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Effect of Cytoarchitecture on the Transport and Localization of Protein Synthetic Machinery

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Abstract The emerging picture of cytoarchitecture imposes constraints on the transport and localization of several components of the protein synthetic machinery. The range in which "free" polysomes can diffuse through the cytoplasm may be restricted to about 50 nm due to obstruction by cytoskeletal barriers. Individual ribosomes and large transcripts will diffuse at least 4–10 times slower in cytoplasm than in dilute aqueous solution and may be sterically excluded from some cytoplasmic domains. The transport of these components from the nucleus to the cell periphery may be restricted to microtubule-containing channels that traverse the excluding domains. In the peripheral cytoplasm, mitochondria, endoplasmic reticulum, and other membrane-bound organelles are found only in nonexcluding channels, while actin, nonmuscle filamin (ABP280), and fodrin are concentrated in excluding domains. This suggests that the cytoplasmic volume may be functionally compartmentalized by local differentiations of cytoarchitecture. Excluding compartments may play a structural role, while nonexcluding compartments are the site of vesicle traffic and protein synthesis.

Key words: cytoplasm, excluding compartments, tracer particles, FRAP data, polysomes

The discovery of site-specific translation demands an explanation of how mRNAs arrive at distant targets. Although several recent papers have examined the mechanism of export of transcripts from the nucleus and the signal sequences required for localization to specific sites in the cytoplasm, relatively little is known about the mechanism of transport once the transcript leaves the nucleus [for recent reviews see Agutter, 1991; Hesketh and Pryme, 1991]. Transcripts encoding membrane and secretory proteins most likely encounter ribosomes and elements of the endoplasmic reticulum in the vicinity of the nuclear pore from which they exit. Some of these transcripts might then be carried into the peripheral cytoplasm by active transport of the endoplasmic reticulum along microtubules [Lee et al., 1989; Vale and Hotani, 1988; Terasaki et al., 1991]. The situation differs for transcripts that are not translated on membrane-bound polysomes. It is often tacitly assumed that these transcripts rely on diffusion for transport to the site of their eventual translation, but the possibility of active transport has not been ruled out. Several features of cytoplasmic organization may be instructive to those who address this question.

Because enzyme activities generally are measured in dilute assay systems, it is tempting to regard the cytoplasm as a dilute, aqueous solution in which diffusion-limited reactions are the norm. Yet the intracellular concentration of many enzymes may be as much as 10 μM [Srere, 1967] and the total concentration of protein in cytoplasm is on the order of 200–300 mg/ml [Lanni et al., 1985]. If even half that amount of protein is "soluble," the cytoplasm is a crowded solution where diffusion of a macromolecule may be hindered by hydrodynamic and excluded volume effects from its neighbors [Minton, 1990]. Additionally, a variety of morphological and biochemical evidence indicates that cytoplasm is a viscoelastic gel network made up of crosslinked actin filaments [e.g., Wolosewick and Porter, 1979; Oster and Perelson, 1987; Stossel, 1989; Janmey et al., 1990; Zahalak et al., 1990; Janson et al., 1992]. The extent of this network, its pore size, and its dynamics could have profound implications for the diffusive transport of macromole-

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cules within the cytoplasmic volume. In the past several years, my laboratory has been engaged in characterizing the architecture of the cytoplasmic gel network in living cells. The results impose some unexpected constraints on the mechanism of transport of transcripts to the site of translation.

