Localization and Active Transport of mRNA in Axons of Sympathetic Neurons in Culture

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In neurons, the establishment and maintenance of distinct somatic, dendritic, and axonal domains has long been known to rely on regulated traffic of organelles and proteins. More recently, the local targeting of specific mRNAs has also been demonstrated, at least in dendrites, to provide a local supply of specific proteins. Here we set out to test directly for the presence of mRNA in axons of cultured chick sympathetic neurons, to examine their distribution during axonal outgrowth, to determine the reliance of this distribution on specific cytoskeletal elements, and to assess whether the axonal and somatic mRNA complements differ. Using fluorescent *in situ* hybridization, we found that sympathetic axons do contain poly(A⁺) mRNA along their length in a pattern that changes gradually as axons elongate, from an evenly dispersed punctate distribution with strong growth cone staining to a distribution focused at

branch points, varicosities, and some growth cones. Selective perturbations of the cytoskeleton revealed that the presence of axonal mRNA was dependent on microtubules (MTs), but not actin filaments, indicating that mRNA transport and/or anchoring within the axon are active processes involving MTs. Finally, reverse transcription-PCR amplification of RNAs from the axonal and somatic compartments showed that β -actin mRNA was present in both compartments, whereas mRNA encoding α -tubulin was restricted to the somatic compartment and entirely absent from the axons. Thus, the mRNA populations in the soma versus the axon are both quantitatively and qualitatively different, and these neurons are able to direct specific mRNAs to the axon.

Key words: axon; axonal transport; mRNA; microtubules; microfilaments; *in situ* hybridization; RT-PCR

An essential aspect of molecular trafficking is the sorting, selective transport, and regional docking of mRNA molecules in cytoplasmic microdomains distant from the nucleus (for review, see Wilhelm and Vale, 1993; Rings et al., 1994; St. Johnston, 1995). Localized mRNAs play essential roles in early development in *Xenopus* (Melton, 1987; Kloc et al., 1991; Elinson et al., 1993; Ku and Melton, 1993; Mosquera et al., 1993; Kloc and Etkin, 1994) and *Drosophila* (Berleth et al., 1988; Ephrussi et al., 1991; Wang and Lehman, 1991; Gavis and Lehman, 1992; Neuman-Silberberg and Schubach, 1993) (for review, see Ding and Lipshitz, 1993). Specific mRNA localization has also been reported in muscle (Merlie and Sanes, 1985; Fontaine et al., 1988; Fontaine and Changeux, 1989; Cripe et al., 1993; Morris and Fulton, 1994), fibroblasts and myoblasts (Hill and Gunning, 1993; Kislauskis et al., 1993), oligodendrocytes (Ainger et al., 1993), epithelia (Hook et al., 1991), and neurons (Garner et al., 1988; Bruckenstein et al., 1990; Burgin et al., 1990; Kleiman et al., 1990; Lyford et al., 1995) (for review, see Steward and Banker, 1992). Although the mechanisms of mRNA sorting and transport are not well understood, mRNA localization before translation can serve as an efficient mechanism to distribute proteins within the cell, and may allow for timely regulation of protein synthesis in response to local

stimuli. In addition, the role of the cytoskeleton in mRNA transport and in the anchorage of most cellular mRNA is established in several systems (for review, see Hesketh and Pryme, 1991; Pachter, 1992; Suprenant, 1993).

Neurons seem to be a likely cell type to use mRNA transport and localization, because their functional and morphological polarization can place a majority of their axonal cytoplasm at a considerable distance from the nucleus, Golgi, and rough endoplasmic reticulum. However, at least in vertebrate neurons that have a distinct and highly elongated axonal compartment, transport along the axon of material synthesized in the soma has long been considered adequate to support axonal growth and maintenance. In addition, conventional ultrastructural methods generally have revealed a paucity of ribosomes in axoplasm (Peters et al., 1976). Thus, there has been little perceived need to invoke local translation in the axon, and axonal transport studies have led to the conclusion that, at least in vertebrates, the axonal compartment is devoid of synthetic capacity (for review, see Grafstein and Forman, 1980). As a result, analyses of the targeting of neuronal mRNAs have focused primarily on the somatodendritic compartment (for review, see Steward and Banker, 1992; Steward, 1994). However, evidence has gradually accumulated favoring the idea that axonal mRNA localization may also be an important feature of the cell biology of neurons (for review, see Mohr and Richter, 1993; Van Minnen, 1994b). Three invertebrate systems show unambiguously the presence of axonal mRNAs, translation machinery, and/or evidence of autonomous protein synthesis (Rapalino et al., 1988; Giuditta et al., 1991; Davis et al., 1992; Kaplan et al., 1992; Crispino et al., 1993a,b; Gioio et al., 1994; Van Minnen, 1994a). In addition, in one vertebrate system, the neuroendocrine cells of the hypothalamo–hypophyseal tract, the presence and transport of axonal mRNAs has been described (Jirikowski et al.,

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1990; Mohr et al., 1991; Mohr and Richter, 1992; Skutella et al., 1994; Trembleau et al., 1994). Olfactory sensory neurons are the only other example in vertebrates in which specific transcripts have been detected in the axons and terminals (Ehrlich et al., 1990; Ressler et al., 1994; Vassar et al., 1994; Wensley et al., 1995). However, these studies seem not to have shaken the dogma that the supply of axonal proteins relies entirely on anterograde transport of material synthesized in the soma (Vale et al., 1992; Alberts et al., 1994). This could be attributable to their analysis of large invertebrate axons or atypical vertebrate axons the cell biology of which is considered to have unique cytotypic features.

In the present study, we probed for axonal mRNAs in vertebrate neurons having a distinct, extended axonal compartment, determined the distribution of axonal mRNAs during axonal outgrowth and its reliance on cytoskeletal elements, and assessed whether the axonal and somatic mRNA complements were identical. We used chick sympathetic neurons grown under culture conditions in which they elaborate axons but not dendrites (Baas and Ahmad, 1992, 1993). To assess whether growing axons contained mRNA, we used two techniques in parallel: *in situ* hybridization of cultured neurons with probes for polyadenylated mRNAs [poly(A⁺) mRNAs], and reverse transcription (RT)-PCR amplification of axonal versus somatic mRNA. Our data indicate that these axons do contain poly(A⁺) mRNAs distributed in a pattern that changes through their development, and that the axonal and somatic mRNA complements differ. Furthermore, selective perturbation of the axonal cytoskeleton showed that the localization of poly(A⁺) mRNAs in axons requires microtubules (MTs), but not microfilaments (MFs), and that it relies on active transport.

