Journal of Neuroscience Research 64:447–453 (2001)

Local Synthesis of Nuclear-Encoded Mitochondrial Proteins in the Presynaptic Nerve Terminal

Anthony E. Gioio,[1] Maria Eyman,[2] Hengshan Zhang,[1] Zeno Scotto Lavina,[1] Antonio Giuditta,[2] and Barry B. Kaplan[1]★

[1]Laboratory of Molecular Biology, NIMH, NIH, Bethesda, Maryland
[2]Department of General and Environmental Physiology, University of Naples, Naples, Italy

One of the central tenets in neuroscience has been that the protein constituents of distal compartments of the neuron (e.g., the axon and nerve terminal) are synthesized in the nerve cell body and are subsequently transported to their ultimate sites of function. In contrast to this postulate, we have established previously that a heterogeneous population of mRNAs and biologically active polyribosomes exist in the giant axon and presynaptic nerve terminals of the photoreceptor neurons in squid. We report that these mRNA populations contain mRNAs for nuclear-encoded mitochondrial proteins to include: cytochrome oxidase subunit 17, propionyl-CoA carboxylase (EC 6.4.1.3), dihydrolipoamide dehydrogenase (EC 1.8.1.4), and coenzyme Q subunit 7. The mRNA for heat shock protein 70, a chaperone protein known to be involved in the import of proteins into mitochondria, has also been identified. Electrophoretic gel analysis of newly synthesized proteins in the synaptosomal fraction isolated from the squid optic lobe revealed that the large presynaptic terminals of the photoreceptor neuron contain a cytoplasmic protein synthetic system. Importantly, a significant amount of the cycloheximide resistant proteins locally synthesized in the terminal becomes associated with mitochondria. PCR analysis of RNA from synaptosomal polysomes establishes that COX17 and CoQ7 mRNAs are being actively translated. Taken together, these findings indicate that proteins required for the maintenance of mitochondrial function are synthesized locally in the presynaptic nerve terminal, and call attention to the intimacy of the relationship between the terminal and its energy generating system. J. Neurosci. Res. 64:447–453, 2001. **Published 2001 Wiley-Liss, Inc.†**

Key words: presynaptic nerve ending; local protein synthesis; nuclear-encoded mitochondrial mRNAs; polyribosomes

The mitochondrial genome encodes only a few proteins thus requiring the mitochondria to import several hundred nuclear-encoded proteins to maintain its structure and functional activity. Numerous studies in yeast and mammalian cells have demonstrated that mitochondrial activity includes a continuous import of nuclear-encoded proteins (for review, see Neupert, 1997).

In neurons, it is well established that mitochondria are translocated via fast axonal transport from the neuronal perikaryon to the axon and presynaptic nerve terminal. Very little is known, however, about the fate of these organelles after they reach their final destinations. If extra-mitochondrial protein synthesis is absent in the axon and nerve terminal, then the resupply of protein for mitochondria located in these neuronal domains must differ significantly from that occurring in the cell soma. Alternatively, the organelles present in the distal domains of the neuron could be short-lived and continuously resupplied by fast transport mechanisms.

Arguments dismissing extra-mitochondrial protein synthesis in the axon and presynaptic nerve terminal are largely based on the apparent absence of rough endoplasmic reticulum and free polyribosomes, as judged by conventional electron microscopic analysis. Yet, extensive biochemical and morphological data exists documenting protein synthesis in these regions both in invertebrates (Giuditta et al., 1991; Crispino et al., 1993a,b, 1997; Martin et al., 1997; Sotelo et al., 1999; Spencer et al., 2000) and vertebrate model systems (Koenig, 1991; Koenig and Martin, 1996). This large body of evidence has been recently reviewed (Koenig and Giuditta, 1999; Alvarez et al., 2000). That local de novo protein synthesis occurs in the neuronal growth cones is also well documented (Davis et al., 1992; Crino and Eberwine, 1996; Eng et al., 1999). In this regard, nucleic acid hybridization data indicate that approximately 100–200 different mRNAs are present in the squid giant axon (Perrone Capano et al., 1987) and by extension the presynaptic nerve terminal. Hence, a significant portion of extra-mitochondrial protein synthesis in the axon and presynaptic nerve ending

Contract grant sponsor: Italian Ministry of the University (MURST); Contract grant sponsor: University of Naples "Frederico II."

★Correspondence to: Barry B. Kaplan, Laboratory of Molecular Biology, National Institute of Mental Health, Clinical Center, Room 4N-222, 9000 Rockville Pike, Bethesda, MD 20892-1381.
E-mail: barry.kaplan@nih.gov

Received 3 January 2000; Revised 14 February 2001; Accepted 15 February 2001

could be responsible for the provision of nuclear-encoded mitochondrial proteins.

In this communication, we report that several mRNAs for nuclear-encoded mitochondrial proteins are present in the squid giant axon and the large presynaptic nerve terminals of the retinal photoreceptor neurons. Evidence is also provided to indicate that these mRNAs are present in synaptosomal polysomes and that a significant portion of the protein locally synthesized in the nerve terminal is associated with the mitochondrial fraction. Taken together, these findings call attention to the intimacy of the relationship that exists between the distal structural and functional domains of the neuron and their energy generating systems.

