SUBCELLULAR COMPARTMENTALIZATION OF mRNAs

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by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

Faculty of Medicine
Memorial University of Newfoundland

May 1986

St. John's Newfoundland

ISBN 0-315-33641-2

ACKNOWLEDGEMENTS

The author wishes to thank Dr. B. Sells, supervisor and mentor, for his constant guidance and encouragement throughout the project. Thanks are also due to Drs. D. Larson, G. Spedding and P. Zahradka for their valuable suggestions and advice during the project. The keen interest shown by Dr. J. Bag, faculty and students at Memorial University of Newfoundland, in the authors academic pursuits is deeply appreciated. The author also acknowledges with gratitude, financial support from Memorial University of Newfoundland during 1982-1983 and from the National Cancer Institute, Canada, during 1983-1986. Facilities extended to the author by the Chairman, Department of Molecular Biology and Genetics and the Dean's Office, College of Biological Science, University of Guelph, is gratefully acknowledged.

ABSTRACT

The importance of the cytoskeleton in protein synthesis was studied in differentiating L6 rat myoblasts. Soluble and cytoskeletal fractions obtained after gentle, non-ionic detergent lysis of myoblasts and myotubes were analysed for the presence of ribosomes and mRNPs. The role of the cytoskeleton in compartmentalization of specific mRNPs in myoblasts and myotubes was investigated.

mRNPs in polysomal arrays were primarily associated with the cytoskeleton. In addition, the cytoskeletal fraction also contained mRNA in the form of free mRNPs. Therefore, the association of mRNA with the cytoskeleton did not seem to depend on the presence of ribosomes for attachment. Furthermore, analysis of specific mRNAs in the various subcellular fractions of myoblasts and myotubes revealed differences in the distribution pattern of these mRNAs.

The effects of depolymerizing the microfilaments with cytochalasin B in myoblasts, was investigated. Treatment of myoblasts with cytochalasin B did not result in movement of ribosomes or specific mRNPs from the cytoskeletal fraction to the soluble fraction. This indicates that in L6 myoblasts, ribosomes and mRNPs are not associated with microfilaments. In addition, it was observed that cytochalasin B inhibited the incorporation of precursor into RNA and not protein. The effect on RNA synthesis, however, was due to an inhibition of uptake of precursor uridine, which was found to be reversible.

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CHAPTER ONE

Introduction

Expression of genetic information involves transcription of RNA molecules from DNA and the subsequent functioning of RNA molecules in protein synthesis. While transcription and translation occur in tandem in procaryotes, eucaryotic cells have evolved to separate these events both spatially and temporally. Eucaryotic gene transcripts are first generated in the nucleus and then transported across the nuclear membrane into the cytoplasm, to participate in protein biosynthesis. An understanding of gene regulation in eucaryotes therefore involves independent analysis of the events of transcription and translation.

A) Transcription

DNA transcription in both procaryotes and eucaryotes is performed by the enzyme DNA-dependent RNA polymerase. Although transcription of the various classes of RNA is performed by the same enzyme in procaryotes, transcription in eucaryotes is more specialized. There are three different RNA polymerases in eucaryotes. Polymerase I transcribes genes coding for ribosomal RNA's (18S, 5.8S and 28S), polymerase II transcribes mainly genes that encode proteins and small nuclear RNAs while polymerase III transcribes genes for tRNA, 5S ribosomal RNA and other small RNAs. Transcription by RNA polymerase I has been found to be species specific in contrast to that by polymerase II and III (Mishima, Financsek, Kominami & Muramatsu, 1982). Besides sequences at the 5' flanking region of the gene, initiation factors responsible for species specific rDNA transcription have been characterized in

HeLa cells and mouse L cells (Miesfeld & Arnheim, 1984). The 5' flanking region of mouse rRNA genes has been shown to contain the promoter and initiator sequences. In contrast, transcription by polymerase III is controlled by promoter sequences in the centre of the gene in the case of 5S rRNA (Sakonju, Bogenhagen & Brown, 1980) and tRNA genes (Galli, Hofstetter & Birnstiel, 1981; Ciliberto, Castagnoli, Melton & Cortese, 1982). Regulation of 5S rDNA transcription apparently is mediated through a 40K dalton protein which interacts specifically with the internal promoter. Although knowledge about the sequences controlling termination of transcription is scarce in the case of polymerase I, Brown & Gurdon (1977) have demonstrated the importance of a stretch of thymidine residues at the 3' terminus, in controlling termination of transcription by polymerase III.

RNA polymerase II transcribes genes coding for proteins besides producing transcripts of small nuclear RNAs. Initiation of transcription is controlled by a 'TATAA' consensus sequence about 30 nucleotides upstream from the transcription start site. Absence of this sequence from certain viral genes, however, does not seem to affect efficient transcription (Soeda, Arrand, Smolar, Walsh & Griffin, 1980; Baker & Ziff, 1981). In addition to transcription initiation control signals, the 5' region of polymerase II transcribed genes also contain sequences which regulate transcription in a specific manner. In contrast to the stretch of thymidine residues that controls transcription termination in case of polymerase III, most polymerase II transcription termination seems to be controlled by a sequence 'ATAAA' at the 3' end of transcriptional units. Although deletion of this sequence has been demonstrated to interfere with accurate transcription (Fitzgerald & Shenk, 1981), transcriptional units such as those for histones are devoid of this sequence and yet yield functional mRNAs

(Hentschel & Birnstiel, 1981).

In most cases the primary transcription products are modified by molecular alterations known as processing. Processing of RNA includes cleavage and trimming of large precursor molecules, terminal addition of nucleotides and various nucleoside modifications depending on the class of RNA. Whereas modification of tRNA and rRNA are equally complex in both procaryotes and eucaryotes, the processing of primary transcripts giving rise to mRNA in eucaryotes is very different from that in procaryotes. In procaryotes, formation of mRNA is a relatively simple process primarily involving transcription of an appropriate gene by RNA polymerase. The primary transcript of the gene is the mRNA and functions as such even before transcription is complete. In eucaryotic cells, the primary transcript must be processed before a mature mRNA is formed. The final processed product must then be transported from the nucleus to the cytoplasm to carry out its function. Experiments performed with both adenovirus and SV 40 indicate that only fully processed molecules enter the cytoplasm (Nevins, 1979; Piper, 1979) and there appears to be no cytoplasmic processing of mRNA. Furthermore, the portions of the primary transcript that are removed during RNA processing are not transported to the cytoplasm (Nevins & Darnell, 1978; Fraser, Nevins, Ziff & Darnell, 1979). It has been reported that prematurely terminated transcripts from the adenovirus late promoter are not transported to the cytoplasm (Ziff & Evans, 1978). It is also known that late in adenovirus infection most cellular RNAs fail to appear in the cytoplasm even though trahscription and RNA processing continue (Beltz & Flint, 1979). Thus there exists a control mechanism that regulates selective transport of functional RNA from the nucleus to the cytoplasm.

