Restricted Distribution of mRNA Produced from a Single Nucleus in Hybrid Myotubes

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Abstract. Although the proteins encoded by a single nucleus in multinucleated myotubes have a wide range of distributions within the myofiber, little is known about the distributions of their mRNAs. We have used hybrid myotubes in which one or a few nuclei are derived from myoblasts that express nonmuscle proteins to investigate this question. We find that three different mRNAs, encoding proteins that are, respectively, nuclear, cytoplasmic, and targeted to the ER, have similar distributions within myotubes. Each is confined to an area within ~100 μm of the nucleus that expresses it.

We report here experiments in which we have determined the distributions of three different mRNAs produced by single nuclei in hybrid myotubes and have compared them with the distributions of the proteins that they encode. Although the distributions of the proteins differed widely, those of their mRNAs were remarkably similar.

Materials and Methods

Cell Culture and Formation of Hybrid Myotubes

Details of the culture of the C2C12 subclone of the C2 mouse muscle cell line (Yaffe, 1977) as well as of the formation of hybrid myotubes and labeling with [3H]thymidine can be found in Ralston and Hall (1989b). Briefly, myoblasts of the appropriate cell lines were plated on multi-well slides (Nunc, Roskilde, Denmark) in growth medium (DME, 20% FBS, 0.5% chick embryo extract, 2 mM glutamine, 100 U/ml penicillin-streptomycin) at a total density of 40,000 cells/cm2. After 24 h they were switched to fusion medium (DME, 5% horse serum, 2 mM glutamine). Two days later, the cultures were fixed for 5 min in 4% para-formaldehyde, rinsed with PBS, and either processed for immunocytochemistry or dehydrated in ethanol, air dried, and stored at -80°C for in situ hybridization.

Transfection of C2 Cells with SI07 κ Genomic Vector

The plasmid pRSV107 κ was a gift from Linda Matsuchi (University of California, San Francisco, CA). It contains the entire genomic SI07 κ light chain gene under the control of the RSV long terminal repeat (Matsuchi et al., 1988). Transfections using the calcium phosphate precipitation technique were performed as described previously (Ralston and Hall, 1989b).

Immunocytochemistry

For β-galactosidase detection, the slides were stained with a mouse monoclonal anti-β-galactosidase antibody (Promega Corp., Madison, WI) followed by a fluorescein-conjugated anti-mouse antibody (Cappel Laboratories, Durham, NC). For κ light chain detection, the first antibody was a biotin-conjugated anti-immunoglobulin (Vector Laboratories, Inc., Burlingame, CA), followed by Texas red-conjugated streptavidin (Molecular Probes, Inc., Eugene, OR). Controls were stained with a rabbit polyclonal antibody to the ER generously provided by Daniel Louvard (Institut Pasteur, Paris) (Louvard et al., 1982), followed by a fluorescein-conjugated anti-rabbit

ONE mechanism by which cells localize proteins and establish polarity is by controlling the distribution of their mRNAs. Although potentially important for all cells, this mechanism is especially useful as a means of achieving regional specialization in large cells such as muscle fibers, neurons, or developing embryos. Adult muscle fibers, for example, can be tens of centimeters long and contain thousands of nuclei. mRNA for the subunits of the acetylcholine receptor, a protein that is localized in the postsynaptic membrane of the neuromuscular junction, is not uniformly distributed throughout the fiber, but is concentrated near nuclei at the endplate, which occupies <0.1% of the cell surface (Merlie and Sanes, 1985; Fontaine et al., 1988; Goldman and Staple, 1989). In spite of the potential importance of mRNA localization, the distribution within cells of relatively few mRNAs has been examined (Lawrence and Singer, 1986; Trapp et al., 1987; Garner et al., 1988; Lawrence et al., 1988; Macdonald and Struhl, 1988; Yisraeli and Melton, 1988; Bruckenstein et al., 1990; Kleiman et al., 1990).

In previous experiments, we have used hybrid myotubes to determine the distribution of proteins produced by a single nucleus within a multinucleated myotube (Ralston and Hall, 1989a,b). Our results, along with those of others (Miller et al., 1988; Pavlath et al., 1989; Rotundo, 1990) have shown that although some proteins have a localized distribution near the nuclei producing the mRNA that encodes them, others are distributed throughout the myotube. These experiments suggested a mechanism by which the proteins are synthesized locally, and diffuse within the cytoplasm or within the plane of the membrane unless they are retained by anchoring to stationary components of the myotube (Hall and Ralston, 1989). Although our results suggest that mRNAs are localized and translated near the nuclei that produce them, no direct information is available on this point.