MATERIALS AND METHODS

Materials

All reagents, tissue culture media, and supplements were obtained from Sigma (St. Louis, MO) unless specified otherwise. Tissue culture plastic was obtained from Dow Corning (Corning, NY). Monoclonal rat anti-tubulin YL1 was a gift from J. Kilmartin (Kilmartin, 1982). Mouse nerve growth factor (2.5S), digoxigenin-11-deoxyuridine triphosphate (digoxigenin-11dUTP), digoxigenin detection kits, digoxigenin-conjugated sheep anti-horseradish peroxidase (HRP), 5S RNA, T4 polynucleotide kinase, and *in situ* blocking reagent were obtained from Boehringer Mannheim (Indianapolis, IN). All other secondary antibodies were from Vector Laboratories (Burlingame, CA), except Cy3-conjugated rabbit anti-digoxigenin (Jackson ImmunoResearch, West Grove, PA). Rhodamine-phalloidin was obtained from Molecular Probes (Eugene, OR). Acetylated bovine serum albumin (BSA) and deoxynucleoside triphosphates (dNTPs) were obtained from Stratagene (La Jolla, CA). AmpliTaq DNA polymerase, AmpliWax PCR Gem50 and oligo(dT)₁₆ were obtained from Perkin-Elmer (Foster City, CA). Ribonuclease inhibitors and Moloney murine leukemia virus (M-MLV)-reverse transcriptase RNase H⁻ recombinant were obtained from Promega (Madison, WI). Vinblastine was stored as a 4 mM stock in 100% methanol, and cytochalasin E was stored as a 4 mg/ml stock in 100% dimethylsulfoxide, both at -20°C.

Cell culture

Sympathetic chain ganglia were dissected out of 9–11 d chicken embryos and grown either as whole ganglia or dissociated neurons in C-medium (Leibowitz L-15 medium supplemented with 10% fetal bovine serum, 0.6 mM glucose, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 ng/ml 2.5S mouse nerve growth factor and 0.5% methylcellulose) as described previously (Hollenbeck, 1993). For immunofluorescence or *in situ* hybridization experiments, neurons were grown at low density in 12-well plates on 15 mm glass round coverslips coated with 1 mg/ml poly-L-lysine overnight at 4°C followed by 0.1 µg/ml laminin treatment for 1 hr at 25°C. For axon isolation, whole ganglia were plated onto poly-L-lysine-coated plastic dishes and grown in C-medium for 4–7 d at 37°C.

Drug treatments

To obtain neurites lacking one cytoskeletal element, dissociated neurons from three sympathetic chains were plated on coverslips in a 12-well plate and incubated at 37°C for 30 min before adding any drug. To obtain axons lacking MTs, medium containing 100 nM vinblastine was then added and cells were grown for 18 hr before fixation (Lamoureux et al., 1990). To obtain axons lacking MFs, medium containing 20 ng/ml cytochalasin E was added at 30 min and cells were grown for 3 d before fixation (Marsh and Letourneau, 1984). To depolymerize MTs of neurons in established cultures, dissociated sympathetic neurons were plated on coverslips in a 12-well plate, grown for 18 hr at 37°C, and treated with 1 µM vinblastine for 1 hr before fixation (Morris and Hollenbeck, 1995). To disrupt MFs of neurons in established cultures, dissociated neurons were plated and grown the same way, but were treated with 20 ng/ml cytochalasin E for 1 hr before fixation (Morris and Hollenbeck, 1995).

Probe preparation

Synthetic oligo(dT) (32 nucleotides) and oligo(dA) (32 nucleotides) were 3' end-labeled with digoxigenin-11dUTP using terminal transferase [0.2 µM oligonucleotide, 60 µM digoxigenin dUTP, 200 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.25 mg/ml BSA, 2.5 mM CoCl₂, 0.1 mM dithiothreitol (DTT), and 1500 U/ml terminal transferase] at 37°C for 1 hr. Probes were then ethanol-precipitated, resuspended in 50 µl of diethyl pyrocarbonate (DEPC)-treated H₂O and purified on a G50 Sephadex column. The incorporation of digoxigenin-11dUTP was quantified by blotting the probes onto nitrocellulose and by detection with an anti-digoxigenin-alkaline phosphatase conjugate.

Fixation, immunofluorescence, and *in situ* hybridization

For immunofluorescence experiments, cells were washed in warm HBSS, fixed for 15 min in the same solution containing 2% formaldehyde, 0.1% glutaraldehyde, 2 mM EGTA, 0.12 M sucrose, and 0.5% Triton X-100, transferred for 10 min to the same fixation solution without detergent, then washed in a large volume of PBS, blocked for 15 min in 3% BSA in PBS, and processed for immunostaining. To visualize tubulin, cells were incubated for 45 min at 37°C with YL1 rat monoclonal anti-tubulin in PBS containing 1% BSA and 0.05% Triton X-100, washed thoroughly in the same buffer, incubated for 30 min at 37°C with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat IgG diluted in the same buffer. To visualize neurofilaments, incubation with a rabbit antiserum against the chick 70K neurofilament subunit (Fekete and Brookes, 1987) was followed by incubation with FITC-conjugated goat anti-rabbit IgG. To visualize F-actin, 5 µg/ml rhodamine-phalloidin was added simultaneously with secondary antibodies for double-labeling experiments. Coverslips were washed and mounted as described previously (Hollenbeck and Bray, 1987).