B) Translation

The decoding of the nucleotide sequences in the mRNA results in the synthesis of a polypeptide. This process of mRNA translation which occurs in the cytoplasm involves ribosomes and various translational factors functioning in the different steps of polypeptide synthesis. Ribosomes are cellular organelles which are the major structures of the translational machinery. The eucaryotic ribosome exists as an 80S ribonucleoprotein particle, composed of two subunits consisting of approximately 80 ribosomal proteins and 4 ribosomal RNA species. While the larger 60S ribosomal subunit consists of about 50 proteins and 3 rRNA species namely 28S, 5.8S and 5S, the 40S subunit consists of 30 proteins and the 18S rRNA. In contrast, ribosomes in procaryotes have been characterized as 70S particles comprising of 21 proteins and the 16S rRNA in the small 30S subunit and 32 proteins and the 23S and 5S rRNAs in the large 50S subunit.

Protein synthesis in both procaryotes and eucaryotes can be divided into three distinct steps characterized as initiation, elongation and termination. Although these steps are similar in both systems, details of the steps involved are much more complex in eucaryotes. Initiation of protein synthesis serves primarily to decode the initiation codon AUG which codes for methionine at the beginning of the cistron. This process involves GTP, ATP, various initiation factors, mRNA, ribosomal subunits and the initiator methionyl-tRNA. The steps that lead to the formation of the 80S initiation complex include ribosome dissociation and subsequent accumulation of ribosomal subunits followed by formation of a ternary complex containing eucaryotic initiation factor (eIF) -2, GTP and initiator methionyl-tRNA. The ternary complex then binds to the 40S subunits to form the 40S preinitiation complex followed by binding to the mRNA and the final joining of 60S ribosomal subunits to the 40S intermediary complex.

Since protein synthesis initiation requires the availability of ribosomes in the form of dissociated subunits, maintenance of ribosomes as subunits is one of the key steps in protein synthesis. Ribosomal subunits may be produced as a result of active dissociation of 80S ribosomes or prevention of spontaneously dissociating subunits from reassociation. Although the precise mechanism underlying this process is unclear, available evidence suggests the participation of eIF-3 isolated from native 40S subunits, in maintaining ribosomes in a dissociated state. eIF-3 preparations from rat liver appear to have both dissociation and anti-association activities (Thompson, Sadnik, Scheinbuks & Moldave, 1977). While in wheat germ extracts eIF-3 prevents only reassociation, a low molecular weight factor from the same extracts actively dissociates 80S ribosomes (Seal, Schmidt & Marcus, 1983). Finally, eIF-4C has been reported to act as an accessory factor to eIF-3 in ribosome dissociation (Goumans, Thomas, Verhoeven, Voorma & Benne, 1980). Whatever the mechanisms involved, it is clear that the interaction of eIF-3 with 40S subunits is an essential prerequisite to the subsequent reactions in forming the preinitiation complex.

Elements involved in forming the ternary complex include initiator methionyl-tRNA, GTP and eIF-2. Interactions between these elements are highly specific so much so that eIF-2 reacts with initiator methionyl-tRNA and 40S subunits but does not recognize any of the other aminoacyl-tRNAs or the 80S ribosome. Although ternary complexes can be formed with nonhydrolyzable analogues of GTP, the resulting complexes are inactive in promoting formation of the final 80S initiation complex. To maintain the cyclic nature of the various steps in protein synthesis, it seems important to have a recycling mechanism to keep both GTP and eIF-2 available for subsequent use in the crucial initiation process. This recycling of GTP and eIF-2 is performed by a polymeric protein

factor eIF-2B (Safer, 1983) which catalyzes guanine nucleotide (GDP/GTP) exchange on eIF-2 and stimulates the catalytic binding of initiator methionyl-tRNA to the initiation complex. The available data (Moldave, 1985) suggests a role for eIF-2B in recycling eIF-2 by converting the inactive fator (eIF-2.GDP) produced in the course of initiation to the active form (eIF-2.GTP). The importance of this step in protein synthesis has been demonstrated in studies with hemin-deficient reticulocytes. Phosphorylation of the alpha subunit of eIF-2 is promoted by hemin deficiency and results in the sequestration of eIF-2B. As a consequence, further initiation of protein synthesis is inhibited (Ranu & London, 1976). A similar sequestration of the recycling factor has also been observed in interferon treated cells where double stranded RNA induces the inhibition of protein synthesis (Petryshyn, Levin & London, 1982).

Formation of the 40S preinitiation complex is the next step in protein synthesis which includes binding of the ternary complex to the 40S subunit. Although no additional initiation factors are required for this step, the reaction is markedly stimulated by eIF-3. Observations made with a temperature sensitive yeast mutant suggest that the formation of the 40S.eIF-3 complex is obligatory for the interaction with the ternary complex (Feinberg, McLaughlin & Moldave, 1982). Regulation of protein synthesis at the level of initiation under various conditions has been encountered mostly at the step of mRNA binding to the 40S preinitiation complex. Besides ATP, several other factors including eIF-4A, eIF-4B, cap binding protein (CBP) I, and CBP II have been implicated in the interaction between mRNA and the preinitiation complex. In addition to increasing the stability of mRNA and its binding to the 40S subunits (Kozak & Shatkin, 1977), the cap structure has been implicated in selective translation of mRNAs in virus infected cells (Helentjaris & Ehrenfeld, 1978). Eucaryotic

mRNAs contain untranslated noncoding regions at both the 5' and 3' ends of the coding sequence. There is speculation implicating two conserved nucleotide sequences 5' to the initition codon in accurate and efficient binding and/or positioning of mRNA to 40S ribosomal subunits (Kozak, 1983). The enzymatic addition of the cap structure to a normally uncapped procaryotic mRNA allows translation in a eucaryotic cell-free system (Paterson & Rosenberg, 1979). At the same time, removal of the cap structure from a normally capped eucaryotic mRNA decreases its translational efficiency in such systems (Lodish & Rose, 1977; Wodnar-Filipowicz, Szczesna, Zan-Kowalczewska, Muthukrishnan, Szybiak, Legocki and Filipowicz, 1978).

The initiation step of protein synthesis results eventually in the formation of the 80S initiation complex. The initiation factor responsible for bringing about the joining of the large 60S ribosomal subunit to the 40S preinitiation complex is a monomeric protein, eIF-5. Interaction of eIF-5 with the preinitiation complex results in the hydrolysis of GTP and the release of eIF-2.GDP and eIF-3. This catalytic effect of eIF-5 takes place even in the absence of 60S subunits (Peterson, Safer & Merrick, 1979). Along with the formation of the 80S initiation complex, the eIF-2.GDP complex reacts with eIF-2B to generate active eIF-2.GTP for the next round of initiation reactions. The importance of GTP hydrolysis in protein synthesis initiation has been demonstrated in experiments with nonhydrolyzable analogues of GTP. In the presence of such analogues, the binding of initiator methionyl-tRNA and mRNA occurs, but the release of factors eIF-2 and eIF-3 and the joining of the two subunits does not take place (Safer, Peterson & Merrick, 1977). Thus the complex interplay of various initiation factors along with GTP and ATP, in the presence of ribosomal subunits, mRNA and initiator methionyl-tRNA finally

result in the formation of the 80S initiation complex which is then ready for the subsequent steps of elongation and termination.

The process of peptide chain elongation is a series of reactions on the 80S ribosome which results in translation of all the codons between the initiation and termination triplets. All the aminoacyl-tRNAs other than the initiator methionyl-tRNA are used in this process which also involves GTP and elongation factors. The reactions which include binding of the appropriate aminoacyl-tRNA to the ribosomal acceptor site, transpeptidation to form a peptide bond and movement of the ribosome on the mRNA, are repeated until a termination codon enters the acceptor site.