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In Situ Hybridization

The probe for β-galactosidase was prepared by subcloning the entire coding sequence of LacZ (without a nuclear localization signal) into the BamHI site of the pcDNA I vector (Invitrogen, San Diego, CA). After linearization with HindIII, the antisense RNA probe was transcribed from the SP6 promoter of pCDNA1 with 35S-labeled CTP. The labeled probe was then reduced to fragments of ~150 bp by alkaline hydrolysis (Cox et al., 1984). The probe for S107 κ was prepared by subcloning the constant region of the mouse κ chain from a pUC19-Ck vector (the generous gift of Tris Parslow, University of California, San Diego, CA) into the pBSII KS vector (Stratagene Inc., La Jolla, CA). After linearization with EcoRI, the antisense probe was transcribed from the T3 promoter of pBS, with either 35S-labeled CTP or digoxigenin-UTP (Boehringer-Mannheim Biochemicals, Indianapolis, IN), and fragmented by alkaline hydrolysis. The in situ hybridization protocol was modified from Deschepper et al. (1988). Slides were treated with 2.5 μg/ml proteinase K and with acetic anhydride in 0.1 M triethanolamine. They were incubated at 52°C for 2 h in a prehybridization mixture containing 50% deionized formamide, 4× SSC, 1× Denhardt’s, 0.1 mg/ml yeast tRNA, 5% dextran sulfate, 25 μg/ml poly-A RNA, 200 μg/ml heparin, and 50 mM DTT, and then overnight in the same solution containing 35S-labeled RNA (100,000 cpm/μl) or dig-RNA (1-2 ng/μl). The slides were covered with Parafilm and the next day were washed in 1× SSC at 52°C for 30 min, treated with 20 μg/ml RNase A at 37°C for 30 min, and washed 4-5 more times with 0.25× SSC at 52°C for 15 min. For the experiments with 35S-labeled probes, all washes contained β-mercaptoethanol (50 mM). The slides were then rinsed in water, dried, and transferred to a darkroom to be covered with Tifford K-5 autoradiographic emulsion (applied by dipping the slide in a 1:1 emulsion-water suspension at 37°C). After drying at room temperature for 4 h the slides were placed at 4°C. After exposure times ranging from a few days to 2 wk they were developed, stained with bisbenzimide, and mounted. Slides hybridized with dig-RNA were incubated with alkaline phosphatase-conjugated antidigoxigenin (Boehringer-Mannheim Biochemicals) and histochemical staining with alkaline phosphate-conjugated antialkaline phosphatase (Boehringer-Mannheim Biochemicals). Hybrid myotubes were stained as described in Materials and Methods to show the localization of the transfected protein. The slides were then examined systematically and, for each hybrid myotube encountered, the range of the protein was measured with a reticle in the microscope eyepiece. For each protein the range was defined as drawn on the figure, and the results plotted as a histogram.

In one of the cell lines (C2-Zn), the nuclear localization signal is constitutive, so that the protein is targeted to the nucleus in the absence of glucocorticoid. In the other cell line (C2-Zc), nuclear localization requires a glucocorticoid; in its absence the protein is cytoplasmic. We have shown that in hybrid myotubes containing a single C2-Zn nucleus, the fusion protein expressed had a restricted distribution limited to the C2-Zn nucleus (the source nucleus) and a few nuclei near it. In contrast, in myotubes containing a single C2-Zc nucleus and grown in the absence of glucocorticoid, the fusion protein was distributed throughout the myotube (Ralston and Hall, 1989b). We now describe the distribution of these proteins more quantitatively by staining cultures containing hybrid myotubes of each kind with an antibody to β-galactosidase and examining the myotubes systematically for the distribution of the protein. For each myotube containing a nucleus expressing the fusion protein, the length of myotube stained for the protein was measured directly using a reticle in the microscope eyepiece. Histograms of the results (Fig. 1, a and b) show the different distribution of the two proteins: Zn is mostly confined to the source nucleus and to neighboring nuclei within a 100-μm area around it; the distribution of Zc, in contrast, is limited only by the observable length of each individual myotube.