MATERIALS AND METHODS

Tissue Preparation and RNA Isolation

Squid (*Loligo pealii*) were obtained during the summer at the Marine Biological Laboratory (Woods Hole, MA). Animals were kept in tanks of running, chilled seawater and were used within 48 hr of their delivery. The giant fiber lobe (GFL), containing the cell bodies of the giant axon, was dissected from the stellate ganglion, and the axoplasm extruded from the giant axon using a Teflon roller (Perrone Capano et al., 1987). Total RNA was isolated from the axoplasm and GFL by extraction using TRISOL reagent (Sigma, St. Louis, MO) following recommended protocols.

Isolation of Synaptosomes and Mitochondria

Synaptosomes were isolated as described previously (Crispino et al., 1997). Briefly, 1 g of optic lobe was homogenized in 10 ml of 0.7M sucrose, 20 mM Tris-Cl (pH 7.4). The sample was centrifuged at 4°C in a JA-20 rotor of a Beckman J2-21M centrifuge (3,000 rpm × 11 min) and the supernatant collected and spun at 12,000 rpm × 30 min. The synaptosome fraction, which forms a pellicle floating on the surface of the preparation, was removed, washed with homogenizing solution, and was used immediately in in vitro labeling reactions and RNA preparations. The supernate on which the pellicle was floating was also recovered and centrifuged at 2°C in rotor SW41 of a Beckman ultracentrifuge (40,000 rpm × 2 hr) to sediment the microsomal fraction.

Total RNA was isolated from the synaptosomal and microsomal fractions by resuspension in TRISOL reagent (Sigma) after recommended protocols.

In in vitro labeling experiments, purified synaptosomes (50 μg/ml) were incubated in artificial seawater containing [^{35}S]methionine (25 μCi/ml) in the absence or presence of chloramphenicol (10–50 μg/ml) or cycloheximide (10 μg/ml) at room temperature (18–20°C) for 90 min. Incorporation was stopped by placing the sample on ice. The preparation was then centrifuged at 14,000 rpm in an Eppendorf microcentrifuge (15 min at 4°C), after that the synaptosomal pellet was resuspended in distilled water using a glass homogenizer. The sample was placed on ice for 30 min to allow for lysis, layered onto a discontinuous sucrose gradient (0.2–1.2 M), consisting of five layers varying by 0.2 M sucrose, and centrifuged in rotor SW55 (21,000 rpm × 2 hr at 2°C). The mitochondrial pellet was re-suspended in 50 μl of 10 mM Tris-Cl (pH 7.4), 100 mM NaCl, and 1 mM EDTA (pH. 8), and acid precipitable, alkaline resistant radioactivity measured on Whatman GFC filter discs (Giuditta et al., 1991).

Isolation and Fractionation of Polysomes

Polysomes were isolated as described previously (Perrone Capano et al., 1986; Giuditta et al., 1991). Briefly, synaptosome and microsome fractions (see above) were homogenized in 0.6 ml of 0.32 M sucrose, 250 mM Tris-Cl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 10 U/ml RNasin (Promega, Madison, WI). The post-mitochondrial supernate was made 0.5% (v/v) with respect to both Triton X-100 and sodium deoxycholate and was clarified by centrifugation in an Eppendorf centrifuge (10,000 rpm × 10 min. at 4°C). Polysomes were purified from the supernatant fraction by sedimentation through 2 M sucrose (0.1 ml) in 50 mM Tris-Cl (pH 7.4), 100 mM KCl, 2 mM $MgCl_2$, 10 mM dithiothreitol, (TKMD buffer) and centrifuged in a SW 50.1 rotor of a Beckman ultracentrifuge (36,000 rpm × 4 hr at 4°C). The polysome pellet was re-suspended in TKMD buffer and layered on a linear 15–45% (w/v) sucrose gradient (5.0 ml) in TKMD buffer and centrifuged in rotor SW 50.1 (36,000 rpm × 1 hr at 4°C). The gradient was divided into monosome and polysome fractions based on UV absorbance (254 nm) using an ISCO gradient fractionator. RNA was extracted from each fraction using TRISOL reagent.

Differential mRNA Display

These studies were carried out using the Delta Differential Display Kit (Clontech, Palo Alto, CA) after recommended protocols. First strand synthesis was carried out using 1 μg of total RNA combined with one of the nine oligo(dT) primers provided, MMLV reverse transcriptase, and incubated at 42°C for 1 hr. Differential display PCR was carried out using pair wise combinations of nine oligo(dT) primers and 10 arbitrary primers, Advantage KlenTaq Polymerase Mix with TaqStart Antibody (Clontech), and [^{33}P] dATP. Three low stringency cycles were carried out at 40°C followed by 25 high-stringency cycles at 60°C. PCR products were displayed by electrophoresis on 5% polyacrylamide gels and exposed to Biomax MR X-ray film (Kodak, Rochester, NY). cDNAs preferentially amplified from axoplasmic RNA were excised from the gel, and reamplified using the same primer sets. The reamplified cDNA was then ligated into the vector pGEM Eazy (Promega) and cloned using standard procedures. Fidelity of the clones was established by amplification of the insert using the original primer sets and comparing the size of the PCR products to the size of the bands excised from the gel.