Peptide chain termination which is the final step in protein synthesis is brought about by an interaction between a recycling factor and the termination codon at the acceptor site. This reaction involves the hydrolysis of the peptidyl-tRNA bond, hydrolysis of GTP and the release of the completed polypeptide chain, mRNA and translational factors from the ribosome.

C) Translational Control

Of central importance to the study of gene expression in both procaryotes and eucaryotes is identifying the mechanisms by which formation of gene products is regulated. Control of the expression of a particular eucaryotic gene is reflected by the concentration of functional mRNA in the cytoplasm. Factors contributing to the ability of an mRNA to be functional eventually regulate its active participation in protein synthesis. For the past two decades it has been known that mRNA exists in the cell in the form of mRNA-protein complexes (mRNPs). In addition to their roles in mRNA processing (Pederson, 1983), mRNP complexes have been implicated in translational regulation.

Although our knowledge about the structure and function of various classes of RNP complexes is limited, progress has been made in understanding the significance of these complexes in translational control.

mRNP complexes are known to exist in the cell either free or in association with polysomes. It is generally believed that mRNPs not associated with polysomes contain a repressed class of mRNAs (Civelli, Vincent, Maundrell, Buri & Scherrer, 1980; Bag & Sells, 1981) while those present in polysomes are active in protein synthesis (Preobrazhensky & Spirin, 1978; Geoghegan, Cereghini & Brawerman, 1979). The repressed population of mRNA may represent either stored mRNA, mRNA in transit to polysomes, 'run off' mRNA released from a polysomal translational complex or simply excess mRNA in the cytoplasm (Spirin & Ajtkhozin, 1985). The repressed nature of the non-polysomal mRNA has been attributed to the presence of macromolecules such as proteins (Bag & Sells, 1979; Liautard & Egly, 1980) or other inhibitory factors including small RNA molecules (Bester, Kennedy & Heywood, 1975; Bag, Hubley & Sells, 1980) in the free mRNP complexes. Evidence currently available, however, does not point to a common translational control mechanism in the systems studied.

The identification of proteins in mRNA-protein particles has been assisted by the isolation of native mRNP complexes by UV crosslinking in vivo of proteins and mRNA. This technique of crosslinking has allowed isolation of mRNP complexes from cells under conditions that ordinarily disrupt mRNA-protein complexes. The information derived from these studies has lent credence to the specificity of mRNA-protein interactions (Greenberg, 1979; Greenberg, 1980). Using a direct approach of UV crosslinking deproteinized mRNA with cytosol RNA-binding proteins and comparing these mRNA-protein complexes with native mRNP complexes, the specificity and dynamic nature of

these interactions has been demonstrated (Gaedigk, Oehler, Kohler & Setyono, 1985). In a similar study (Greenberg & Carroll, 1985), proteins UV crosslinked to mRNA in reticulocyte polysomes were shown to be similar to those obtained by UV irradiating mouse L- and HeLa-cell polysomes. This same study revealed that the UV crosslinked protein composition of mRNP varied with the translational state of the mRNA. These results suggest that these proteins play a role in translation. Ruzdijic, Bag & Sells (1984) examined the protein composition of a specific mRNP in two different subcellular fractions. They demonstrated differences in proteins associated with histone mRNPs from polysomal and free mRNP fractions. A more compelling role for mRNP proteins in translation has been further supported by the work of Schmid, Kohler & Setyono (1982). They reported translational factor activity of mRNP proteins in a cell-free translation system devoid of translational factors.

Among the well known examples of translational control involving mRNP complexes are those described for globin and myosin heavy chain. In duck erythroblasts, mRNA for globin exists as a translationally active polysome-associated 15S mRNP and a free 20S mRNP that is translationally repressed. While both classes of mRNPs yield translationally active mRNA, only the 15S polysomal mRNP is translatable in vitro (Civelli, Vincent, Buri & Scherrer, 1976). Extensive characterization of the two types of mRNPs has revealed that specific sets of proteins are associated with globin mRNA in each of these functional states (Vincent, Goldenberg & Scherrer, 1981). A comparison of the proteins associated with the 20S globin mRNP and a 35S mRNP containing heterogenous non-globin mRNAs has revealed that specific polypeptides are associated with one of the two mRNA types while others are common to both mRNPs. This specificity of proteins associated with different

subpopulations of mRNA suggests a role for mRNP proteins in mRNA recognition and selective translational repression.

Participation of small molecular weight RNAs in translational control has been postulated in the embryonic chick muscle system. Kennedy, Siegel & Heywood (1978) have reported the association of an oligo(U)-containing RNA (termed translational control RNA) with free myosin heavy chain mRNP's in embryonic muscle. Using purified preparations of this RNA in an in vitro translation system, they reported selective inhibition of myosin heavy chain mRNA when compared with globin mRNA. Similar findings of a translational inhibitory RNA (iRNA) has been reported as a component of a novel cytoplasmic RNP complex (iRNP) in chick embryonic muscle. Both the iRNA and iRNP, however, have been shown to inhibit in vitro translation of mRNAs in a non-specific manner (Sarkar, 1984). A 4.4S RNA has also been described by Bag et al. (1980) as part of a 10-15S RNP particle. While examining the effect of this RNA in a cell-free translation system, they observed that it inhibits both capped and uncapped messages. They further demonstrated that inhibition of translation by this RNA occurs at the level of initiation.

Although the number of instances implicating mRNP complexes is small, there is growing evidence suggesting the involvement of translational control in gene regulation. Mobilization of pre-existing mRNAs into polysomes resulting in protein synthesis has been shown to be triggered by various factors. It is well known that unfertilized invertebrate and vertebrate eggs harbour a set of mRNAs that are maternally derived and are unavailable for translation. The sudden increase in the rate of protein synthesis triggered by fertilization is believed to involve a shift of this maternal mRNA from free mRNPs to polysomes (Davidson, 1976). The fact that the egg does not undergo mitosis until

after fertilization and that new proteins are necessary immediately after fertilization (Timourian & Uno, 1967; Wilt, Sakai & Mazia, 1967; Young, Hendler & Kanofsky 1969; Wagenaar, 1983) for the first cell division strongly suggests that maternal masked mRNAs specify these proteins. One such protein, ribonucleotide reductase, is necessary for a major cellular function: DNA replication (Thelander & Reichard, 1979; Holmgren, 1981). The activity of this enzyme is very low or undetectable prior to fertilization, and the appearance of enzyme activity requires protein synthesis but not transcription (Noronha, Sheys & Buchanan, 1972). Recent reports (Standart, Bray, George, Hunt & Ruderman, 1985) describe the abundance of the ribonucleotide reductase small subunit mRNA amongst the maternal masked mRNAs in clam and sea-urchin eggs. That this is one of the polypeptides synthesized immediately following fertilization, strongly implies control of gene expression at the level of translation.

Translational control has also been observed for mRNAs of ribosomal proteins during early developmental stages in Drosophila. Analysis of mRNA from various stages of Drosophila development suggests that while their levels are abundant, the association of at least two ribosomal protein mRNAs with polysomes is maximal during oogenesis, minimal during early embryogenesis and intermediate during late embryogenesis. In contrast, the levels of polysomal mRNAs for non-ribosomal proteins is relatively constant throughout these stages (Al-Atia, Fruscoloni & Jacobs-Lorena, 1985). Such selective translation of stored mRNAs has also been reported in Acetabularia differentiation (Li-Weber & Schweiger, 1985), Volvox development (Kirk & Kirk, 1985) and pea chloroplasts (Inamine, Nash, Weissbach & Brot, 1985).