The procedure used for *in situ* hybridization was derived from the protocol of Bassell et al. (1994). Cells were fixed with 4% *p*-formaldehyde in PBS containing 4% sucrose and 5 mM MgCl₂. Fixed cells were kept in PBS containing 5 mM MgCl₂ (PBS/MgCl₂) overnight at 4°C, then washed in PBS/MgCl₂ and permeabilized in 0.3% Triton X-100 in PBS/MgCl₂ for 5 min at 25°C. Coverslips were then successively incubated in 1× SSC/10 mM sodium phosphate, pH 7.0, 2× SSC/10 mM sodium phosphate, pH 7.0, for 5 min at 25°C and, finally, in 15% formamide/2× SSC/10 mM sodium phosphate, pH 7.0, for 10 min at 37°C. Five microliters of probe (20 ng) were dried down with *Escherichia coli* tRNA (20 µg) and resuspended in 15 µl of 30% formamide/20 mM sodium phosphate, pH 7.0. Probes were mixed with 15 µl of hybridization buffer (4× SSC, 0.4% BSA, 20 mM vanadyl adenosine complex, 10 mM EDTA, 20 mM sodium phosphate, and 20% dextran sulfate, pH 7.0). Coverslips were placed cell side-down on Parafilm containing 30 µl of probe mixture and hybridized overnight at 37°C. After hybridization, coverslips were successively washed at 37°C for 20 min in 15% formamide/2× SSC/10 mM sodium phosphate, then for 10 min in 15% formamide/1× SSC/10 mM sodium phosphate, followed by three 10 min washes in 1× SSC/10 mM sodium phosphate at 25°C. Cells were then equilibrated in maleate buffer (100 mM sodium maleate, pH 7.0, and 150 mM NaCl) and incubated for 15 min at 25°C in maleate buffer with 3% blocking reagent. Probes were detected using HRP-conjugated sheep anti-digoxigenin and Cy3-conjugated rabbit anti-HRP. Antibody incubations were for 45 min at 37°C in 1% blocking reagent, 0.1% Triton X-100 followed by several washes in PBS/MgCl₂. Cy3 was visualized with a Nikon microscope using a rhodamine filter set and a 63× plan-Neofluar objective and photographed with Kodak TMAX 400 film. As controls for the specificity of *in situ* hybridization,

digoxigenin-labeled oligo(dA) probes were hybridized in parallel to digoxigenin-labeled oligo(dT) probes in all experiments. For RNase treatment, fixed and permeabilized cells were incubated with a mixture of RNases (RNase A, 0.2 mg/ml; RNase T1, 0.2 mg/ml; RNase T2, 100 U/ml) for 30 min at 37°C before hybridization.

Isolation of pure axons

After 4–7 d in culture, whole explanted ganglia developed a radial halo of axons long enough to be mechanically isolated from the somatic mass by microdissection as described by Peng et al. (1986). After several gentle washes with warm HBSS, axons of selected halos were cut out, detached from the dish by gentle pipetting with HBSS, and immediately put into Eppendorf tubes containing ice-cold HBSS. Their corresponding somatic mass was also recovered and placed into a separate tube. To prevent any contamination from the cell bodies, axons of each halo were cut at a distance from the somatic mass. The proliferation of fibroblasts was avoided almost completely by treatment of the dish with 1 mg/ml poly-L-lysine. The residual fibroblasts remained attached to the dish when halos of axons were removed from the dish by gentle pipetting.

RNA extraction

Twenty to fifty axonal halos were required to detect specific, ethidium bromide-stained bands on a polyacrylamide gel after the extraction of total axonal RNA and 30 cycles of amplification by RT-PCR. Total RNAs from axons and the somatic mass were prepared based on the rapid one-step procedure of Chomczynski and Sacchi (1987). Axonal halos and somatic masses were pelleted in separate tubes for 5 min at $500 \times g$, washed in ice-cold PBS, resuspended in 200 μ l of denaturing solution (4 M guanidium thiocyanate, 42 mM sodium citrate, 0.83% *N*-lauryl sarcosine, and 0.2 mM 2-mercaptoethanol), and transferred to DEPC-treated Eppendorf tubes, in which the material was lysed on ice by pipetting. The first extraction of the total RNA was performed by adding 20 μ l of 2 M sodium acetate, pH 4.0, and 200 μ l of phenol/chloroform/isoamyl alcohol (25:24:1). After 5 min on ice, the lysate was centrifuged for 20 min at $10,000 \times g$ at 4°C, and RNAs were precipitated from the upper aqueous phase with an equal volume of isopropanol overnight at –20°C using DNase/RNase-free *E. coli* 5S rRNA (10 μ g) as carrier. An additional purification step for the RNAs was performed by resuspending the pellet in 200 μ l of denaturing solution. After an isopropanol precipitation at –20°C for 30 min, RNA pellets were washed with ice-cold 75% ethanol, dried, resuspended in 50 μ l of DEPC-treated H₂O and stored at –20°C until use.

RT-PCR amplification

Reverse transcription. To perform the RT-PCR amplification of axonal and somatic total RNA under conditions and with RNA quantities as similar as possible, a sample of somatic RNA equivalent to that of the somatic mass of 1 ganglion was reverse-transcribed into cDNA in parallel with the RNA from 20–50 axonal halos. RNA pellets were resuspended in 20 μ l of RT solution [50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 mM of each dNTP, and 2.5 μ M oligo(dT)₁₆]. This mixture was heated to 80°C for 5 min and slowly cooled to 42°C to optimize the primer annealing. Twenty units of RNasin ribonuclease inhibitors and 200 U of M-MLV-reverse transcriptase RNase H[–] recombinant were then added to each tube. The mixture was incubated for 30 min at 42°C. The reaction was stopped by inactivation of the enzyme at 95°C for 5 min.