DNA Sequencing and RT-PCR Analysis

DNA sequencing was carried out on a PE Applied Biosystems model 310 sequencer using Big Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA). Comparative sequence analyses were conducted using The BLAST X computer program to screen GENE BANK and SWISSPRO databases. RT-PCR was carried out using Superscript Preamplification System (Gibco BRL, Gaithersburg, MD). First strand cDNA was reverse transcribed from 1.0 μg of total microsomal or synaptosomal RNA using oligo-dT primers, and subsequently amplified using the primer

TABLE I. cDNA for Nuclear-Encoded Mitochondrial Proteins and HSP70

cDNA	Length cDNA (bp)	Blastx E value	Sequence similarity (%)	PCR primers	Amplicon size (bp)
COX 17	305	2.0E-14	78	[F] GCCCCAGACCTCAAAAGCTA [R] CGAAACCCATTGTTTTCATTC	108
CoQ7	513	4.0E-47	80	[F] CCTCACTAAATGCTGGGGAA [R] TTGCAGCCTCTTTGCCTATT	247
PCC	314	2.0E-18	78		
DLD	303	6.0E-09	91		
HSP70	321	1.0E-37	93	[F] CACAACCATTCCAACAAAACA [R] ACATTAAGAATGCCGTTGGC	214

pairs described in Table I. PCR conditions were 35 cycles, 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec for all samples. In experiments that employed polysomal RNA, 25% of the sample recovered from the monosomal and polysomal fractions of the sucrose gradient were used in the RT-PCR analysis.

RESULTS

Identification of mRNAs for Nuclear-Encoded Mitochondrial Proteins in the Axon

In previous work, we reported that the squid giant axon contains a diverse mRNA population that encodes key elements of the cytoskeleton, axon transport system and local energy metabolism to include: β-actin, β-tubulin, neurofilament proteins, kinesin and enolase (Giuditta et al., 1991; Kaplan et al., 1992; Gioio et al., 1994; Chun et al., 1995). In each case, the axonal localization of these mRNAs was confirmed by in situ hybridization histochemistry. Results of quantitative RT-PCR analysis established that the relative abundance of these mRNAs in the axon differed markedly from that in the parental cell bodies, suggesting that these mRNAs were differentially transported into the axonal compartment (Chun et al., 1996).

To further define the composition of this unique mRNA population, we have used differential mRNA display to compare mRNAs present in the axon and the cell bodies located in the GFL. The comparisons yielded 150 sequences with a higher relative abundance in the axon (data not shown). These fragments were excised from the gels, PCR-amplified using the same primer pairs employed for differential display, and affixed to Nytran Plus filters (Schleicher and Schuell, Keene, NH). The cDNAs were subsequently screened for evolutionary sequence conservation using [^{32}P] cDNA reverse transcribed from mouse brain mRNA as a probe. Fifty of the squid axoplasmic cDNAs cross-hybridized to the mouse brain cDNA under conditions of moderate stringency (0.6 M Na^+ at 55°C; data not shown). The length of the cDNAs ranged in size from 250–800 bp. The mRNAs identified fell into several broad categories to include cytoskeletal proteins, molecular motors, ribosomal proteins and translation factors as well as several novel mRNAs.

Surprisingly, DNA sequence analysis yielded four messages for nuclear-encoded mitochondrial proteins: COX 17, propionyl-CoA carboxylase (PCC; EC 6.4.1.3), dihydrolipoamide dehydrogenase (DLD; EC 1.8.1.4) and CoQ7 (Table I). In addition, the cDNA for HSP70, a chaperone protein, was also identified. The results of a BLASTX analysis showed a high level of sequence conservation for all five clones, with E-values ranging from 6.0E-09 for DLD to 4.0E-47 for CoQ7 (Table I). The percent sequence similarity between the squid sequences and the closest related species ranged from 78–93% at the amino acid level. An example of this extensive evolutionary sequence conservation is seen in Figure 1.

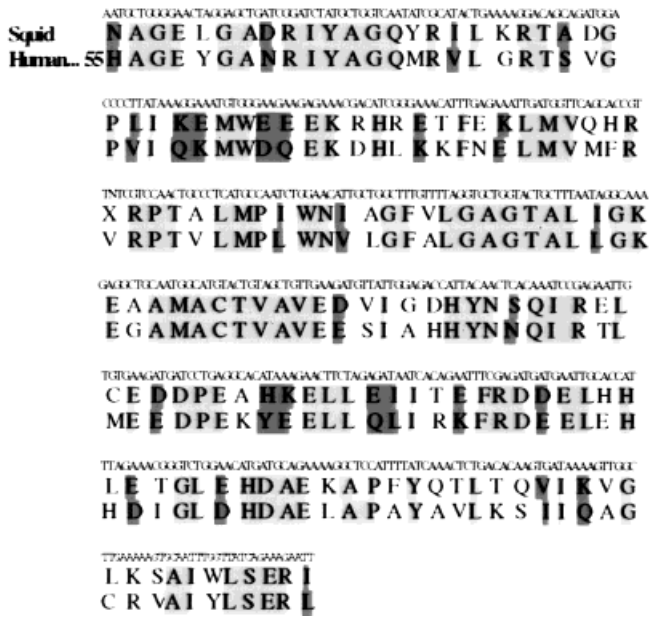

Fig. 1. Comparison of the partial amino acid sequence of squid CoQ 7 with human CoQ7 (Asaumi et al., 1999). The squid sequence is missing the first 55 NH_2-terminal amino acids of the human sequence. Lightly shaded regions signify sequence homology and darkly shaded areas indicate conservative substitutions.