D) Cellular Localization of Specific mRNAs

Another aspect of translational regulation which may play a significant role in controlling gene expression is the cellular localization of a specific mRNA. Ascidian eggs contain different pigmented ooplasmic regions of specific morphogenetic fate. These regions are rearranged after fertilization and are differentially segregated between the various embryonic cell lineages during cleavage. Using the technique of in situ hybridization, Jeffery, Tomlinson & Brodeur (1983) demonstrated that the general population, as well as specific mRNAs (actin and histone), are uniquely distributed in the various ooplasmic regions. Whereas most of the poly(A)-containing RNA is concentrated in the ectoplasm, actin mRNA is localized in the ectoplasm and myoplasm and histone mRNA is uniformly distributed between the different ooplasms. Following fertilization, these mRNAs migrate with their respective ooplasms and are differentially partitioned between the various cell lineages.

In a similar kind of investigation, Merlie and Sanes (1985) examined the distribution of acetylcholine receptors and poly(A)- containing RNA at the neuromuscular junctions of skeletal muscle fibres. The postsynaptic membranes in the neuromuscular junctions of skeletal muscle contains high levels of acetylcholine receptors. Fertuck and Salpeter (1976) and Salpeter and Harris (1983) showed that while the receptors are packed at a density of less than 30 per um^2 in extrasynaptic membranes, their density in postsynaptic membranes is over 15000 per um^2. Analysis of poly(A)-containing RNA from synapse-rich and synapse-free areas of mouse diaphragm muscle (Merlie & Sanes, 1985) revealed that there is a distinct enrichment of receptor mRNA in the synapse-rich region. This demonstration that acetylcholine mRNA is concentrated near synapses in adult muscle tissue provides evidence that the receptors are

preferentially synthesized in synaptic areas. This preferential distribution of mRNAs may reflect increased stability of mRNA or preferential transport of mRNA to its site of utilization.

E) Cytoskeleton

It is now well established that most eucaryotic cells contain a highly complex and structured meshwork of filamentous and irregularly connecting elements that extends throughout the interior of the cell (Fulton, 1984). This meshwork operationally defined as the cytoskeleton or cytoskeletal framework is resistant to extraction by non-ionic detergents. Immunofluorescence studies and ultrastructural analyses show the cytoskeleton to be composed of three major filament systems, namely microfilaments (Weber & Groeschel-Stewart, 1974; Lazarides, 1976; Heggeness, Wang & Singer, 1977), intermediate filaments (Jorgensen, Subrahmanyan, Turnbull & Kalnins, 1976; Osborn, Franke & Weber, 1977) and microtubules (Brinkley, Fuller & Highfield, 1975; Osborn & Weber, 1976). In addition, studies using electronmicroscopy have revealed a 2-3 nm diameter filament interconnecting the major cytoplasmic filaments giving rise to a 'microtrabecular network' (Wolosewick & Porter, 1979; Schliwa & van Blerkom, 1981).

Microfilaments which are 5-7 nm in diameter found as bundles, sheets or meshworks are composed largely of actin protein in the form of linear polymers. They have been implicated in various cellular functions including cell spreading and movement (Albrecht-Buehler & Goldman, 1976; Albrecht-Buehler, 1977) and exocytosis (Orr, Hall & Allison, 1972). It has been demonstrated that in addition to its involvement in cell spreading and movement of 3T3 cells, the microfilament containing 'microspikes' also mediate particle transport towards

the cell body (Albrecht-Buehler & Goldman, 1976).

The second major component of the cytoskeleton are the microtubules which are proteinaceous, 25 nm diameter filaments formed by linear polymerization of tubulin subunits. Unlike microfilaments wherein the subunit is a monomer, the tubulin subunit is a dimer containing alpha- and beta-tubulins. A variety of cellular functions and processes have been attributed to the microtubules. They are known to function in movement and positioning of nuclei during fusion of cells (Wang, Cross & Choppin, 1979), pigment translocation in erythrophores (Luby & Porter, 1980) besides other saltatory movements in cultured cells (Freed & Lebowitz, 1970). Although the exact mechanism is not clear, they perform an important function during mitosis. They form the structural basis of mitotic spindle and also assist in chromosomal movement between the two poles during mitosis (Pickett-Heaps, Tippit & Porter, 1982; Mitchison & Kirschner, 1984).

The third major class of filament systems termed intermediate filaments are 10 nm in diameter and function to integrate the various structures of cytoplasmic space. Unlike the microfilaments and microtubules, the intermediate filaments display a much larger range of tissue specific variation in their sequence (Lazarides, 1980). Five distinct classes, each characteristic of a particular cell type have been identified in higher eucaryotes. These include keratin filaments in epithelial cells, desmin filaments in muscle cells, vimentin filaments in cells of mesenchymal origin, neurofilaments in neurones and glial filaments in all types of glial cells.

The fourth class of cytoskeletal structure, the microtrabeculae, has not yet been assigned to any particular protein. Analyses using electronmicroscopy have revealed this element to be 2-3 nm in diameter and 30-300 nm long, and

interspersed between the other filaments described above. Due to their heterogeneity in size, it has been argued that this could be a group of proteins functioning in crosslinking the entire cytoskeletal network (Satir, 1984).

F) Role of the Cytoskeleton in Protein Synthesis

i) Association of Ribosomes with the Cytoskeleton

In addition to the above mentioned functions of the various cytoskeletal elements, reports over the past decade have suggested a role for the cytoskeleton in protein synthesis (Nielsen, Goelz & Trachsel, 1983). Examination of intact and unextracted cells by electronmicroscopy has revealed the existence of ribosomes at junctions of microtrabecular lattices, on filaments, or attached to membranes (Wolosewick & Porter 1979). Gentle extraction of cells with non-ionic detergent removes cellular lipids and soluble proteins. Analysis by high voltage electronmicroscopy indicated that such preparations correspond fairly well to those of unextracted cells (Lenk, Ransom, Kaufmann & Penman, 1977; van Venrooij, Sillekens, van Eekelen & Reinders, 1981). Although there is a complete absence of structures resembling mitochondria and endoplasmic reticulum, the strong retention of polysomes and membrane-depleted nuclei along with the filamentous network of the cytoskeleton are evident. In HeLa cells (Lenk et al., 1977) and human KB cells (van Venrooij et al., 1981), polysomes are not uniformly distributed throughout the cell space, but found in clusters around the nucleus and excluded from regions rich in intermediate filaments. Furthermore, Fulton, Wan & Penman (1980) observed that acridine orange staining of both intact and detergent extracted 3T3 cells reveals polysomes in the more central region of the cytoplasm and in close proximity to the microtrabeculae. This observation is substantiated by the fact that in cells

treated with ribonuclease or polysome disaggregating agents, RNA-specific stained regions disappear.

ii) Association of other Translational Components with the Cytoskeleton

It is evident from the literature that the process of protein synthesis involves many translational initiation factors. Polysomes being the active site of protein synthesis, would be expected to have translational initiation factors associated with them. Attachment of polysomes to the cytoskeleton suggests the possibility of a transient association of initiation factors with the cytoskeleton. Evidence for such an association of initiation factors with the cytoskeleton has been reported. Using a monoclonal antibody against a 50,000 MW cap binding protein, Zumbe, Stahli & Trachsel (1982), demonstrated the association of cap binding protein with the cytoskeleton in BHK cells. They further suggested this association to be most likely with the intermediate filaments. In similar studies on HeLa cells, Howe and Hershey (1984) have reported the interaction of ribosomes and protein synthesis initiation factors with the cytoskeletal framework. Comparing the distribution of initiation factors to that of total protein in both soluble and cytoskeletal fractions, they reported that except for several subunits of eIF-3, all factors were enriched in the cytoskeletal fraction.