Results

Distributions of Cytoplasmic or Nuclear Proteins Produced by Single Nuclei

We have previously described (Ralston and Hall, 1989b) two muscle cell lines derived from C2C12 cells that express a fusion protein containing a nuclear localization signal from the glucocorticoid receptor attached to E. coli β-galactosidase.

Figure 1. Distribution of Zn, Zc, and κ protein ranges. Cultures of hybrid myotubes were stained as described in Materials and Methods to show the localization of the transfected protein. The slides were then examined systematically and, for each hybrid myotube encountered, the range of the protein was measured with a reticle in the microscope eyepiece. For each protein the range was defined as drawn on the figure, and the results plotted as a histogram.
Figure 2. The mRNAs for Zn, Zc, and k show a similar distribution around their respective source nuclei. Cultures of hybrid myotubes were hybridized with an antisense probe to localize the message for the nonmuscle protein. (a–d) Myotube containing a single Zn nucleus (a–b) or Zc nucleus (c–d). The probe was labeled with 35S and detected by autoradiography. The same field was observed with a dark-field condenser under white light to show the autoradiographic grains, and UV fluorescence to show the Hoechst-stained nuclei (a and c), or under phase and fluorescence optics (b and d). (e) Myotube containing single C2-k nucleus. The probe was labeled with digoxigenin and detected by immunocytochemistry with alkaline phosphatase; the field was observed under phase and fluorescence optics. Bar, 20 μm.

C2-Zn or C2-Zc nuclei was then examined. 2–3 d after fusion, cultures were processed for in situ hybridization. The antisense probe for the Lac Z sequence common to Zn and Zc was labeled with 35S. The distributions of the two mRNAs were remarkably similar. In hybrid myotubes, mRNAs for the two proteins were found largely around the source nucleus (Fig. 2, a–d), though they extended, in many cases, to nearby nuclei. A systematic examination of a large number of myotubes (Fig. 3, a and b) showed that in 50% of the cases, the range was <50 μm for Zn mRNA and <70 μm for Zc mRNA. The two mRNAs were restricted to a 100-μm domain in ~90% of the examples encountered.

Distribution of a Protein Synthesized and Retained in the ER

Both Zn and Zc fusion proteins are synthesized on free ribo-
somes. In view of the similarity of the distributions of their mRNAs, we wished to compare them with that of an mRNA for a protein synthesized on membrane-bound ribosomes. We constructed a cell line that expresses a mouse myeloma \( \kappa \) light chain (S107 \( \kappa \)) that, in other cells, has been shown to accumulate in the ER (Matsuuchi et al., 1988). C2 myoblasts were transfected with a vector containing a genomic clone for the S107 \( \kappa \) light chain and a subclone that expressed the protein was selected (see Materials and Methods). Both myoblasts and myotubes from this line (referred to subsequently as C2-\( \kappa \)) expressed high levels of the light chain that could be detected by immunofluorescence (Fig. 4). The staining pattern was that expected for a protein localized in the ER (Gu et al., 1989), as shown by double staining with an antibody specific for membranes of the ER (Fig. 5).

Hybrid myotubes were then formed and the distribution of protein around the source nucleus determined. C2-\( \kappa \) myoblasts were incubated with \(^{3}H\)thymidine before fusion to allow identification of their nuclei in the resulting myotubes (Ralston and Hall, 1989b). The distribution of immunofluorescence in hybrid myotubes revealed that the protein occupied the ER, not only around the source nucleus, but also around neighboring nuclei (Fig. 6). The range of the \( \kappa \) light chain was \( \sim 200 \mu m \) (Fig. 1 c); it was intermediate between that of the Zn and Zc proteins. The distribution of the \( \kappa \) light chain resembles that of a protein in the Golgi complex that we have described earlier (Ralston and Hall, 1989a).