EXISTENCE AND MEAN PORE SIZE OF A CYTOPLASMIC GEL NETWORK IN LIVING CELLS

Plotting the relative diffusion coefficient of tracer particles as a function of particle size is a common biophysical approach to characterizing the architecture of complex matrices [e.g., Sellen, 1987; Poitevin and Wahl, 1988; Newman et al., 1989]. We have used fluorescence recovery after photobleaching (FRAP) to measure the cytoplasmic diffusion coefficient of size-fractionated, inert fluorescent particles that have been microinjected into living tissue culture cells [Luby-Phelps et al., 1986, 1987]. The Stokes-Einstein relation predicts that in a simple, aqueous solution, the diffusion coefficient of the tracer relative to its diffusion coefficient in water will be a constant function of particle size, equal to the inverse relative viscosity of the solution. Instead, the relative diffusion coefficient of tracer particles in cytoplasm is a steeply decreasing function of particle size [Luby-Phelps et al., 1987]. The simplest nontrivial explanation for this is the presence of a network of obstructions that hinders diffusion by hydrodynamic and steric effects. In the case of a statically crosslinked network, long-range diffusion in the cytoplasm would be impossible for nondeformable particles larger than the pore size of the network. By linear extrapolation of the data to a relative diffusion coefficient of zero, we estimate that the mean pore size of the cytoplasmic network would be 26 nm in radius or 52 nm in diameter. So far, we have been unable to test this hypothesis directly due to the unavailability of suitable test particles.

Existing theoretical treatments of diffusion in complex media do not allow us to extract a more detailed model for cytoarchitecture from these data. As an alternative, we have studied the diffusion of our tracer particles in model systems of defined geometry in attempt to reconstitute this behavior in vitro. The results show that the hindered diffusion of tracers in cytoplasm cannot be accounted for by a very high concentration of soluble globular proteins or by

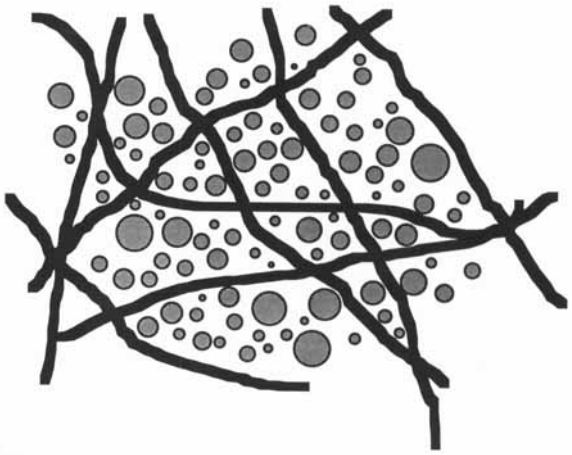

Fig. 1. Schematic diagram of a model for cytoarchitecture derived from tracer diffusion experiments in living tissue culture cells. The data are consistent with an entangled network of long filaments in a background solution containing protein-size particles. The model predicts a filament volume fraction of 0.11 and particle concentration of 12.4 g/100 ml.

an uncrosslinked network of entangled filaments [Hou et al., 1990]. We find that the diffusion of tracers in cytoplasm qualitatively resembles the diffusion of tracers through a network of entangled filaments interpenetrated by a crowded solution of protein-size, spherical particles (Fig. 1). By curve fitting and extrapolation of the model data to filament concentrations unattainable in vitro, we can obtain quantitative agreement with the data from living cells [Hou et al., 1990]. The filament concentrations required are far higher than the combined concentrations of all biochemically characterized cytoskeletal filaments, although the volume fraction of filaments required is very similar to the volume fraction reportedly occupied by the microtrabecular lattice [Gershon et al., 1985; Hou et al., 1990]. We are testing several other model systems in which the filaments are noncovalently crosslinked to see if we can obtain better agreement with our in vivo data.

FUNCTIONAL DIFFERENTIATION OF CYTOARCHITECTURE

The FRAP results described above represent an average of data taken from several positions in the cytoplasm of each of a large number of cells. Closer examination of the data reveals that the architecture of the cytoplasm is not homogeneous. A significant immobile component appears in the averaged FRAP data for tracer particles greater than 14–18 nm in radius (28–36 nm in diameter), and some individual data re-