The presence of mRNA on the cytoskeleton correlates with a functional role in protein synthesis in a number of systems. mRNA was isolated from the soluble and cytoskeletal fractions of HeLa cells (Cervera, Dreyfuss & Penman, 1981) and human KB cells (van Venrooij et al., 1981), and translated in an in vitro protein synthesizing system. Analysis of the resulting polypeptides by two-dimensional gel electrophoresis suggests a mechanism by which specific

mRNAs may be seggregated by being bound to the cytoskeleton. In VSV-infected HeLa cells (Cervera et al., 1981), adenovirus infected human KB cells (van Venrooij et al., 1981) and SV 40-infected BSC-1 cells (Ben-zeev, Horowitz, Skolnik, Abulafia, Laub & Aloni, 1981), host mRNAs were replaced with viral mRNAs on the cytoskeleton with a concomittant synthesis of viral proteins. These observations suggest that mRNA interaction with the cytoskeleton is a pre-requisite for protein synthesis. In a similar study analysing various classes of eucaryotic and viral mRNAs, Bonneau, Darveau & Sonenberg (1985) have concluded that association of mRNA with the cytoskeleton is necessary but not sufficient for protein synthesis and that the interaction of mRNA with the cytoskeleton is not dependent on the 5' terminal cap structure or the 3' poly(A) tail.

Although experiments described so far suggest an association of mRNA with the cytoskeleton as obligatory for protein synthesis, the nature of this association is still unclear. To determine whether mRNA and/or ribosomes are involved in this binding, van Venrooij et al. (1981) analysed the distribution of polysomes and mRNA on the cytoskeleton after treating human KB cells with various inhibitors of protein synthesis initiation. Pactamycin and NaF are potent inhibitors of protein synthesis initiation resulting in complete disaggregation of polysomes. Incubation of cells with pactamycin prior to extraction had minimal effect on the distribution of mRNA among the various fractions. Similarly, pre-treatment of cells with NaF failed to displace mRNA from the cytoskeleton, suggesting that ribosomes are not responsible for retaining mRNA on the cytoskeleton but rather the mRNA itself is involved. Extending such studies, Cervera et al. (1981) demonstrated that while polysomes containing viral specific mRNAs are associated with the cytoskeleton following viral infection of

HeLa cells, these polysomes have been found to disaggregate at higher temperatures, leaving the viral mRNA on the cytoskeleton. On the other hand, Howe and Hershey (1984) reported that in HeLa cells, ribosomal subunits continue to be associated with the cytoskeleton even after mild RNase treatment of the cytoskeletal fraction, thus questioning whether polysomes are attached to the cytoskeleton via mRNA.

iii) Cytoskeletal Component Involved in Anchoring Translational Machinery

Investigations into the cytoskeletal component involved in interacting with the translational machinery involves treatment of cells with a cytoskeletal disrupting agent and examining the distribution of ribosomes, translational initiation factors and mRNA in the various fractions. Cytochalasin B disrupts microfilaments in a wide variety of systems. In HeLa cells, Lenk et al. (1977) demonstrated that treatment of cells with cytochalasin B disrupted the association of mRNA and polysomes with the cytoskeleton. In similar studies, Howe and Hershey (1984) demonstrated that treatment of HeLa cells with cytochalasin B results in a pronounced shift of translational initiation factors and mRNA from the cytoskeleton to the soluble fraction suggesting a direct or indirect association of these components with microfilaments. Parallel studies by Jeffery (1984) in Ascidian eggs, however, suggested that such an association was independent of the integrity of microfilaments. Similar observations were made by Welch and Feramisco (1985) using rat embryo fibroblasts. Their results demonstrate that disruption of all three cytoskeletal elements failed to affect the ability of these cells to undergo an apparently normal heat shock response, implying normal activity of the protein synthetic apparatus.

G) Statement of the Problem

Eucaryotic cells can be separated into soluble and cytoskeletal fractions. These studies were designed to test the hypothesis i) that mRNA was attached to the cytoskeleton through its association with mRNP complexes and not through its association with ribosomes ii) that the mRNAs are attached to the microfilament element of the cytoskeleton and iii) that differentiation results in redistribution of mRNA between the soluble and cytoskeletal fractions in L6 muscle cells.

CHAPTER TWO

Materials and Methods

A) Growth and Maintenance of Cells

The L6 rat myoblast cell line used in these studies was originally obtained from Dr. C. P. Stanners of McGill University, Montreal. A subclone, L6-5 (Jacobs, Bird & Sells, 1985)(referred to as L6 in this thesis), derived from a single cell was chosen for these studies. Cells were grown on 15 cm diameter plastic tissue culture dishes in Growth Medium consisting of alpha-MEM (alpha modification of minimum essential medium) (90%, v/v), fetal bovine serum (10%, v/v), 5 international units per ml of penicillin, 5 ug per ml of streptomycin and $NaHCO_3$ (0.2%, v/v). The alpha-MEM was sterilized by filtration through a sterile 0.22 micron filter (Millistack, Millipore). Addition of supplements was done under sterile conditions. The cells were cultured at 37°C in a controlled environment of CO_2 (6%, v/v in air) and 100% humidity.

Continuity of the cell line was maintained by replating the proliferating myoblasts. The cells growing in a 15 cm diameter tissue culture dish were trypsinized with 2.5 ml of a sterile solution containing Hank's balanced salts, trypsin (0.25%, w/v), 10 mM EDTA and 10 mM Hepes (pH 7.2) at 37°C for 3 minutes. The cells were replated onto new tissue culture dishes at a density of approximately $2x10^5$ cells per ml of fresh medium. The cells were allowed to grow until 70% confluency, at a mean generation time of approximately 16 hours. At 70% confluency, a 15 cm diameter tissue culture dish contained approximately $2x10^7$ cells.

Differentiation was induced by changing the Growth Medium to Differentiation Medium when the cells had reached about 70% confluency. The

Differentiation Medium contained alpha-MEM (97.5%, v/v), donor horse serum (2.5%, v/v), 5 international units per ml of penicillin, 5 ug per ml of streptomycin and $NaHCO_3$ (0.2%, w/v). The Differentiation Medium was changed on the second day to give fully differentiated myotubes within 4 days. By this time, more than 90% of the myoblasts had fused to form myotubes.

Stocks of L6 myoblast cells were maintained in Growth Medium containing dimethyl sulfoxide (10%, v/v) under liquid nitrogen or in a -70°C freezer. Freshly trypsinized cells were pelleted (1Kxg for 3 minutes) and resuspended in the above mentioned medium at a concentration of $5x10^6$ cells per ml. A one ml aliquot of this suspension was placed in a 2 ml cryo-vial and immersed in ice for 30 minutes. The vials then were stored at -70°C overnight before removing to a liquid nitrogen freezer. To revive a frozen stock, a vial of frozen cells was thawed immediately at room temperature and pelleted (1Kxg for 3 minutes). The pellet was resuspended in 2 ml of Growth Medium and plated onto a culture dish at a final concentration of $2x10^5$ cells per ml of Growth Medium. All tissue culture materials were obtained from Flow Laboratories.