β -actin and α -tubulin cDNA PCR coamplification. To avoid an additional ethanol precipitation, axonal and somatic single-strand cDNA were diluted 1:5 and 1:100, respectively, in 50 mM KCl, 10 mM Tris-HCl, pH 8.3, and used as stocks for each reaction of PCR amplification. Second-strand synthesis and coamplification of β -actin and α -tubulin cDNA by PCR were performed by using, respectively, oligonucleotide primers (5' primer CBA1: 5'-ATGGATGATGATATTGCTCCC-3' corresponding to nucleotides 1–21 of the β -actin coding sequence; 3' primer CBA2: 5'-GGTGACAATACCGTGTTCAT-3' complementary to the β -actin coding sequence 531–552), and oligonucleotide primers (5' primer TUB1: 5'-ATCGCCACCATCAAGACCAA-3' corresponding to codon 331–338 of the α -tubulin coding sequence; 3' primer TUB2: 5'-CAGGTCAAACCTTGTGGTCCA-3' complementary to the α -tubulin coding sequence 390–396), defined from the chicken cDNA sequences (Valenzuela et al., 1981; Kost et al., 1983). Each primer (10 pmol total) was ³²P 5' end-labeled in a single tube using the T4 polynucleotide kinase and purified on a G50 Sephadex column. PCR coamplifications were

done in a 50 μ l reaction volume using hot start conditions: a first “lower” mix (12.5 μ l) containing 0.2 mM of each dNTP, 2.0 mM MgCl₂, 0.1 mg/ml acetylated BSA, and 0.2 μ M of each unlabeled primer CBA2 and TUB2 was added to each tube, topped with an AmpliWax PCR Gem50 plug, which was melted to form a tight seal by incubating the tubes at 70°C for 2 min. An “upper” mix (37.5 μ l) containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.2 μ M of each unlabeled primer CBA1 and TUB1, 1.25 U AmpliTaq DNA polymerase, 5 μ l of the stock single strand cDNA sample and 1 μ l (150,000 cpm/ μ l) corresponding to 1:50 of the ³²P-labeled primer mix, was added on the top of the solid AmpliWax and covered with 25 μ l of mineral oil. Tubes were placed in a TwinBlocks DNA thermal cycler (Ericomp, San Diego, CA) and reactions started with one-step denaturation at 95°C for 2 min, followed by 32 cycles of amplification (30 sec at 95°C, 30 sec at 55°C, 1 min at 72°C) and one final extension for 5 min at 72°C. Five microliter aliquots were taken every 2 cycles from each reaction mixture after 20–32 cycles and loaded on a 5% polyacrylamide/7 M urea gel that was dried by vacuum suction and exposed to Kodak XAR-OMAT film at –80°C for 5 d, or analyzed by PhosphorImaging (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Polyadenylated mRNA distribution in cultured sympathetic neurons

We tested for the presence of poly(A⁺) mRNA in growing sympathetic axons using fluorescence *in situ* hybridization with digoxigenin-labeled oligo(dT) probes, followed by primary and secondary antibodies. The high sensitivity and resolution of the technique were mainly attributable to the use of secondary antibodies conjugated to the highly fluorescent dye Cy3. Under our culture conditions, sympathetic neurons elaborate axonal processes (Baas and Ahmad, 1992, 1993) extending well over 200 μ m in length by 24 hr, and sometimes branching. When these cells were subjected to *in situ* hybridization with labeled oligo(dT) probes, the strongest signal was in the soma, but it was also detected along the axons of most neurons in a bright, punctate pattern suggesting a clustering of mRNAs (Fig. 1*A,B*). The hybridization signal was always more intense in the proximal part of the axon; however, brightly labeled clusters of mRNA were clearly detected in the distal regions, particularly at axonal branch points, varicosities, and at some growth cones (Figs. 1*A,B*, 3). This signal was abolished by treatment of the cultures with RNases before incubation with oligo(dT) probes (Fig. 1*C,D*) and was absent from cultures incubated with labeled oligo(dA) probes (Fig. 1*E,F*). Thus, the oligo(dT) hybridization signal is specific and reports the location of poly(A⁺) mRNA, although it is possible that other RNAs with unusually long A-rich regions may also contribute to the signal. Moreover, the mRNA clusters were clearly detected in most axonal branches but absent from others (Figs. 1*B*, 3*B*), providing additional evidence that the hybridization is highly specific.

We then analyzed the distribution of poly(A⁺) mRNA within the axon in neurons fixed at specific stages of development and found that it varied with the extent of axonal outgrowth (Fig. 2). Axons of neurons cultured for only 2 hr were ~50 μ m long, unbranched, and possessed elaborate growth cones in which the poly(A⁺) mRNA signal was routinely concentrated (Fig. 2*A,B*). The signal intensity within these short axons was not significantly greater than that obtained in the proximal portion of longer axons. As neurons were cultured for 8 hr and axons extended farther, poly(A⁺) mRNA became distributed in a punctate pattern along the axon shaft, and although it persisted in many growth cones, became less intense there (Fig. 2*C,D*). After >8 hr of growth, axons of most neurons started to form collateral branches and, although poly(A⁺) mRNA in these neurons remained distributed in a punctate pattern along the axon, the signal

Figure 1. *In situ* hybridization of sympathetic neurons with fluorescently labeled oligo(dT) probes and corresponding controls. A differential interference contrast (DIC) image (A, C, E) and fluorescence image (B, D, F) are shown for each field. Comparison of the DIC image in A with the corresponding fluorescent *in situ* image in B revealed bright staining in the soma, as well as staining in the axonal growth cones, branch points, and along the axonal shafts in a punctate pattern. Cultures treated with RNase before *in situ* hybridization (C, D) showed a gross reduction in somatic staining and no axonal staining. Cultures probed with identically labeled oligo(dA) (E, F) showed no staining above background. Scale bar, 20 μ m.

became particularly concentrated at axonal branch points and was sparser elsewhere (Fig. 2E,F). After >1 d in culture, neurons generated very long axons in which the total signal in the distal regions appeared to decrease, but axonal branch points and varicosities remained brightly labeled and were the principal sites of poly(A⁺) mRNA localization (Fig. 3). The range of patterns of hybridization observed in longer neurons is reflected in the two examples shown in Figure 3, which illustrate both punctate staining along the axon shaft (Fig. 3B) and concentrated staining at branch points and varicosities (Fig. 3D). It should be noted that much longer axons, which could not be shown over their entire

length, had similar patterns of staining, such that the poly(A⁺) mRNA was detected at distances considerably greater than 300 μ m from the soma.