The regions of marked sequence similarity revealed by BLASTX greatly facilitated the identification of these cDNAs with a high degree of certainty even though some of the sequences were relatively short. The cDNAs for COX17 and CoQ7 were almost full-length, encoding for all but the first 14 and 19 NH_2-terminal amino acids,

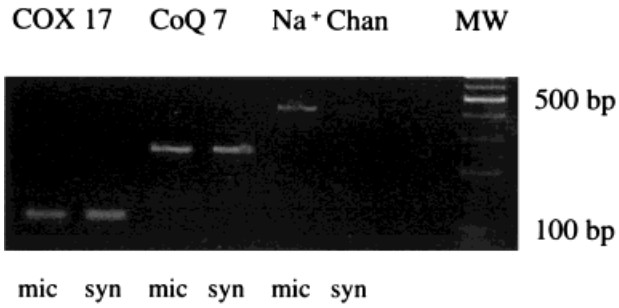

Fig. 2. RT-PCR analysis of total RNA in the synaptosomal and microsomal fractions. Total RNA (1.0 μg) was used in RT-PCR reactions containing the gene-specific primer sets provided in Table I. Conditions for each reaction are given in the Materials and Methods. mic, microsomal fraction; syn, synaptosomal fraction.

respectively. In the remaining three clones, the isolated sequences encoded a smaller portion of the full-length protein. Specifically, HSP70 coded for 103 amino acids of 646 residues comprising the full-length protein, PPC encoded 79 of 595 amino acids, and DLD encoded 34 residues of the component 478 amino acids.

Nuclear-Encoded Mitochondrial mRNAs are Present in Synaptosomes

Total RNA isolated from synaptosomal and microsomal fractions prepared from optic lobe were analyzed by RT-PCR to establish the presence of these mRNAs in the presynaptic nerve ending. A primer set for squid Na^+ channel, an mRNA shown previously to be relatively abundant in the giant fibre lobe and optic lobe but absent from the giant axon (Gioio et al., 1994; Chun et al., 1995), was used as a positive control. As seen in Figure 2, using gene-specific primers for COX 17 and CoQ7 amplicons were generated from synaptosomal and microsomal RNA, indicating that mRNAs for these proteins are present both in the perikaryon (microsomes) and presynaptic nerve terminals (synaptosomes). In contrast, RNA for Na^+ channel although present in the microsomal fraction was absent in the synaptosome. The absence of Na^+ channel amplicons in the synaptosomal PCR reactions demonstrates that the signals obtained for COX 17 and CoQ7 are not due to contamination by microsomal RNA.

To determine if synaptosomal polysomes contain nuclear-encoded mitochondrial mRNAs, polysomes were isolated from the synaptosomal fraction and were displayed on a linear 15–45% sucrose gradient. Two fractions were collected, the monosome fraction comprised of RNPs and monosomes, and the polysome fraction (Fig. 3A), and each fraction was subsequently analyzed by RT-PCR using a CoQ7 primer set. As shown in Figure 3B, CoQ7 mRNA was readily detected in the polysomal fraction, providing strong evidence that this message is undergoing translation. Similar results were obtained for HSP 70 and COX17 mRNAs (data not shown). The absence of amplicons generated from the Na^+ channel primers indicate that the polysome fraction is free of microsomal contamination as discussed above.

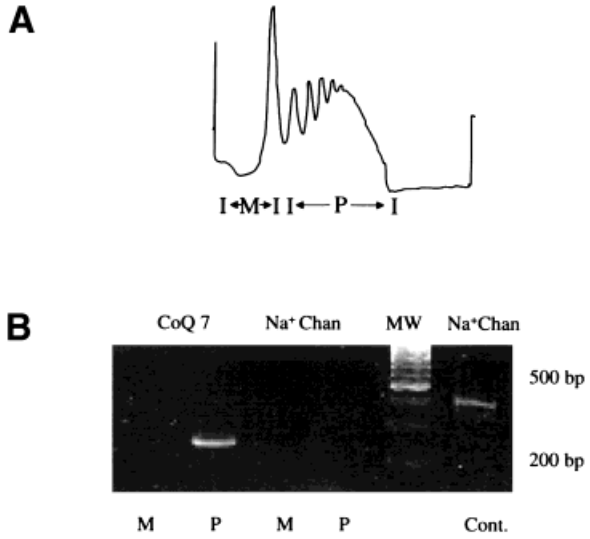

Fig. 3. Analysis of synaptosomal polysomes. **A:** Sedimentation profile of polysomes prepared from the synaptosomal fraction on a 15–45% sucrose gradient, UV absorbance 254 nm. Gradient was divided into monosome (M) and polysome (P) fractions. **B:** RT-PCR analysis of monosome and polysome fractions. PCR conditions found in Materials and Methods.