B) Cell Labelling Studies

To study the effect of cytochalasin B on RNA and protein syntheses, cells were grown in a 24 well tissue culture plate. Cells incubated in Growth Medium were treated with various concentrations of cytochalasin B and labelled with 5 uCi per ml (^{3}H)uridine (46 Ci per mmol, New England Nuclear) or 10 uCi per ml (^{3}H)lysine (100 Ci per mmol, New England Nuclear) for various periods of time. Each determination was performed in duplicate.

Following removal of the incubation medium, cells were lysed and

incorporation arrested by the addition of 60 ul of a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and SDS (1%, w/v). Aliquots (40 ul) of cell lysate were dried onto 2.5 cm Whatman #540 filter paper circles and washed in ice-cold trichloroacetic acid (20%, w/v) for 10 minutes. The (^{3}H)lysine labelled samples were further washed in trichloroacetic acid (20%, w/v) at 95°C for 10 minutes. All filters were subsequently rinsed in ethanol, washed with ether for 15 minutes and rinsed again in ethanol. The filters were then air dried and their radioactivity content was measured.

In determining the effect of cytochalasin B on uptake of (^{3}H)uridine into acid soluble material, analysis of acid soluble pool was performed as described by Hauschka (1973). Labelling and treatment of cells was essentially as above except that they were grown in 5 cm diameter tissue culture dishes. Following removal of incubation medium, cells were rinsed twice with cold phosphate buffered saline (PBS, 2.68 mM KCl, 1.47 mM KH_2PO_4, 137.0 mM NaCl and 8.09 mM Na_2HPO_4) containing unlabelled uridine (0.1%, w/v) and extracted with 1.5 ml of 0.4M $HClO_4$ for 30 minutes on ice. The extract was removed and neutralized by adding 0.75 ml of an ice-cold solution containing 0.72 N KOH and 0.6 M $KHCO_3$. After 5 minutes on ice, the neutralized extract was centrifuged at 12Kxg for 10 minutes and aliquots of the supernatant were taken to measure radioactivity.

To estimate the amount of (^{3}H)lysine in the acid soluble material following treatment of cells with cytochalasin B, cells were grown and labelled as above. Following removal of incubation medium, cells were rinsed twice with cold PBS containing unlabelled lysine (0.1%, w/v) and lysed with a solution containing trichloroacetic acid (20%, w/v) and unlabelled lysine (0.1%, w/v). The extract was placed on ice for 20 minutes and collected by centrifugation at

12Kxg for 10 minutes. Aliquots of the supernatant were then taken for radioactivity measurements.

C) Fluorescence Microscopy

NBD-phallacidin obtained from Molecular Probes Inc. was used to stain microfilaments according to the manufacturer's instructions. Cells were grown on tissue culture chamber slides. After two rinses in PBS, cells were fixed with a solution containing 3.7% (v/v) formaldehyde in PBS for 10 minutes at room temperature. The cells were again rinsed twice with PBS before extraction with acetone at -20°C for 5 minutes. After air-drying, a solution containing 33 ng of NBD-phallacidin in 200 ul of PBS was added to each slide and incubated for 20 minutes at room temperature. The cells were then mounted in a solution containing glycerol (50%, v/v) and PBS (50%, v/v) and viewed under a Nikon microscope with epifluorescent optics using the appropriate filters.

D) Subcellular Fractionation of L6 Myoblasts and Myotubes

To obtain soluble and cytoskeletal fractions, the cells were exposed to gentle detergent lysis. Polysomal and free mRNP material from each of these fractions were obtained by differential centrifugation. Lysis and extraction conditions were as described by Fey et al. (1984) with minor modifications incorporated as described below.

Monolayer cultures were incubated with emetine dihydrochloride, an inhibitor of protein synthesis (0.2%, w/v), at 37°C for 5 minutes prior to extraction. The Growth Medium from a 15 cm culture dish of L6 cells was decanted and the monolayer rinsed while maintained on an ice-slurry, with 5 ml of an ice-cold solution of PBS. The cells then were treated with 8 ml of an

ice-cold Extraction Buffer containing 20 mM Pipes (pH 6.8), 10 mM KCl, 2.5 mM $MgCl_2$, 0.3 M sucrose, 0.3 mM phenylmethyl sulfonyl fluoride, 0.1 mM aurintricarboxylic acid, 10 mM dithiothreitol, Triton X-100 (0.1%, v/v) and 5 units per ml of human placental ribonuclease inhibitior (HPRI) (Amersham) for 2 minutes. The extracted material obtained from the cells under these conditions was referred to as the 'soluble fraction'. The remaining unextracted material contained cell remnants including the 'cytoskeletal fraction'. To obtain polysomes and free mRNPs from the cytoskeletal fraction, this latter material was scraped with a rubber 'policeman' and homogenized in a Dounce homogenizer with 8 ml of ice-cold Cytoskeletal Extraction Buffer containing 20 mM Pipes (pH 6.8), 0.25 M $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.3 M sucrose, 0.3 mM phenylmethylsulfonyl fluoride, 0.1 mM aurintricarboxylic acid, 10 mM dithiothreitol, Triton X-100 (0.5%, v/v) and 5 units per ml of HPRI. Both the soluble and cytoskeletal fractions thus obtained were centrifuged (2.5Kxg for 5 minutes) to remove nuclei and thereby obtain the post-nuclear supernatants.

After the addition of polyvinyl sulphate (PVS) (0.01 volumes of a stock solution (1%, w/v), each extract was layered over 4 ml of a solution containing sucrose (30%, w/v), 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$ and 0.1 mg per ml of PVS, and centrifuged (176Kxg for 70 minutes) to obtain the polysomal pellet. PVS was again added to the post-polysomal supernatant and further centrifuged (176Kxg for 18 hours) to obtain the free mRNP material. Both the polysomal and free mRNP pellets were stored at -70°C until needed.

E) Distribution of Macromolecules in the Soluble and Cytoskeletal Fractions

To determine the distributions of RNA and protein in the soluble and

cytoskeletal fractions, L6 myoblasts were labelled (separately) overnight with 5 uCi per ml (^{3}H)uridine (46 Ci per mmol, New England Nuclear) and 5 uCi per ml (^{35}S)methionine (800 Ci per mmol, Amersham) respectively, prior to extraction of the two cytoplasmic fractions. Aliquots of the post-nuclear supernatants were analysed for incorporation of radioactivity into acid insoluble material as described in section B.

F) Analysis of Polysomes

Post-nuclear supernatants obtained from the two cytoplasmic fractions were subjected to velocity sedimentation on a sucrose gradient to separate monosomes and polysomes. L6 myoblasts which had been incubated overnight in Growth Medium containing 2 uCi per ml (^{3}H)uridine (46 Ci per mmol, New England Nuclear) were separated into soluble and cytoskeletal fractions as described in section D. Equal volumes of the post-nuclear supernatant from each fraction were analysed on a 15-40% linear sucrose density gradient.