It was notable that poly(A⁺) mRNA was not found to be distributed equivalently in the different collateral branches of the same axons: signal was clearly detected in some axonal branches (e.g., Fig. 3B, *main shaft*), whereas in others, little if any hybridization was observed [Fig. 3, B (*small branches*) and D (*upper right branches*)]. Moreover, in long axons, the intensity of staining in the growth cones was lower in axons with larger numbers of branches (not shown).

Figure 2. Distribution of poly(A⁺) mRNA at different stages in axonal outgrowth. After 2 hr in culture (*A, B*) fluorescent *in situ* hybridization showed that the growth cone and axon shaft contained mRNA. By 8 hr (*C, D*), the signal was distributed along the growing axon shaft and was less prominent in the growth cone. After >16 hr of growth (*E, F*), axons showed the signal distributed in a punctate fashion along their shafts and concentrated at branch points. Scale bar, 20 μ m.

β -actin mRNA, but not α -tubulin mRNA, is detected in axons by RT-PCR amplification

We chose β -actin mRNA as a candidate for axonal localization because it is found to be selectively located at the leading edge of motile non-neuronal cells, where its translation presumably supports the prolific actin polymerization characteristic of this region (Lawrence and Singer, 1986; Taneja and Singer, 1990; Hill and Gunning, 1993). The axonal growth cone has very similar structural and motile properties to the leading edge of other polarized cells (Bray and Hollenbeck, 1987; Heidemann and Buxbaum, 1991), including a dense network of locally polymerized actin (Sobue, 1993). In addition, because the extreme length of the axon places the growth cone at a considerable distance from the synthetic capacity of the neuronal soma, we reasoned that β -actin mRNA could be an important mRNA species within the axon. To obtain adequate sensitivity to detect an individual axonal RNA species, we used RT-PCR amplification of total axonal RNA. Using RNA isolated from either axons or cell bodies of sympathetic neurons as described in Materials and Methods (Fig. 4), we amplified the β -actin mRNA by RT-PCR using β -actin-specific primers. After 30 cycles of amplification, PCR products of the

expected size (231 nucleotides) were detected by ethidium bromide staining of polyacrylamide gels (not shown). Then, to determine whether the axonal mRNA pool differed significantly from the somatic pool, as well as to assess the possibility of minor contamination from cell bodies in the axonal RNA preparation, we coamplified β -actin mRNA and α -tubulin mRNA from both the axonal and somatic RNA extracts. Tubulin was chosen because its mRNA has been reported to be exclusively located in the soma of sympathetic developing neurons (Bruckenstein et al., 1990) and because β -actin and α -tubulin are both major structural proteins of the neuron.

For the β -actin/ α -tubulin cDNA coamplification to report accurately the ratio of the respective mRNAs in the original compartments, (1) a set of α -tubulin-specific primers was chosen that would generate PCR fragments of similar size to those obtained with the β -actin primers, because the rate of amplification is directly related to the size of the DNA to be amplified; (2) experimental conditions were established under which β -actin and α -tubulin cDNAs were the only fragments amplified without significantly affecting their respective amplification rates; (3) a number of PCR cycles was chosen that was low enough to provide

Figure 3. Changing distribution of mRNA in very long axons. Paired DIC (*A*, *C*) and fluorescent *in situ* images (*B*, *D*) of axons after >1 d of outgrowth showed that the punctate mRNA signal distributed along the axon shafts was decreased in quantity compared with earlier stages but still present (*B*, *D*), whereas axonal branch points and varicosities were brightly labeled (arrowheads). In addition, most branches contained mRNA label, whereas a fraction lacked label entirely (arrows). Scale bar, 20 μ m.

optimal amplification for both primers, but high enough to detect the β -actin PCR products in the axonal compartment. To amplify under conditions as similar as possible from the axonal and somatic compartments, we adjusted the starting quantities of the axonal and somatic mRNA in the RT procedure, and of the resulting single-stranded cDNAs in the PCR procedure, to use comparable starting quantities of material at both steps (see Materials and Methods). 32 P-labeled β -actin and α -tubulin primers were added to the PCR reaction mixture to detect β -actin PCR products after as few as 20 cycles of amplification of the

axonal compartment cDNA. Aliquots of the PCR reaction mixture taken at 22, 24, 26, 28, and 30 cycles of the amplification were directly loaded on a 5% polyacrylamide/urea denaturing gel, and the separated PCR labeled products were analyzed using PhosphorImaging. As shown in Figure 4C, whereas β -actin PCR products were found in similar amounts in both the axonal and somatic compartments, α -tubulin PCR products were detected only in the somatic compartment even after 30 cycles of amplification. Over the range of cycles examined, the rates of β -actin amplification were virtually identical for the axonal and somatic material. Al-

Figure 4. Analysis of β -actin and α -tubulin in somatic versus axonal mRNA populations. *A*, Phase-contrast micrograph of a portion of the axonal halo emanating from one cell body mass (left). Scale bar, 500 μ m. *B*, Schematic diagram of the physical separation of extended axonal halos (periphery) from their corresponding cell bodies (center) to obtain material for the extraction of somatic versus axonal RNA. *C*, PhosphorImager analysis of the coamplification of the β -actin and α -tubulin fragments from cell body (*C* lanes) and axonal (*A* lanes) cDNA samples. After the number of cycles shown at the bottom, 5 μ l aliquots of the PCR products were withdrawn from each sample, separated on polyacrylamide/urea gels, and visualized. Both the β -actin fragment (231 nucleotides) and the α -tubulin fragment (197 nucleotides) were amplified from the cell body sample, with no significant change in their ratio throughout this range of amplification, whereas the β -actin PCR fragment alone was amplified from the axonal sample with no evidence of the α -tubulin fragment even after 30 cycles of amplification.

though the rate of the amplification was linear for both sets of primers in the range of 24–30 cycles, the β -actin: α -tubulin ratio in the somatic preparations remained nearly unchanged throughout the procedure, and the small difference in the rate of amplification actually favored α -tubulin. Thus, the fact that α -tubulin mRNA was not detected in the axonal compartment leads us to conclude that mRNA encoding β -actin, but not α -tubulin, is one of the poly(A⁺) mRNAs detected in axons of peripheral neurons and is not a somatic contaminant. These results indicate that α -tubulin mRNA, although prominently expressed in the soma, is not represented in the axonal mRNA population, and that β -actin mRNA is a bona fide resident of the axonal compartment. Thus, the two compartments differ in their mRNA complement of two major structural proteins of the cell, and the absence of α -tubulin products apparently constitutes, in this system, an appropriate control for somatic contamination of axonal preparations.