TABLE II. Translational Activity of the Synaptosomal and Mitochondrial Fractions

		Total CPM		
Exp.	Fraction	Control	Chloramphenicol	% inhibition
1	Syn	350,000	339,000	5.3
	Mit	89,720	71,583	20.0
2	Syn	100,000	94,000	6.0
	Mit	29,200	24,800	15.1
3	Syn	387,150	368,350	4.9
	Mit	43,225	34,755	19.6

Translational Activity of the Synaptosomal and Mitochondrial Fractions

Synaptosomes prepared from optic lobe were incubated with [^{35}S]methionine, lysed by osmotic shock, and the mitochondria subsequently isolated by differential centrifugation. Translational inhibition by chloramphenicol was used to discriminate between endogenous mitochondrial translational activity and cytoplasmic translation of mRNA. As shown in Table II, the translational activity associated with the mitochondrial fraction isolated from the synaptosomal preparation represents approximately 20% of the total translational activity of the synaptosomes. In the presence of chloramphenicol, an inhibitor of mitochondrial protein synthesis, total synaptosomal protein synthetic activity was inhibited an average of 5.3%, whereas inhibiting mitochondrial protein synthetic activity by an average of 18.2% (Table II, Exp. 1–3). These findings suggest that the majority (~80%) of the newly synthesized protein present in the mitochondrial fraction derive from cytosolic polyribosomes.

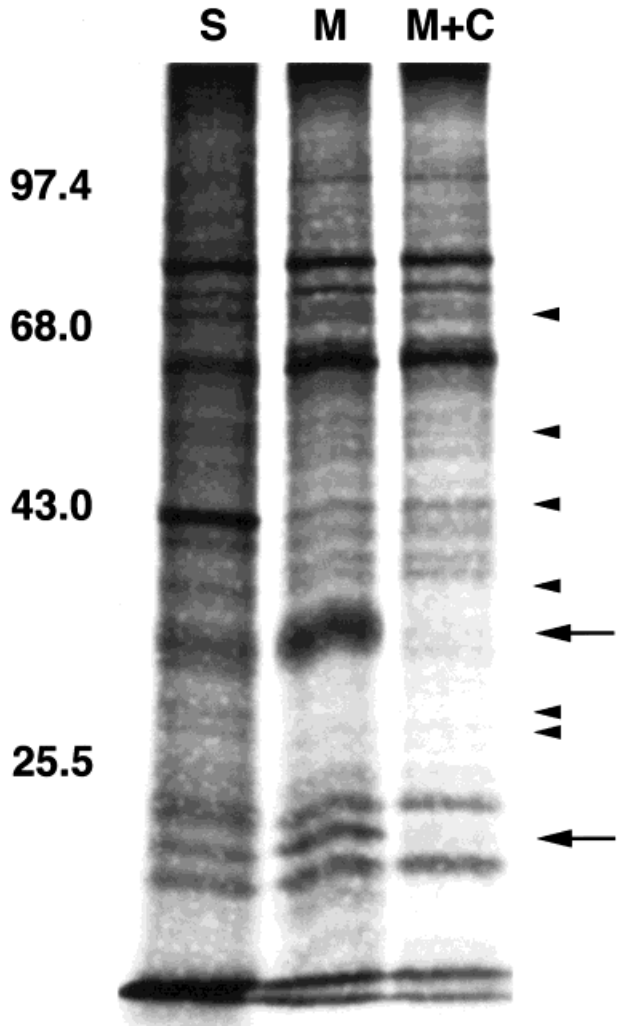

Fig. 4. Translational activity of the synaptosomal and mitochondrial fractions. Equal amounts of [^{35}S]met-labeled synaptosomal and mitochondrial protein were applied per lane (10,000 cpm) and were fractionated on a 10% polyacrylamide-SDS gel. S, synaptosomal fraction. M, mitochondrial fraction. M+C mitochondrial fraction isolated from synaptosomes incubated in the presence of 50 μg/ml chloramphenicol. Arrows indicate chloramphenicol sensitive translation products. Arrowheads mark differences in synaptosomal and mitochondrial translation products.