Linear gradients of 15-40% (w/v) sucrose were prepared with a linear gradient former (Hoefer Scientific Instruments). The reservoir chamber of the gradient former contained 5.5 ml of a solution containing sucrose (15%, w/v), 10 mM Tris-HCl (pH 7.5), 1.5 mM phenylmethylsulfonyl fluoride, 100 mM KCl, 5 mM $MgCl_2$ and heparin (0.01%, w/v). The mixing chamber contained 5.5 ml of a solution containing sucrose (40%, w/v) dissolved in the aforementioned buffer. A 12 ml Beckman 'SW 41' polyallomer tube was filled with the sucrose gradient from the bottom upwards and cooled to 4^{o}C before the sample was loaded. After centrifugation in a Beckman 'SW 41' rotor (40K rpm at 4^{o}C for 120 minutes), the gradients were fractionated from the bottom using a peristaltic pump and the radioactivity in each fraction was measured.

G) Purification of RNA from L6 Myoblasts and Myotubes

i) Preparation of RNA from Subcellular Fractions

RNA was prepared from polysomal and free mRNP pellets obtained from both the soluble and cytoskeletal fractions. RNA was isolated essentially by the method of Maniatis, Fritsch & Sambrook (1982). The mRNP pellets (obtained in section D) were resuspended in 2 ml of a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and SDS (1%, w/v). Following addition of Protease K (0.05%, w/v) (0.1 volumes of a freshly prepared stock solution of 1%, w/v), the resuspended material was incubated for 60 minutes at 37°C in a rotating water bath to digest the proteins. The digests were placed in 15 ml sterile polypropylene tubes and 1 volume of phenol which was pre-equilibrated with 0.1 volumes of a stock solution of 1 M Tris base, was added to the incubation mix. The contents were mixed thoroughly by either horizontal or vertical agitation for 5 minutes after which, one volume of chloroform was added. The contents in the tube were mixed thoroughly again for 1 minute. The organic and aqueous phases were separated by centrifugation (2Kxg for 5 minutes). The lower organic phase was removed and discarded without disturbing the interphase. Following 2 more chloroform extractions, the aqueous phase was removed, leaving the interphase material behind. RNA in the aqueous phase was precipitated by the addition of ammonium acetate (0.5 volumes of a 7.5 M stock solution) and ethanol (2 volumes) and stored at -20°C overnight.

The RNA preparation was pelleted by centrifugation (10Kxg. for 30 minutes) and washed once with 70% ethanol. The pellet was dissolved in a solution containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Quantitation of RNA was done by measuring absorbance at 260, 280 and 320 nm. The amount of

RNA was calculated using the following formula:

$$(OD_{260} - OD_{320}) \times 40 = \text{ug per ml.}$$

An $(OD_{260} - OD_{320}) : (OD_{280} - OD_{320})$ ratio of 2 was considered to be pure RNA.

ii) Oligo(dT)-cellulose Chromatography of RNA

Preparation of poly(A)-containing RNA from total purified RNA was accomplished using oligo(dT)-cellulose chromatography (Bantle, Maxwell & Hahn, 1976). Approximately 200 mg of oligo(dT)-cellulose (Type III, Collaborative Research) was suspended in sterile RNase-free water. Following removal of fines, the resin was poured into a 0.7 cm i.d. x 10 cm RNase-free column (Bio-Rad). The column was washed with 1 bed volume of a solution containing 0.1 M NaOH, followed by 10 bed volumes of Binding Buffer containing 10 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA and SDS (1%, w/v).

The RNA sample to be chromatographed was taken up in Binding Buffer. The sample was heated to 65°C for 1 minute, NaCl added (0.1 volumes of a 5 M stock solution) and the sample brought to room temperature immediately by immersing in an ice-water bath. Following passage of the RNA sample through the oligo(dT)-cellulose column 3 times, the column was washed with 10 bed volumes of Binding Buffer. The material bound to the oligo(dT)-cellulose column was eluted with 3 bed volumes of Elution Buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and SDS (0.1%, w/v).

Following elution, the oligo(dT)-cellulose column was re-equilibrated with 10 bed volumes of Binding Buffer and the once chromatographed RNA sample was rechromatographed 2 more times, after bringing up the salt concentration to 0.5 M NaCl each time. The eluted poly(A)-containing RNA was then

precipitated by incubation overnight at -20°C, with ammonium acetate (0.5 volumes of a 7.5 M stock solution) and 2 volumes of ethanol.

H) Electrophoresis of RNA

RNA samples were size fractionated by electrophoresis in an agarose (1.2%, w/v) gel. The Gel Buffer contained 25 mM Mops (pH 7.5), 5 mM Na acetate and 2 mM EDTA. The RNA samples obtained above were centrifuged (12Kxg for 30 minutes in a Beckman Microfuge II). The pellet was rinsed once with 70% ethanol and dissolved in 15 ul of Sample Buffer containing freshly deionized formamide (50%,v/v), Gel Buffer (25%, v/v) and formaldehyde (25%, v/v) and heated at 60°C for 15 minutes. Tracking dye (5 ul) containing glycerol (50%, v/v), bromophenol blue (0.1%, w/v) and xylene cyanol (0.1%, w/v) was added to the samples before electrophoresis. Electrophoresis was conducted at 100 volts towards the anode until the bromophenol blue dye moved through 80% of the gel.

I) Northern Blot Analysis

After size fractionation, the RNA was immobilized by electroblotting onto a solid support for further qualitative and quantitative analyses of specific RNA species. The solid support used in these studies was DPT-paper and was prepared as described in the following section.

i) Preparation of DPT-Paper

Diazotized aminophenylthioether paper (DPT-paper) used as the solid support was obtained by freshly diazotizing aminophenylthioether paper (APT-paper) (Seed, 1982). APT-paper was prepared by treating 5 sheets of 20

cm^2 filter paper (Type 589 WH, Schleicher & Schuell) with 100 ml of a solution containing 350 mM NaOH and butanediol diglycidyl ether (30%, v/v, Aldrich Chemicals) in a Super Sealobag (Phillips Electronics) overnight with constant agitation at room temperature. After discarding the reaction mixture, the paper was further treated with a solution containing 11.5 ml of aminophenylthioether and 40 ml of acetone. The reaction was carried out in the same bag containing the filter paper and the bag was resealed and agitated at room temperature for 24 hours. The APT-paper was washed twice for 15 minutes, once in ethanol, then in 0.1 M HCl. After a final rinse with ethanol, the APT-paper was air dried and stored in a Super Sealobag in the dark. For further use, the APT-paper was diazotized in 200 ml of a solution containing 1.2 M HCl and 25 mg $NaNO_2$ at 0°C for 15 minutes. After 5 brief rinses in ice-cold water followed by 2 in Transfer Buffer containing 25 mM KH_2PO_4 (pH 5.0), the DPT-paper was ready for use. The diazotized paper thus prepared was used immediately.