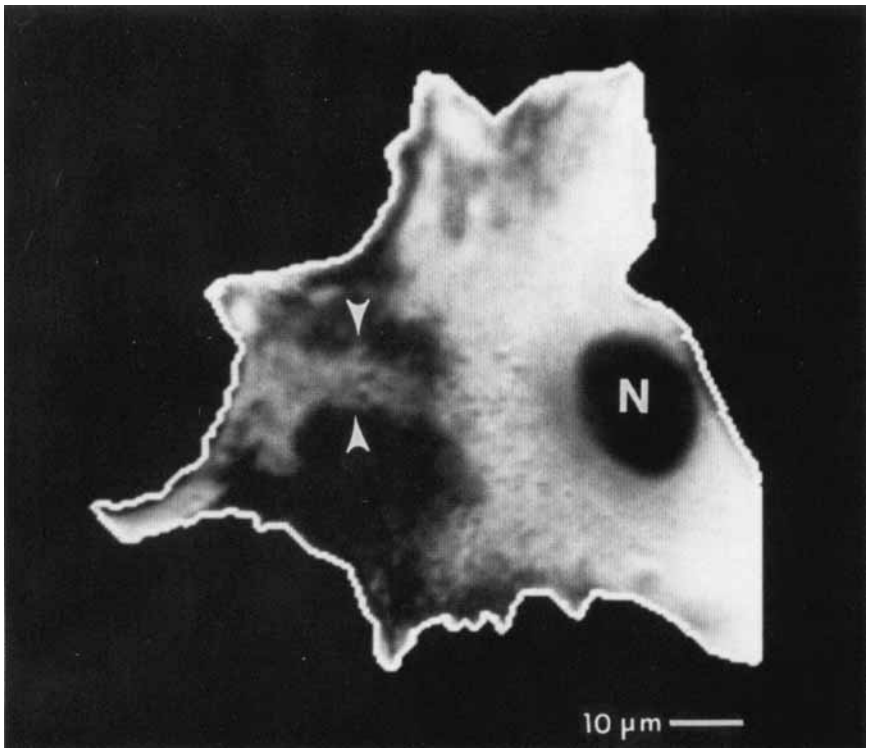

Fig. 2. Functional compartmentalization of the cytoplasmic volume Fluorescence ratio image of the relative distribution of 24 nm and 3 nm tracer particles in a living cell Regions of low intensity are cytoplasmic compartments from which particles ≥13 nm in radius are excluded These compartments are traversed by channels that contain both sizes of tracer particle (arrowheads) Multiparameter fluorescence microscopy and electron microscopy show that mitochondria, endoplasmic reticulum, vesicles, intermediate filaments, and microtubules are found only in these non-excluding channels Actin, ABP280, and fodrin are relatively concentrated in excluding compartments The edge of the cell is outlined in white N = nucleus

cords show no recovery at all, suggesting that a portion of the tracer is trapped in subdomains of the cytoplasm [Luby-Phelps et al., 1986; Luby-Phelps and Taylor, 1988]. Visual confirmation of this is provided by fluorescence ratio imaging of the distribution of large tracer particles relative to small in single living cells. We have found that sizable expanses of the peripheral cytoplasm exclude particles greater than or equal to 26 nm in diameter [Luby-Phelps and Taylor, 1988; Provance et al., submitted]. The excluding compartments are penetrated or traversed by channels of nonexcluding cytoplasm radiating from the cell center (Fig. 2). Islands containing large tracer particles are sometimes found completely surrounded by excluding cytoplasm.

The leading edge of a migrating cell is one prominent example of an excluding compartment [Luby-Phelps and Taylor, 1988]. Since it is well established that the leading lamellipodium is occupied by a three-dimensional network of actin filaments [Abercrombie et al., 1971; Small et al., 1982; Hartwig and Shevlin, 1986], one likely mechanism for the exclusion of tracer from excluding compartments is inability to percolate through an actin-based cytoplasmic network of finite pore size. Although excluding compartments generally are thinner than adjacent nonexcluding regions, we have demonstrated that close apposition of the upper and lower cell cortices is not the mechanism by which large tracer particles are restricted from entering excluding compartments [Provance et al., submitted]. Electron microscopy of carefully prepared whole mounts shows that excluding compartments are readily distinguishable from adjacent nonexcluding compartments by the higher crosslink (or overlap) density of the cytoplasmic filament network. Stereomorphometry shows that the mean pore diameter of the network in excluding domains is 31 nm, comparable to the percolation cutoff of 26 nm measured by fluorescence ratio imaging. In contrast, the pore size in adjacent nonexcluding domains is sufficient to allow free diffusion of particles up to 51 nm in diameter [Provance et al., submitted]. This is

very similar to the mean pore size of 52 nm estimated from the FRAP data [Luby-Phelps et al., 1987].