### The Distribution of \( \kappa \) mRNA Is Similar to That of Zn and Zc mRNAs

The distribution of \( \kappa \) mRNA was then examined in hybrid myotubes by in situ hybridization. Both \(^{35}S\)-labeled and digoxigenin-labeled probes were used to detect the \( \kappa \) mRNA. The distributions obtained with the two techniques were very similar. When compared with the \(^{35}S\) histogram (not shown), the digoxigenin histogram (Fig. 2 e) was shifted to the left by 5–10 \( \mu m \). This shift presumably reflects the tight colocalization of the histochemical precipitate with the mRNA; autoradiographic grains for \(^{35}S\) extend beyond the range of the mRNA, and thus give a slight overestimate of the range. In each case the mRNA was found mostly around the source nucleus. When hybrid myotubes were examined systematically for quantitation of the results, in 50% of the cases, the range was \(<65 \mu m\), similar to the 50–\( \mu m\) range of Zn mRNA and that of 70 \( \mu m\) for Zc mRNA. Like Zn and Zc mRNAs, \( \kappa \) mRNA was limited to a 100–\( \mu m\) domain in \( \sim 90\% \) of the cases surveyed (Fig. 3 c).

### Figure 3. Distribution of Zn, Zc, and \( \kappa \) mRNA ranges. In each case, cultures of hybrid myotubes were hybridized with an antisense probe as described in Materials and Methods to show the localization of the nonmuscle mRNA synthesized by the source nucleus. The slides were then examined systematically and, for each hybrid myotube encountered, the range of the mRNA was measured with a reticle in the microscope eyepiece. For each mRNA the range was defined as drawn on the figure and the results plotted as a histogram.

![Figure 3](image)

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### Figure 4. Expression of \( \kappa \) light chain by transfected C2 cells. Myoblasts (a) and myotubes (b) of the cell line C2-\( \kappa \) were stained as described in Materials and Methods with a biotinylated anti-IgG followed by Texas red-conjugated streptavidin. Bars, 20 \( \mu m \).

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### Figure 5. The \( \kappa \) light chain is retained in the ER in C2-\( \kappa \). Myoblasts of the cell line C2-\( \kappa \) were stained with biotinylated anti-IgG and Texas red streptavidin (a) or with an antibody to the ER followed by fluorescein-conjugated second antibody (b). Bar, 10 \( \mu m \).

![Figure 5](image)

**Figure 5.** The \( \kappa \) light chain is retained in the ER in C2-\( \kappa \). Myoblasts of the cell line C2-\( \kappa \) were stained with biotinylated anti-IgG and Texas red streptavidin (a) or with an antibody to the ER followed by fluorescein-conjugated second antibody (b). Bar, 10 \( \mu m \).
**Discussion**

The principal observation that we have made is that three different mRNAs, each made by a single nucleus within multinucleated myotubes, have remarkably similar distributions within the myotubes. Two of the mRNAs are presumably translated on free ribosomes, and the third on ribosomes bound to the ER. Each of them is restricted to a region within ~100 μm of the nucleus in which it is synthesized. In contrast to the mRNAs, the proteins that they encode have widely different distributions. One is a cytoplasmic protein that is found throughout the length of the myotube, another is a nuclear protein, and the third is accumulated in the ER.

Generally, the products made by a single nucleus within a myotube or myofiber cannot be distinguished from those made by other nuclei. For this reason, we used hybrid myotubes in which one or a few nuclei express an mRNA not normally synthesized in muscle cells; we then determined the distribution of the nonmuscle mRNA to obtain an estimate of how far within the cell the product of a single nucleus can range. In contrast to most muscle protein mRNAs, the mRNA encoding subunits of the AChR, and perhaps other synaptic proteins as well, is expressed at high level only by nuclei near the endplate (Merlie and Sanes, 1985; Fontaine and Changeux, 1988; Goldman and Staple, 1989; Brenner et al., 1990). In addition, only a minority of nuclei within myotubes that are stimulated on a purified preparation of ARIA (acetylcholine-receptor inducing activity) (Harris et al., 1989) or of ascorbic acid (Horovitz et al., 1989) synthesize high levels of the α subunit. Because the mRNA is produced by only one or a few nuclei, in situ hybridization of acetylcholine receptor subunit mRNA can thus provide an estimate of how far endogenous muscle cell mRNA ranges from its nucleus of origin. The distributions found in these studies, which are on the order of 100 μm, are similar to those that we have seen for exogenous mRNAs.

Although the distributions of the three mRNAs that we examined were generally similar, there may be small differences between them (Fig. 3). More refined methods of analysis, however, will be required to examine this question.