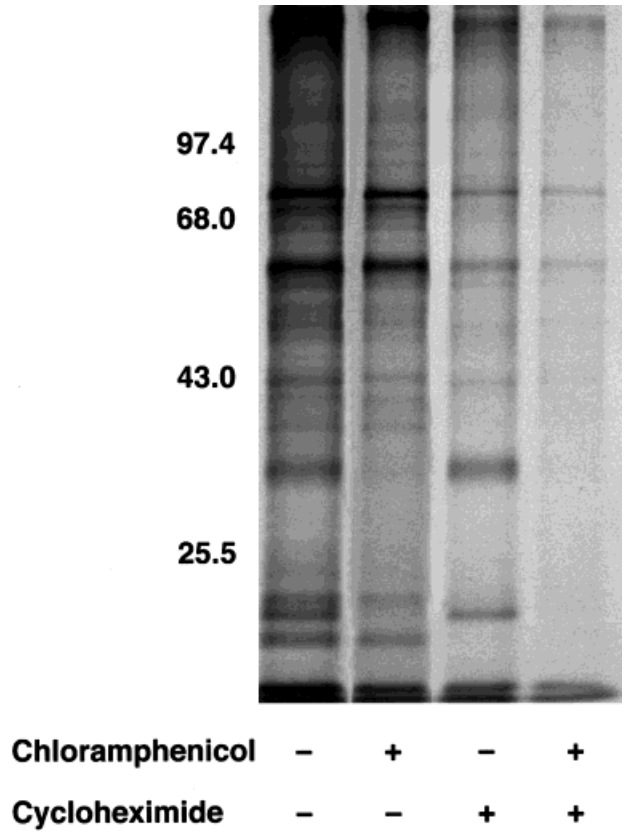

Fig. 5. Effects of chloramphenicol and cycloheximide on locally synthesized synaptosomal proteins associated with the mitochondrial fraction. All samples were isolated from synaptosomes incubated with [^{35}S]met. Ten μg of total protein was loaded per lane. **Lane 1:** Mitochondrial fraction from synaptosomes incubated without antibiotics; **lane 2:** mitochondrial fraction from synaptosomes incubated in the presence of 10 μg/ml chloramphenicol; **lane 3:** mitochondrial fraction isolated from synaptosomes incubated in the presence of 10 μg/ml cycloheximide; **lane 4:** mitochondrial fraction from synaptosomes incubated with both chloramphenicol and cycloheximide (10 μg/ml each).

Nuclear-Encoded Proteins Are Synthesized and Become Associated With Mitochondria in the Nerve Terminal

Comparison of the electrophoretic banding patterns of newly synthesized protein in the synaptosomal fraction and in the mitochondrial fraction isolated from the synaptosomes revealed several qualitative and quantitative differences, the most prominent examples being bands located at 69, 56, 42, 36 and a doublet at 28 kDa, (Fig. 4, arrowheads). In addition, the marked reduction in the abundance of β-actin (42 kDa) in the mitochondrial fraction, in conjunction with the enrichment of the mitochondrial-encoded 32 kDa protein (see below) suggests that the mitochondrial fraction was not heavily contaminated by cytosolic protein.

We next compared the banding patterns of newly-synthesized mitochondrial proteins when synaptosomes were incubated in the presence of chloramphenicol or cycloheximide, an inhibitor of cytoplasmic protein synthesis. In the presence of chloramphenicol, at least two major bands at 32 and 22 kDa (Fig. 4, arrows) were eliminated, whereas the synthesis of the majority of proteins remained unchanged. In contrast, cycloheximide blocked the synthesis of an entirely different set of proteins (Fig. 5). For example, the synthesis of the two major bands that were inhibited by chloramphenicol, (Fig. 4, arrows) was not affected by cycloheximide, whereas the synthesis of the majority of the bands was greatly diminished.

The results clearly indicate that the protein synthetic activity that had been demonstrated previously in synaptosomal preparations (Hernandez et al., 1976; Crispino et

al., 1997) is indeed comprised of both mitochondrial and eucaryotic components, supporting the hypothesis that active cytosolic polysomes are present in the nerve terminals. In addition, when comparing the effects of chloramphenicol and cycloheximide on the synthesis of protein in the mitochondrial fraction, the electrophoretic patterns indicate that both mitochondrial and cytosolic translated proteins cofractionate with mitochondria. This finding indicates that these nuclear-encoded, cytosolic proteins have been either transported into the mitochondria or alternatively are firmly attached to their outer surface.

DISCUSSION

Local protein synthesis followed by transport into the mitochondria is the accepted model for mitochondrial protein import in non-neuronal cells and the neuronal perikaryon. This may also be the case in the distal structural/functional domains of many large asymmetric sensory and motor neurons. This postulate is based upon data derived from two experimental approaches used to investigate this possibility. The first involved the identification of mRNAs for several squid nuclear-encoded mitochondrial proteins. Differential display of RNA from the GFL and axon identified the presence of four such proteins in the giant axon. Two of the proteins, PPC and DHD, are enzymes involved in synthesis of substrates for the tricarboxylic acid cycle. Two other proteins, COX17 and CoQ7, are both components of the electron transport chain. The association of these mRNAs with the polysomes in the presynaptic nerve terminal was subsequently established by RT-PCR, using synaptosome polysomal RNA from the optic lobe as a template. The demonstration that mRNAs for COX17 and CoQ7 are associated with translationally active polysomes provides strong evidence for their local synthesis. We also identified a mRNA for HSP-70, a chaperone protein involved in the import of proteins into mitochondria (Neupert, 1997), and established that the mRNA was present in synaptosomal polysomes.