Poly(A⁺) mRNA distribution in neurons grown lacking MTs or MFs

The previous result indicated the presence of one major neuronal mRNA species in the axon and the exclusion of another, suggesting that axonal mRNAs constitute a subpopulation of the total mRNA expressed in these neurons in culture, and that their presence within the axon must be the result of an active and selective transport. Because recent studies in neurons and non-neuronal cells have implicated MTs, MFs, and intermediate filaments in the translocation and anchoring of mRNAs (Yisraeli et al., 1990; Sundell and Singer, 1991; Ainger et al., 1993; Bassell et al., 1994; Ferrandon et al., 1994; Litman et al., 1994; Pokrywka

and Stephenson, 1995), we selectively disrupted elements of the cytoskeleton in cultured neurons by the use of specific drugs and examined the effects on the distribution of poly(A⁺) mRNA.

First, we grew sympathetic neurons in the continuous presence of vinblastine, to prevent MT formation, or cytochalasin, to prevent formation of a coherent MF network, and fixed cultures in parallel for immunofluorescence and *in situ* hybridization with oligo(dT) probes. In the presence of low concentrations of vinblastine in the culture medium, previous studies have already demonstrated the ability of neurons to extend axonal processes lacking MTs, with a decreased axonal caliber, but a normal density of the other cytoskeletal elements (Lamoureux et al., 1990; Morris and Hollenbeck, 1995). The double staining of vinblastine-treated cells with rhodamine-phalloidin and anti-tubulin confirmed that neurons in these conditions were slowly able to extend straight axon-like processes over 100 μ m in length (Fig. 5), containing robust MFs but lacking MTs. The poly(A⁺) mRNA distribution in these cells was found to be exclusively restricted to the soma (Fig. 5D), suggesting that MTs are involved in the positioning of mRNA in the neurite. This result also indicates that the presence of poly(A⁺) mRNA in short axons of control cells (Fig. 2A,B) is unlikely to be attributable to simple diffusion and is likely to rely on active transport, probably involving MTs. Moreover, the mRNA signal in the soma was significantly reduced in intensity compared with controls, suggesting that at least a fraction of poly(A⁺) mRNA in the soma may also interact with the MT network. Neurons grown in the continuous presence of low concentrations of cytochalasin E also extend neurites, but with a characteristic curly morphology (Fig. 5E), and requiring 3 d to achieve the lengths seen in the previous experiment (Marsh and Letourneau, 1984; Morris and Hollenbeck, 1995). Immunofluorescence and rhodamine-phalloidin staining confirmed that these neurites contained normal MT densities but lacked coherent MF arrays (Fig. 5E,F). Poly(A⁺) mRNA in cytochalasin-treated neurons was detected all along the processes (Fig. 5H), in a pattern very similar to that seen in control neurons with axons of similar length (Fig. 2C,D). This result indicates that MF integrity is not necessary for the access and/or transport of mRNA into the neurite.

Effects on the poly(A⁺) mRNA distribution of cytoskeletal disruption in established neuronal cultures

The previous data raised the question of whether the MTs or MFs are also necessary to maintain the axonal localization of mRNA in neurons with established axons. To address this, neurons were grown for 24 hr and then treated with 1 μ M vinblastine or 20 μ g/ml cytochalasin E for 1 hr before the fixation. Vinblastine treatment eliminated virtually all MTs from the axon (Fig. 6A) (Morris and Hollenbeck, 1995), without affecting the densities of MFs (Fig. 6B) or of neurofilaments (Morris and Hollenbeck, 1995). This treatment abolished detectable poly(A⁺) mRNA from the axon, and hybridization was restricted to the soma of all neurons (Fig. 6C,D). Conversely, cytochalasin treatment, which disrupted MFs almost to the point of elimination from the axon while leaving a robust MT array in place (Fig. 6E,F) (Morris and Hollenbeck, 1995), had no apparent effect on the axonal mRNA distribution as compared with control axons of similar length (Figs. 6G,H, 2). These observations implicate the MTs, but not the MFs, in the maintenance of the axonal localization of mRNA.

Figure 5. MTs are involved in the positioning of poly(A⁺) mRNAs in axons. Sympathetic neurons were grown in the presence of either vinblastine (*A–D*) or cytochalasin E (*E–H*), to generate neurites lacking either MTs or F-actin, respectively. Double-staining of α -tubulin (*A, E*) and F-actin (*B, F*) shows the selective inhibition of cytoskeletal development in neurons grown continuously for 18 hr in vinblastine (*A, B*) or for 3 d in cytochalasin (*E, F*). *In situ* hybridization, shown as DIC/fluorescence pairs, illustrates that mRNA is absent from MT-deficient processes (*C, D*), whereas the mRNA distribution in processes of similar length that contain MTs but have grossly disrupted F-actin (*G, H*) is indistinguishable from control cells (compare Figs. 1, 2). Scale bars, 20 μ m.

DISCUSSION

Our purpose in this study was to test directly for the presence of specific axonal mRNAs in growing axons of vertebrate neurons and to gain an initial insight into the mechanisms of their transport. Using fluorescence *in situ* hybridization and RT-PCR amplification, we have provided direct evidence that axons of chick sympathetic neurons do contain a selective and presumably heterogeneous population of poly(A⁺) mRNAs, including mRNA encoding β -actin but not α -tubulin. Our results also indicate that the distribution of axonal mRNAs changes during axonal outgrowth and that the transport and/or localization of mRNAs in these axons occurs via an active MT-dependent process.