Immunofluorescence localization shows that filamentous actin, nonmuscle filamin (ABP280) [Provance et al., submitted], and fodrin (unpublished observation) are concentrated in the excluding compartments of nonmotile cells. ABP280 is an ubiquitous protein that crosslinks actin filaments into an isotropic network [Niederman et al., 1983]. Fodrin is the nonerythroid form of spectrin and most likely links actin to the plasma membrane [Hartwig and Kwiatkowski, 1991]. The concentration of these three proteins in excluding compartments suggests that an actin-ABP280 gel network, linked to the cell membrane by fodrin, may be responsible for exclusion. Since networks formed by actin and ABP280 have a rigidity and elasticity similar to covalent networks [Janmey et al., 1990], one function of excluding compartments may be to maintain the spread morphology of adherent cells and to provide mechanical resistance to deformation. Preliminary observations suggest that excluding compartments are remnants of lamellipodia extended during cell spreading. In well-spread interphase cells, they appear to be static differentiations of the cytoarchitecture, remaining virtually unchanged in morphology over several hours of intermittent observation.

Like the leading lamellipodium of a migrating cell [Abercrombie et al., 1971], excluding compartments are virtually free of recognizable organelles. Simultaneous labeling with a specific fluorescent vital dye shows that mitochondria are excluded from the cytoplasmic compartments that exclude large tracer particles [Luby-Phelps and Taylor, 1988]. By electron microscopy it can be seen that endoplasmic reticulum, microtubules, mitochondria, and vesicles are confined to central regions of the cell and to the same islands and channels in which large tracer particles are found [Provance et al., submitted]. Immunofluorescence localization of tubulin confirms the impression that microtubules are generally lacking in excluding domains, although the boundary between an excluding and a nonexcluding domain often is marked by a microtubule. A bundle of microtubules usually courses down the middle of a nonexcluding channel. These morphological observations suggest that vesicle traffic and membrane-bound protein synthesis occur primarily, if not exclusively, in nonexcluding domains and channels. In fibroblast cell lines such as SW3T3 and CV-1, the distribution of vimentin is similar to tubulin. It is rare or absent in excluding compartments, ruling out intermediate filaments as structural elements of cytoarchitecture in excluding domains.

IMPLICATIONS OF CYTOARCHITECTURE FOR THE TRANSPORT AND LOCALIZATION OF PROTEIN SYNTHETIC MACHINERY

The transport of small molecules, including tRNAs, to the site of protein synthesis will be largely unobstructed by the cytoplasmic gel network and will depend more heavily on the viscosity of the fluid that interpenetrates the network and crowdedness of this solution. Many reports in the literature have suggested that cytoplasmic solvent viscosity is considerably higher than water [for review see Cameron et al., 1988; Clegg, 1988]. However, results from our laboratory and a recent report from another laboratory indicate that the solvent viscosity of cytoplasm is not significantly different from water [Fushimi and Verkman, 1991; Luby-Phelps, et al., in press]. Even if the diffusion of ions and small molecules were retarded by as much as 2- to 3-fold due to elevated solvent viscosity and crowding (Kao et al., 1993), tRNA could still sample the entire cytoplasmic volume within seconds of leaving the nucleus.

In contrast, the data for diffusion of tracer particles in cytoplasm suggest that the diffusion of ribosomes and polysomes would be severely hindered by the cytoarchitecture. The particle size of a mammalian cytoplasmic ribosome is 26 × 32 nm [Verschoor and Frank, 1990]. A tracer particle this size diffuses about 10 times slower in cytoplasm than in dilute solution [Luby-Phelps et al., 1987]. In a static gel network, long-range diffusion of polysomes would be possible only for the completely extended configuration and would be extremely slow. Neither individual ribosomes nor polysomes would be small enough to diffuse into excluding compartments, raising the possibility that protein synthesis might be restricted entirely to nonexcluding compartments. Inspection of published micrographs showing immunofluorescence localization of ribosomal proteins and fluorescent labeling of rRNA supports the idea that ribosomes in the peripheral cytoplasm are primarily, if not exclusively, localized in channels radiating from the perinuclear region [Fulton et al., 1980; Hesketh and Pryme, 1991; Terasaki, 1991], although the identity of these channels

