensely labeled cisterns of endoplasmic reticulum, sometimes at the base of dendritic spines (Fig. 3A) or beneath postsynaptic specializations on the dendritic shaft. No ribosomes were identified in these studies, but Steward and Reeves (1988) noted that almost half of the polyribosomes at the base of dendritic spines were juxtaposed to membrane cisterns reminiscient of RER. The possibility of RER in dendrites is interesting, because it suggests that some post-translational modification of proteins is possible at sites distant from the cell body. The presence of dendritic RER may explain why the mRNA for some integral membrane proteins has been observed in dendrites. In addition to the glycine receptor, Miyashiro and colleagues (1994), using highly sensitive amplification methods in isolated dendrites, provided evidence that mRNAs for some glutamate receptor isoforms are found within dendrites, although others have not been able to localize such transcripts in synaptosomes (Chicurel et al., 1993). In another study, Furuichi et al. (1993) described strong labeling for the Type 1 IP3 receptor, a calcium release channel localized to the smooth endoplasmic reticulum, within the dendrites of Purkinje neurons. No evidence for the presence of Golgi apparatus has yet been provided for dendrites, although some have suggested that the spine apparatus may be involved in posttranslational modification of proteins (Spacek, 1985; Steward and Reeves, 1988, Gordon-Weeks, 1988). A recent study by Torre and Steward (1996) showed that glycosylation of proteins does occur in dendrites isolated from the cell body in culture. This study also indicated that some of the enzymes necessary for posttranslational modification were present in proximal dendrites, but not in distal dendrites (*see also* discussion in Racca et al., 1997).

Conclusions

The application of high-resolution *in situ* hybridization has allowed the visualization of mRNA processes within both live and fixed cells. The presence of protein synthetic machinery near post-synaptic sites in neuronal dendrites has raised the interesting possibility that activity-dependent protein synthesis is

involved in generating synaptic plasticity (Kang and Schuman, 1996). Analysis of the distribution of mRNA and ribosomes in dendrites indicates that they are located near and in dendritic spines and in glial processes surrounding synaptic complexes. Both dendritic spines and glial processes are noteworthy for their dynamic responses to experimental or environmental manipulation: dendritic spines can rapidly change in shape and number (Woolley et al., 1990; Harris and Kater, 1994; Weiler et al., 1995) and glial processes change their extent of association with neuronal surfaces (Wenzel et al., 1991). Because protein synthetic machinery is most common in dendrites during times of active growth, the presence of local protein synthetic machinery in spines, at the base of spines, and in glial processes may indicate that these components drive the dynamic changes in size, shape, and number characteristic of these structures, even in mature neurons. A role for local protein synthesis in spine maturation has been proposed recently based on an analysis of the fragile X protein. A mutation in the gene for this protein is responsible for fragile X syndrome, a common form of inherited mental retardation (Weiler et al., 1997; Comery et al., 1997). Dendrites in knockout mice deprived of this gene exhibited a spine morphology characteristic of immature or experience-deprived synapses (Comery et al., 1997), implying that this protein is required for normal maturation of dendritic spines. A study of synapto-neural preparations suggested that the fragile X mRNA is present in dendrites and is translated in response to synaptic stimulation (Weiler et al., 1997).

The regulation of protein synthesis is ultimately controlled by the ribosomes and the factors that regulate their activity. The studies of mRNA transport in vitro have suggested that dendritic mRNAs are transported as parts of macromolecular complexes containing ribosomes and other protein synthetic components, such as elongation factors. Some of these factors, such as eEf-2, are sensitive to changes in calcium and phosphorylation (Nygard et al., 1991). Nilsson and Nygard (1995) have recently reported the surprising finding that the capacity to phosphorylate and thereby inactivate eEF-2 is highest when the need for protein synthesis is greatest. Inhibition of the elongation cycle of the ribosome stimulates translation of certain mRNAs (Ryazanov et al., 1991), suggesting that the calcium-dependent phosphorylation of eEF-2 causes a translational advantage. We are intrigued by the possibility that regionally distributed mRNAs with ribosomes poised could respond to local fluxes of Ca^{2+} and changes in phosphorylation induced by synaptic activation, so that new proteins are made on demand at the synapse where they are needed, but the rest of the dendrite is unaffected. Such local protein synthetic domains could be an important determinant of synaptic plasticity, both in the developing and adult animal (Kang and Schuman, 1996; Frey and Morris, 1997). Now that the groundwork has been laid, protocols for combined live imaging and ultrastructural analysis will likely yield important new insights into the control and significance of mRNA targeting in neuronal cells.

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