Presence and distribution of poly(A⁺) mRNA in axons of sympathetic neurons

Using oligo(dT) probes and appropriate controls, we observed a significant and specific hybridization signal within axons at all

stages of outgrowth, clearly establishing the presence of poly(A⁺) mRNAs in axons of sympathetic neurons. Moreover, because the axonal localization of these mRNAs is dependent on the integrity of MTs (discussed further below), these data suggest that axonal mRNAs are actively transported and anchored to specific binding sites along the axon. Few studies report the subcellular localization of RNA molecules within the axonal domain, and these studies concern the localization of neuropeptide mRNAs by *in situ* hybridization at the ultrastructural level (for review, see Van Minnen, 1994b). Our results, obtained using light microscope *in situ* hybridization, not only demonstrate the presence of mRNAs in axons but also show how and where they are localized along the axon shaft during outgrowth. The punctate, granular pattern of mRNA that we observed was similar to that reported in other cases of localized mRNAs (Ainger et al., 1993; Bassell et al., 1994; Ferrandon et al., 1994; Forristall et al., 1995), suggesting that this

Figure 6. MT integrity is necessary to maintain poly(A⁺) mRNA in axons. Established cultures of sympathetic neurons were treated for 1 hr with either vinblastine (*A–D*) or cytochalasin E (*E–H*) to selectively disrupt the MTs or F-actin, respectively. Double-staining of α -tubulin (*A, E*) and F-actin (*B, F*) shows the selective perturbation of the axonal cytoskeleton. *In situ* hybridization, shown as DIC/fluorescence pairs, demonstrates that depolymerization of MTs eliminates the mRNA signal (*C, D*), whereas disruption of F-actin does not disturb the mRNA distribution (*G, H*). Scale bars, 20 μ m.

organization may be a common feature of localization by active mRNA transport (for review, see St. Johnston, 1995). Although we observed a decrease of the total hybridization signal along the axon during axonal outgrowth, prominent clusters of mRNA remained detectable in axons after several days of growth, hundreds of micrometers from the soma, and became progressively localized to specific regions—virtually all of the branch points and

varicosities. Thus, the axons maintain during outgrowth the capacity both to transport and to specifically localize mRNAs. The absence of signal from some branches of individual axons (Figs. 1*A*, 3*D*) suggests a functional significance for the mRNA localization at branch points: some form of sorting or selective targeting seems likely to occur at branch points, probably soon after their formation. This would be consistent with recent evidence

that in hippocampal neurons grown in culture different neurites of the same neuron contain a different populations of mRNA (Miyaishi et al., 1994). The presence of mRNAs at varicosities distributed along the axon shaft may also have a biological significance. These structures correspond to local dilations of the axon where mRNAs could be either trapped temporarily among organelles that aggregate at these sites, or specifically docked and sequestered by localized binding sites; the latter seems more likely in view of the tight association of a majority of cellular mRNA with the cytoskeleton (Hesketh and Pryme, 1991; Pachter, 1992; Suprenant, 1993).

The question arises whether axonal mRNA is a general feature of all neurons or a cytotypic specialization of some, because until recently the prevalent view was that protein synthesis occurred exclusively in the somatodendritic domain and that molecules required for the growth, maintenance, and compartmental specificity of the axon were targeted via undetermined intracellular mechanisms (Black and Smith, 1988; Black and Baas, 1989; Kelly and Grote, 1993) for anterograde axonal transport. The rigor and elegance of the body of work characterizing anterograde transport (for review, see Grafstein and Forman, 1980; Brady, 1991; Cleveland and Hoffman, 1991; Nixon, 1991; Vallee and Bloom, 1991; Hirokawa, 1993) have dominated our thinking about the formation of the axonal compartment, and the sheer volume of this flow would seem to obviate any need for local protein synthesis within axons. However, there is a growing body of evidence that in addition to proteins, mRNAs are transported within the axon in invertebrates and vertebrates (for review, see Van Minnen, 1994b). The evidence from invertebrates includes the detection in the squid giant axon of tRNA, rRNA, and active polysomes—contradicting the history of negative evidence for translational machinery in the axon—as well as several mRNAs (Perrone Capano et al., 1987; Rapallino et al., 1988; Crispino et al., 1993b), including those encoding the cytoskeletal proteins β -actin and β -tubulin (Kaplan et al., 1992), neurofilament protein (Giuditta et al., 1991), and the motor protein kinesin (Gioio et al., 1994). In addition, axons of molluscan peptidergic neurons were found to contain substantial amounts of neuropeptide-encoding mRNAs at a considerable distance from the soma (Landry et al., 1992; Van Minnen, 1994a), suggesting the existence of a sorting and transport mechanism for axonal mRNAs in these types of neuroendocrine cells (for review, see Van Minnen, 1994b). Finally, in another mollusk, *Heliosoma trivolis*, isolated growth cones from cultured neurons have been reported to possess the autonomous capacity for protein synthesis (Davis et al., 1992), providing indirect evidence for axonal transport of mRNAs. These latter axons are very short (only a few cell-body diameters), and one could dispute in cell biological terms whether they constitute a separate compartment from the soma.

In vertebrates, the transport of RNAs to the distal portion of axon has been demonstrated in just two types of neuron. In the magnocellular neurons of the hypothalamic neurosecretory system in the rat, mRNAs encoding neuropeptides have been identified and their axonal transport indirectly demonstrated (Jirikowski et al., 1990; Mohr et al., 1991, 1993; Mohr and Richter, 1992; Trembleau et al., 1994). Transcripts encoding several proteins (Mohr and Richter, 1992; Skutella et al., 1994) have also been found in the axonal domain of these cells, as has the untranslated BC1 RNA (Tiedge et al., 1993). In olfactory sensory neurons, the presence (Rogers et al., 1985; Ehrlich et al., 1990; Ressler et al., 1994; Vassar et al., 1994) and transport (Wensley et al., 1995) in axons of mRNA encoding olfactory marker protein

have been reported. Thus, varied evidence for the presence of axonal mRNAs now exists for both invertebrate and vertebrate neurons. However, the existence of axonal mRNA transport and localization as a general mechanism to provide a local supply of specific proteins in the distal axon is still not widely accepted. This may be because the extant evidence comes from large invertebrate axons, neurosecretory vertebrate neurons, and neurons that continuously regenerate. By providing direct evidence that axons of vertebrate peripheral neurons in culture do contain a heterogeneous and selective population of poly(A⁺) mRNAs, the present study shows that at least one type of cultured neuron with a more conventional axonal compartment possesses the capacity to select and transport mRNAs to the distal portion of axons.