with non-excluding channels has not been established. The fact that the mRNA for actin has been found proximal to, but excluded from, the leading lamellipodia of migrating cells, which is an excluding compartment [Sundell and Singer, 1990], is consistent with our observations, although there is no evidence yet to show that the actin message is being translated in this location.

The exact effect of the gel network on the diffusion and localization of mRNAs will depend on the configuration of the transcripts and the form in which they are packaged. Little is yet understood about the secondary structure of mRNA, although this is an active area of research. Stem-loop structures due to local, intramolecular base pairing have been demonstrated at the 5′ and the 3′ ends of several transcripts, but opinions differ on the contribution of longer-range interactions to secondary structure. Assuming that the average transcript is largely a random flight molecule, the mean external diameter ($\bar{X}$) of the molecule can be calculated according to the equation, $\bar{X} = (8nb^2/3\pi)^{1/2}$, where n is the number of bases and b is the root mean squared (RMS) length of each base [Casassa, 1985]. Taking the length of a base to be 7 Å [Filson and Bloomfield, 1968], a naked 1 kb transcript would have a diameter of 20.3 nm, causing it to diffuse about 6 times slower in cytoplasm than in water [Luby-Phelps et al., 1987]. Transcripts ≥ 1.6 kb would be unable to enter excluding compartments. According to the Einstein relation[1] it would take a 1 kb transcript 6 min to diffuse 50 μm from the nucleus. The existence of multiple stem-loop structures and long-range base pairing might either increase or decrease the diffusion coefficient of the molecule depending on whether they stabilize a more extended or a more compact configuration.

Many transcripts appear to be stored in the cytoplasm as prosomes until they are needed for translation. Prosomes are small ribonucleoprotein particles (RNPs), measuring 12 × 17 nm [Coux et al., 1992]. These particles should diffuse about 5 times slower in the cytoplasm than in water, taking about 4 min to travel 50 μm.

Recent electron microscopic evidence suggests that secretory mRNPs emerging from a nuclear pore immediately become associated with ribosomes [Mehlin et al., 1992]. If this is also true for transcripts destined for translation at distant sites in the cytoplasm, active transport to these sites would be a necessity, since polysomes are unlikely to percolate through a cytoplasmic network with a pore diameter of 52 nm.

[1]The Einstein relation states that $\bar{x} = (2Dt)^{1/2}$ when $\bar{x}$ is the RMS displacement of a Brownian particle, D is the diffusion coefficient, and t is the time in seconds.

IS mRNA ACTIVELY TRANSPORTED?

A mRNA too large to diffuse into excluding compartments could simply be "shepherded" to its eventual binding site by diffusion down non-excluding channels. Alternatively, the message may be actively transported to its target in direct or indirect association with cytoskeleton. Agutter [1991] has suggested that mRNA becomes associated with the cytoskeleton immediately upon exiting the nucleus and is transported to distant sites without ever entering a soluble compartment. It also has been suggested that all polysomes become associated with the endoplasmic reticulum in the perinuclear region and that cytoplasmic polysomes are transported with the endoplasmic reticulum and then "dropped off" at appropriate sites in the peripheral cytoplasm [Branes and Pogo, 1975]. This mechanism recently has been proposed for localization of the bicoid transcript in *Drosophila* embryos [Pollock et al., 1990].

There is evidence in the literature to suggest that at least some mRNAs are actively transported. Davis and colleagues [1987] have shown that transport of dendrite-specific messages from the soma into the dendrites of hippocampal neurons in culture is energy requiring. The apparent diffusion coefficient of these messages calculated from the RMS displacement of ^{3}H-uridine label 5 h after a pulse is 3×10^{-9} cm^2/s [Davis et al., 1990], comparable to the cytoplasmic diffusion coefficient of a fluorescent tracer particle 23 nm in radius [Luby-Phelps et al., 1987]. However, the RMS displacement of labeled message as a function of time is constant and cannot be modeled by diffusion kinetics [Davis et al., 1990].