Even when the mRNA synthesized by a single nucleus does not range far from the nucleus that synthesizes it, the domain that it occupies may extend over a volume of cytoplasm encompassing several nuclei. This distribution could explain recent results by Bursztajn et al. (1989) and Berman et al. (1990). They found that although intrinsic probes for the α subunit of the acetylcholine receptor bound to only ~10% of the nuclei in myotubes, exonic probes showed a relatively uniform distribution of the mRNA throughout the fiber.

Not all mRNAs are concentrated near the nuclei that produce them. In many cells, different mRNAs have distinctive distributions, suggesting that specific mechanisms direct localization. In neurons, for example, mRNAs for some proteins (e.g., α- and β-tubulin, GAP-43, NF 68) remain in the cell body, while mRNA for others (e.g., MAP2) is transported to the dendrites (Kleiman et al., 1990; Bruckenstein et al., 1990).

The cytoskeleton is most likely involved both in retention of the message near the nucleus and in its transport to specific sites (for review see Singer, 1992). After detergent extraction mRNAs remain bound to a cellular matrix (Lenk et al., 1977), and both microtubules and microfilaments have been implicated in the correct localization of mRNAs within cells (Edgar et al., 1987; Yisraeli et al., 1990; Sundell and Singer, 1991). Recently mRNA attachment to the cytoskeleton has been visualized at the ultrastructural level (Singer et al., 1989). This association appears to be independent of ribosomes and may occur through direct attachment of the RNA (Sundell and Singer, 1990). 3' untranslated sequences have been implicated in the transport of bicoid mRNA in *Drosophila* oocytes (Macdonald and Struhl, 1988) and of *Vg1* mRNA in *Xenopus* oocytes (Mowry and Melton, 1992).

In our experiments, each of the proteins had a significantly wider distribution within the myotube than that of its mRNA. This difference was most pronounced for the Zc protein which is cytoplasmic and appears to occupy the entire volume of the cytosol (Ralston and Hall, 1989b). We have suggested earlier that this protein is made locally in hybrid myotubes and achieves its final distribution through diffusion. The ranges of both Zn protein and κ light chain were only slightly larger than that of their mRNAs. In the case of Zn, the protein is presumably rapidly captured by sites on nearby nuclei after its synthesis in the cytoplasm. For the κ light chain, the slightly more extended distribution shown by the protein compared to the mRNA may result from diffusion of the protein through the lumen of the ER after its synthesis. The similarity between mRNA and protein distributions, however, suggests that such diffusion must be limited. This conclusion is consistent with that of Rotundo (1990) who investigated the association in hybrid myotubes of allelic forms of acetylcholinesterase, a protein that is assembled in the ER. He found that acetylcholinesterase subunits encoded by different nuclei do not associate randomly, suggesting that proteins synthesized in the ER surrounding different nuclei do not mix freely.

Others have also observed that mRNAs and the proteins that they encode can have different cellular locations. For example, although bicoid mRNA is concentrated at the anterior pole of the syncytial *Drosophila* embryo, the bicoid...
protein forms a gradient throughout the embryo (Driever and Nüsslein-Volhard, 1988). Also, in neurons, proteins that occupy the axons and nerve terminals are synthesized from mRNA in the cell body, and then transported to their final destinations. In several other instances, the cellular distributions of mRNAs and the proteins that they encode are the same. Thus vimentin mRNA aligns with intermediate filaments in muscle cells (Cripe et al., 1992) and $\beta$-actin mRNA is localized to the mobile lamellipodia of myoblasts and fibroblasts (Lawrence and Singer, 1986). The actin mRNA appears to be localized by mechanisms that are distinct from those responsible for the localization of the protein (Sundell and Singer, 1990). In at least one other case, the similarity of protein and mRNA distributions has been shown to result from the very short half-life of the message (Edgar et al., 1987).

Our experiments are thus consistent with a model in which mRNA, shortly after its appearance in the cytoplasm of muscle cells, binds to a cytoskeletal or membrane component that restricts its further movement. The similarity that we have observed between the distribution of several different mRNAs may reflect a general mechanism that limits the spread of mRNAs from the source nucleus. In multinucleated muscle cells, this mechanism may prevent general mixing of mRNAs from different nuclei and so give rise to nuclear domains (Hall and Ralston, 1989).

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