mRNAs are transported and anchored in axons of sympathetic neurons in culture via an MT-dependent process

The mechanism commonly invoked to explain mRNA localization is active transport along the cytoskeleton (St. Johnston, 1995) followed by anchorage there (Pachter, 1992). To test whether this was the case for axonal mRNA in sympathetic neurons, we selectively disrupted the cytoskeletal elements by growing neurons in the continuous presence of specific inhibitors. Cultures were grown under conditions in which they extend processes devoid of MTs but possessing robust MFs or, conversely, under those conditions that give rise to processes lacking coherent MF arrays but containing a typical density of MTs (Fig. 5) (Marsh and Letourneau, 1984; Lamoureux et al., 1990; Morris and Hollenbeck, 1995). We found that mRNAs were restricted to the soma in all neurons with axons lacking MTs, whereas in axons that lacked MFs the signal was undiminished compared with control axons of similar length. These data indicate that MTs are involved in the access and transport of mRNAs into the axons, whereas MFs are not, and they also strongly favor active transport of mRNAs into the axon over entry by diffusion. This is consistent with reports from a variety of invertebrate and vertebrate cell types, in which mRNA localization is achieved by MT-dependent transport (Yisraeli et al., 1990; Ainger et al., 1993; Bassell et al., 1994; Ferrandon et al., 1994; Litman et al., 1994; Pokrywka and Stephenson, 1995). The access of mRNA to the axon in the presence, but not the absence, of MTs also argues against hypotheses that invoke the dense MT array of the axon hillock as an exclusion “filter” for mRNAs or mRNP particles (Steward and Banker, 1992). In any case, the entry of large organelles such as mitochondria into and along the axon (Morris and Hollenbeck, 1993) makes it unlikely that the axonal cytoskeleton serves as a physical barrier to active transport.

MT integrity was also necessary to maintain mRNA localization in axons, suggesting anchorage of mRNAs to axonal MTs. In established cultures, selective disruption of MFs had no significant effect on the mRNA localization in axons, whereas MT depolymerization abolished detectable axonal mRNA. The disappearance of signal that had been present throughout the axon within 1 hr of MT disruption seems likely to involve one or both of two phenomena: the displacement from focal anchoring sites may have caused mRNAs to become too diffusely distributed to be detected by our methods, or it may have increased their rate of degradation, perhaps through increased accessibility to RNases as reported by Berleth et al. (1988). This could also explain the significant diminution of signal that we also observed in cell bodies under conditions of MT disruption (Fig. 6D).

Only a subpopulation of mRNA is transported in axons of sympathetic neurons in culture

In view of our data showing that axons contain actively transported poly(A⁺) mRNAs, we examined whether the system of mRNA transport in axons showed some specificity. Among many possible candidates for axonal localization are mRNAs encoding proteins required in the distal axon, such as cytoskeletal proteins, those used mainly there, such as synapsins, or those associated with outgrowth, such as growth-associated protein-43. We chose to coamplify β -actin and α -tubulin mRNAs by RT-PCR to address the question of the selectivity of axonal transport because they are both major neuronal proteins, tubulin mRNA is reported to be restricted to the somatodendritic compartment (Bruckenstein et al., 1990; Kleiman et al., 1990), and β -actin mRNA is specifically translocated and translated at the leading edge of migrating fibroblasts (Lawrence and Singer, 1986; Taneja and Singer, 1990), a cytoplasmic domain having similar properties to the axonal growth cone (Bray and Hollenbeck, 1987). Using carefully chosen and controlled amplification conditions, we found that mRNA encoding β -actin was present in axons of peripheral neurons, whereas α -tubulin mRNA was restricted to the somatic compartment. These results show that the mRNA populations in the axonal and somatic compartments are qualitatively different: whereas both β -actin and α -tubulin are major axonal proteins transported by slow axonal transport mechanisms, only β -actin mRNA is selected and transported to the axonal domain.

The differential distribution of mRNAs encoding for specific cytoskeletal proteins that we report here for neurons has been demonstrated in other cell types. Recent studies have shown that myoblasts can select and target mRNA encoding the specific actin isoforms to their correct distinct sites: whereas the localization of α - and γ -actin mRNAs is restricted to the perinuclear region, β -actin mRNA is targeted and anchored to specific binding sites at the cell periphery (Hill and Gunning, 1993; Kislauskis et al., 1993). Similarly, peripheral neurons in our cultures possess the capacity to specifically select and target a subset of poly(A⁺) mRNAs to the axonal domain that include mRNA encoding β -actin but not α -tubulin. Because we obtained a bright hybridization signal in axons using oligo(dT) probes, but failed to detect β -actin mRNA consistently using specific probes (data not shown), we assume that the axonal mRNA population contains a variety of mRNAs. Analysis of axonal mRNA complexity is under investigation using other approaches.

What is the role of axonal mRNA?

Because axonal mRNAs are actively transported and specifically sorted, the presence of even small amounts of mRNA in distal axons seems likely to be physiologically significant. Although we lack direct evidence that mammalian axons carry out protein synthesis, local translation of these mRNAs in axons is an obvious hypothesis. However, it has been proposed recently that in magnocellular neurons axonal localization of specific mRNAs could serve to physically exclude them from the somatic translation machinery. Selected mRNAs, therefore, could be stored in axons in an untranslatable form and later, in response to specific stimuli, returned to the soma by retrograde transport and translated there (Mohr et al., 1993; Skutella et al., 1994). This hypothesis seems unlikely to explain the presence of mRNAs at the very distal portion of the axon. However, it is clear that different neurons may use axonal transport for different purposes depending on the cell type and the nature of encoded proteins. Whatever the

function of axonal mRNA in any particular neuron, our data extend previous studies showing that mRNAs can be localized within axons and open the question of the mechanism of their transport.

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