Some remarkable micrographs from Singer's laboratory suggest that the β-actin message also is actively transported. In tissue culture cells transfected with a cDNA containing a β-galactosidase reporter sequence and the 3′ end of the actin gene, an occasional cell shows reaction product extending from the nucleus down an organelle-containing channel toward the cell periphery [Kislauskis and Singer, 1992, personal communication]. If the transcript were freely diffusible, a reaction product should have been

present in the entire perinuclear region and all nonexcluding channels.

The possible role of the cytoskeleton in the transport of mRNA is indicated indirectly by several sets of experimental data. Somatodendritic transport of message is blocked by nocodazole, which depolymerizes microtubules [Davis et al., 1987]. Treatment of neurons with cytochalasin, a drug that disrupts the actin cytoskeleton, results in randomization of message. This most likely reflects the role of microfilaments in anchoring the message at the site of localization [for recent review see Hesketh and Pryme, 1991], but could also be due to disassembly of the actin ABP280 network in excluding domains. Similar results were obtained in *Xenopus* oocytes, where it was found that microtubule poisons inhibit localization of the Vg1 message to the cell cortex during the early stages of oocyte maturation, while cytochalasin B treatment of late stage oocytes resulted in randomization of already localized message [Yisraeli et al., 1990]. In bicaudal *Drosophila* embryos, a mutation in a gene that encodes a myosin-like protein results in aberrant localization of the posteriorizing morphogen, *nanos* [Wharton and Struhl, 1989]. Since the mRNA for *nanos* colocalizes with its gene product, one possible explanation for the redistribution of *nanos* in the bicaudal mutant is that a myosin-like motor is responsible for active transport of the *nanos* message to its correct position in the embryo.

FUTURE DIRECTIONS

Several missing pieces of data prevent a definitive description of how cytoarchitecture affects the transport and localization of the protein synthetic machinery. For example, better understanding of whether mRNAs can undergo long-range diffusion in the cytoplasm or percolate into excluding compartments requires a clearer picture of the form and size of the particle containing the transcript. This question could also be addressed directly in intact living cells. It has been shown that naked transcripts encoding Vg1 become correctly localized following microinjection into the *Xenopus* oocytes [Yisraeli and Melton, 1988]. If fluorescently labeled transcripts for specific proteins, such as β-actin, also become correctly localized when microinjected into living tissue culture cells, the diffusion of the transcript could be studied by FRAP and its subcellular localization with respect to excluding compartments could be observed by digital fluorescence imaging microscopy.

Even if transcripts are small enough to diffuse freely throughout the cytoplasmic volume, some or all may be actively transported. This problem will benefit from higher resolution time courses of the transport of specific transcripts from nucleus to target, including colocalization with organelles and cytoskeletal structures along the way. Discovery and characterization of additional mutants similar to *nanos*, whose phenotype involves the abnormal distribution of mRNA, may implicate cytoskeletal or motor proteins in the transport of message to specific sites. The question of whether mRNA is actively transported can be addressed more directly by microinjection of function-blocking antibodies to known motor proteins as they become available. Once the form in which transcripts are transported is known, researchers could also take advantage of actin-based or microtubule-based motility assays to see whether active transport can be demonstrated in vitro [e.g., Kron et al., 1991; Vale et al., 1985].

The question of whether protein synthesis occurs in excluding compartments can be addressed with available technology. It is relatively straightforward to compare the distribution of ribosomes with the location of excluding compartments using fluorescent dyes that bind rRNA or antibodies to ribosomal subunits. If ribosomes are present in excluding compartments, high resolution immunoelectron microscopy can be used to see whether they occur as polysomes, which would imply that translation does occur in these regions. It is possible that translation of certain messages may be carried out on small populations of ribosomes sequestered at specific sites in the excluding compartments by entrapment in the filament network and/or binding to actin filaments.

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