The second approach took advantage of the protein synthetic system found in synaptosomes prepared from the squid optic lobe. This preparation, comprised predominately of axon–axonic synapses, has proven to be an invaluable system for the study of a wide range of physiological activities that occur at the presynaptic nerve terminal. (for review, see Cohen et al., 1990). Previous biochemical studies, as well as electron spectroscopic imaging and EM autoradiographic analyses have demonstrated that the large presynaptic nerve terminals present in the synaptosomal preparations contain polyribosomes and readily incorporate amino acids into proteins in a process sensitive to inhibition by cycloheximide (Hernandez et al., 1976; Crispino et al., 1993a,b, 1997). In this regard, it has been estimated that approximately two-thirds of the newly synthesized protein in the synaptosomal fraction is localized to the large nerve endings derived from the retinal photoreceptor cells (Crispino et al., 1997).

We confirm that synaptosomal protein synthetic activity is comprised of two components, cytoplasmic and mitochondrial, and present new data documenting the differences between the proteins synthesized on the cytoplasmic and mitochondrial ribosomal components. In addition, our data indicate that a subset of the cycloheximide sensitive synaptosomal proteins become associated with mitochondria.

The local translation of both nuclear-encoded mitochondrial proteins and chaperone proteins necessary for their import into mitochondria supports a model similar to that found in the soma and non-neuronal cells and suggests that a substantial portion of the protein synthesized in the presynaptic terminal may be directed toward fulfilling the biological demands of its energy generating system. Mitochondria import hundreds of different proteins encoded by nuclear genes. The mechanism of protein import into the mitochondria shows a large degree of similarity between lower eukaryotes, such as yeast, and higher mammalian species. The prevailing view has been that proteins are synthesized locally on free ribosomes, and that these proteins contain amino terminal targeting sequences that allow them to specifically bind to the surface of mitochondria and be translocated across the mitochondrial membrane. Cytosolic factors such as HSP 70 and mitochondrial import factor (MSP) are also involved in the folding and targeting of these proteins to the mitochondria (Neupert, 1997).

There is also evidence to suggest, however, that there is a direct attachment of ribosomes to the outer membrane of the mitochondria, and a direct link between translation and translocation. Studies in both yeast and rat liver using homologous in vitro translation systems and in vivo systems, demonstrate that the majority of the imported proteins may occur by a co-translational import system (Kellems et al., 1975; Fujiki and Verner, 1993; Crowley and Payne, 1998; Ni et al., 1999). What is clear in both models is that there exists an intimate relationship between the local synthesis and import of nuclear-encoded proteins into mitochondria. Our data suggests that a similar system exists in the squid giant axon and nerve terminal. Hence, the viability of the mitochondria located in the distal compartments of these large asymmetric neurons may be dependent on a local supply of cytosolic translated proteins.

Synapse function is highly dependent on mitochondrial oxidative phosphorylation. If pre-synaptic terminal mitochondria are dependent on a local supply of cytoplasmic proteins, it is likely that regulation of the local translation activity is tightly coordinated with synapse function and plasticity. Factors effecting the neurons ability to deliver or maintain a pool of functional mRNA in the axon and presynaptic terminal may also serve as a sites of regulation. Future investigations of the transport and translation of nuclear-encoded mRNAs for mitochondrial proteins may provide insights into neuronal function and possible involvement of the local protein synthetic system in neuropathy.

In conclusion, we have presented evidence that: 1) mRNAs for nuclear-encoded mitochondrial proteins are present in the axon and are associated with translationally active polysomes in the presynaptic nerve terminal; 2)

nuclear-encoded proteins are being actively translated in the synaptosomal fraction; and 3) that a portion of the newly synthesized nuclear-encoded synaptosomal proteins are associated with the mitochondria. Taken together, these findings provide strong evidence in support of the hypothesis that protein synthesis occurs in the distal domains of the neuron, and suggest that a major physiological function of this protein synthesis is for the maintenance of mitochondrial activity. In this regard, we suspect that the 19.5 kDa newly synthesized, cycloheximide sensitive, synaptosomal protein is CoQ7 (Fig. 4). The estimate of molecular weight based on the putative amino acid sequence of the protein is consistent with this observation. Western blot analysis of total synaptosomal protein using an antibody raised against a peptide corresponding to amino acid residues 90–108 of squid CoQ7 also exhibits a band of the correct size (data not shown). Definitive proof of this postulate will, however, require the immunoidentification of radiolabeled CoQ7 protein from the synaptosomal fraction.

ACKNOWLEDGMENTS

The authors thank Ms. Maria Grazia Migliardi and Ms. Angela Squillace for their excellent technical assistance, Ms. Simran Kalra for her assistance in the preparation of the article, and Dr. Howard Nash (NIMH) for his critical comments on the study. Portions of this work were carried out at the Marine Biological Laboratory (Woods Hole, MA), whose staff we thank for their kind cooperation and support. Financial support was obtained, in part, from the Italian Ministry of the University (MURST) and the University of Naples "Federico II".

REFERENCES

Alvarez J, Giuditta A, Koenig E. 2000. Protein synthesis in axons and terminals: significance for maintenance, plasticity and regulation of phenotype. With a critique of slow transport theory. Prog Neurobiol 62:1–62.

Asaumi S, Kuroyanagi H, Seki N, Shirasawa T. 1999. Orthologues of the *Caenorhabditis elegans* longevity gene clk-1 in mouse and human. Genomics 58:293–301.

Chun JT, Gioio AE, Crispino M, Giuditta A, Kaplan BB. 1995. Characterization of squid enolase mRNA: sequence analysis, tissue distribution, and axonal location. Neurochem Res 20:923–930.

Chun JT, Gioio AE, Crispino M, Giuditta A, Kaplan BB. 1996. Differential compartmentalization of mRNAs in the squid giant axon. J Neurochem 67:1806–1812.

Cohen RS, Pant HC, Gainer H. 1990. Squid optic lobe synaptosomes: structure and function of isolated synapses. In: Gilbert DL, Adelman WJ, editors. Squid as experimental animals. New York: Plenum Press. p 213–233.

Crino PB, Eberwine J. 1996. Molecular characterization of the dendritic growth cone: regulated mRNA transport and local protein synthesis. Neuron 17:1173–1187.

Crispino M, Castigli E, Perrone Capano C, Martin R, Menichini E, Kaplan BB, Giuditta A. 1993a. Protein synthesis in a synaptosomal fraction from squid brain. Mol Cell Neurosci 4:366– 374.

Crispino M, Perrone Capano C, Kaplan BB, Giuditta A. 1993b. Neurofilament proteins are synthesized in nerve endings from squid brain. J Neurochem 61:1144–1146.

Crispino M, Kaplan BB, Martin R, Alvarez J, Chun JT, Benech JC, Giuditta A. 1997. Active polysomes are present in the large presynaptic endings of the synaptosomal fraction from squid brain. J Neurosci 17: 7694–7702.

Crowley K, Payne M. 1998. Ribosome binding to mitochondria is regulated by GTP and the transit peptide. J Biol Chem 273:17278–17285.

Davis L, Dou P, DeWit M, Kater SB. 1992. Protein synthesis within neuronal growth cones. J Neurosci 12:4867–4877.

Eng H, Lund K, Campenot RB. 1999. Synthesis of beta-tubulin, actin, and other proteins in axons of sympathetic neurons in compartmented cultures. J Neurosci 19:1–9.

Fujiki M, Verner K. 1993. Coupling of cytosolic protein synthesis and mitochondrial protein import in yeast. Evidence for cotranslational import in vivo. J Biol Chem 268:1914–1920.

Gioio AE, Chun JT, Crispino M, Perrone Capano C, Giuditta A, Kaplan BB. 1994. Kinesin mRNA is present in the squid giant axon. J Neurochem 63:13–18.

Giuditta A, Menichini E, Perrone Capano C, Langella M, Martin R, Castigli E, Kaplan BB. 1991. Active polysomes in the axoplasm of the squid giant axon. J Neurosci Res 28:18–28.

Hernandez AG, Langor GM, Martinez JL. 1976. Protein synthesis by synaptosomes from the head ganglion of the squid *Loligo pealli*. Acta Cient Venezolana 27:120–123.

Kaplan BB, Gioio AE, Perrone Capano C, Crispino M, Giuditta A. 1992. β-Actin and β-tubulin are components of a heterogeneous mRNA population present in the squid giant axon. Mol Cell Neurosci 3:133–134.

Kellems R, Allison V, Butow R. 1975. Cytoplasmic type 80S ribosomes associated with yeast mitochondria. J Cell Biol 65:1–14.

Koenig E, Giuditta A. 1999. Protein synthesizing machinery in the axon compartment. Neuroscience 89:5–15.

Koenig E. 1991. Evaluation of local synthesis of axonal proteins in the goldfish Mauther cell axon and axons of dorsal and ventral roots of the rat in vitro. Mol Cell Neurosci 2:384–394.

Koenig E, Martin R. 1996. Cortical plaque-like structures identify ribosome-containing domains in the Mauther cell axon. J Neurosci 16:1400–1411.

Martin KC, Casadeo A, Zhu H, Yapeng E, Rose JC, Chen M, Bailey CH, Kandel ER. 1997. Synapse-specific long-term facilitation of aplysia sensory to motor synapses: a function for local protein synthesis in memory storage. Cell 91:927–938.

Neupert W. 1997. Protein import into mitochondria. Annu Rev Biochem 66:863–917.

Ni L, Heard TS, Weiner H. 1999. In vivo mitochondrial import: a comparison of leader sequence charge and structural relationships with the in vitro models resulting in evidence for co-translational import. J Biol Chem 274:12685–12691.

Perrone Capano C, Gioio AE, Giuditta A, Kaplan BB. 1986. Complexity of nuclear and polysomal RNA from squid optic lobe and gill. J Neurochem 46:1517–1521.

Perrone Capano C, Giuditta A, Castigli E, Kaplan BB. 1987. Occurrence and sequence complexity of polyadenylated RNA in squid axoplasm. J Neurochem 49:698–704.

Sotelo JR, Kun A, Benech JC, Giuditta A, Morillas J, Benech C R. 1999. Ribosomes and polyribosomes are present in the squid giant axon: an immunocytochemical study. Neuroscience 90:705–715.

Spencer GE, Syed NI, van Kesteren E, Lukowiak K, Geraerts WPM, van Minnen J. 2000. Synthesis and functional integration of a neurotransmitter receptor in isolated invertebrate axons. J Neurobiol 44:72–81.