ii) Electroblotting of RNA

After electrophoresis, the RNA gel to be electroblotted was treated with 100 ml of 0.2 M NaOH for 20 minutes. The gel was then briefly rinsed 3 times with distilled water followed by 2 washes in 100 ml 8x Transfer Buffer for 10 minutes each and then two 10 minutes washes in 1x Transfer Buffer. A piece of freshly diazotized APT-paper and 4 pieces of chromatography paper (3MM, Whatman) were cut to the size of the gel to be electroblotted and were soaked in Transfer Buffer for 10 minutes. The gel and DPT-paper were placed between 2 sheets each of chromatography paper and was then sandwiched between 2 layers of Scotch-Brite pad (3M Co.). The sandwich was then placed in an

electroblot chamber (E-C Apparatus) and transfer carried out for more than 6 hours at 4°C. Transfer was performed at 3.6 volts per cm towards the anode with recirculation of the Transfer Buffer. The DPT-paper was thoroughly washed with Transfer Buffer to remove any residual agarose, prior to pre-hybridization.

iii) Pre-hybridization

The RNA electroblotted onto DPT-paper was pre-hybridized to reduce non-specific binding sites. Pre-hybridizaion was carried out in a solution containing freshly deionized formamide (50%, v/v), 5x Modified Denhardt's Solution, 5x SSC, 100 ug per ml of poly(A), 100 ug per ml of yeast tRNA, SDS (0.2%, w/v), glycine (1%, w/v) and 25 mM Tris-HCl (pH 7.5). Deionized formamide was obtained by treating formamide (Ultrapur, Bethesda Research Laboratory) with 0.2 volumes of ion-exchange resin (RG 501-X8, Bio-Rad), with constant agitation for 1 hour at room temperature. Modified Denhardt's Solution (1x) contained Ficoll (0.2%, w/v), polyvinylpyrrolidone (0.02%, w/v), 5 mM Mops (pH 7.5) and 1 mM EDTA. SSC (1x) contained 0.15 M NaCl and 0.3 M tri-sodium citrate (pH 7.0). The pre-hybridization solution added at 10 ml per 100 cm^2 blot area was sealed in a Super Sealobag containing the Northern blot. Pre-hybridization was carried out at 42°C overnight, with constant mixing on a rotator.

iv) Hybridization

After pre-hybridization, the RNA in the Northern blots was hybridizd with (^{32}P)-labelled specific plasmid DNA. Hybridization was performed in the same solution used for pre-hybridization except that poly(A) and glycine were

omitted. Labelled plasmid DNA (see section J (iii)) was included at a specific activity of 4×10^6 cpm per ml of hybridization solution. Immediately prior to use, plasmid DNA was denatured by incubating in 0.2 M NaOH for 15 minutes at room temperature. The denatured plasmid DNA was neutralized with 1 volume of 1 M Tris-HCl (pH 7.5) followed immediately by the addition of deionized formamide and the remaining constituents of the hybridization solution. The pre-hybridization solution in the Super Sealobag was then replaced with an equal volume of the hybridization solution and hybridization carried out at 42°C for 48 hours, with constant mixing on a rotator.

Following hybridization, the Northern blot was washed with 5 changes in 200 ml of a solution containing 2x SSC and SDS (0.1%, w/v) at room temperature over 100 minutes. This was followed by 20 minute washes in 200 ml of a solution containing 0.2x SSC and SDS (0.1%, w/v) until the washes contained less than 50 cpm of Cerenkov radiation per 10 ml of wash solution.

v) Reprobing Northern Blot

The (^{32}P)-labelled DNA probe was removed from the Northern blot before subsequent reprobings of the blot with other (^{32}P)-labelled DNA probes. To remove the old probe, the blot was washed in a solution containing formamide (95%, v/v), 10 mM EDTA, 50 mM Tris-HCl (pH 7.5) at 50°C for 60 minutes. This was followed by washing twice in a solution containing 2x SSC and SDS (0.1%, w/v) for 20 minutes at room temperature, prior to pre-hybridization.

J) Plasmid DNA

i) Purification of Plasmid DNA

Plasmid DNA was purified according to the procedure described by

Birnboim & Doly (1979). Bacteria, transformed with various plasmid DNAs containing specific sequences, were available in the laboratory. A 500 ml solution of LB medium (125 mM NaCl, yeast extract (0.5%, w/v) and tryptone (1%, w/v)) containing the appropriate antibiotic was inoculated with a 5 ml aliquot of an overnight culture of bacteria containing the desired plasmid. The culture was grown at 37°C with constant agitation until it reached an optical density of 100 Klett units (approximately 1 x 10^8 bacteria per ml). Amplification of plasmid production was effected by addition of chloramphenicol (500 ul of a 20 mg per ml stock solution in ethanol) and further incubation at 37°C overnight with constant agitation.

The plasmid DNA amplified bacterial culture was harvested by centrifugation (5Kxg for 5 minutes) and washed once with 25 ml of a solution containing 10 mM Tris-HCl (pH 7.5) and 10 mM EDTA. The bacteria were resuspended in 4 ml of a solution containing 25 mM Tris-HCl (pH 7.5), 10 mM EDTA and 2 mg per ml of freshly prepared lysozyme (Boehringer Mannheim). The suspension was incubated on ice for 30 minutes and the bacteria lysed by the addition of 8 ml of a solution containing 0.2 M NaOH and SDS (1.0%, w/v). After 5 minutes on ice, the lysate was further treated with 6 ml of a solution containing 3 M sodium acetate (pH 4.8). The reactants were gently mixed and allowed to stand on ice for 1 hour. Following centrifugation (16Kxg for 30 minutes) at room temperature, the plasmid DNA in the supernatant was precipitated with 2 volumes of ethanol and allowed to stand on ice for 1 hour. The precipitate was recovered by centrifugation (10Kxg for 20 minutes) and resuspended in 10 ml of a solution containing 10 mM Tris-HCl (pH 7.5) and 100 mM NaCl before the plasmid DNA was precipitated again by the addition of 20 ml of ethanol and left on ice for 1 hour. The precipitated DNA was recovered

by centrifugation (10Kxg for 20 minutes) and resuspended in 4 ml of a solution containing 10 mM Tris-HCl (pH 7.5), 10 mM EDTA and 100 units per ml of RNase A (pre-boiled for 5 minutes, Boehringer Mannheim) and incubated at 37°C for 1 hour. Following addition of SDS (0.5 volumes of a stock solution, 20%, w/v), the incubation mixture was extracted with phenol and chloroform as described in section G. The aqueous phase thus obtained was made up to a final volume of 11.5 ml with distilled water followed by the addition of sodium acetate (0.375 ml of a 4 M stock solution, pH 6.3) and ethanol (16 ml). The DNA was allowed to precipitate at room temperature for 1 hour before being recovered by centrifugation (10K x g for 20 minutes).

The plasmid DNA was further purified by gel filtration chromatography on a Biogel A 15-m (Bio-Rad) column in the presence of a solution containing 0.3 M ammonium acetate, 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Fractions containing purifid plasmid DNA (as assayed by agarose-gel electrophoresis, described below) were pooled and the plasmid DNA was precipitated overnight at -20°C with 2 volumes of ethanol. Purified plasmid DNA was recovered by centrifugation (10Kxg for 20 minutes) and resuspended in a solution containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. The yield of plasmid DNA was estimated by measuring absorbance at 260 nm and using the formula:

(OD_{260}) x 50 = ug DNA per ml. The DNA was stored at 4°C.

ii) Electrophoresis of DNA

Plasmid DNA samples were analysed by electrophoresis in an agarose (0.8%, w/v) gel. The Gel Buffer contained 40 mM Tris-acetate (pH 7.8), 2 mM EDTA and 0.2 ug per ml of ethidium bromide. DNA samples (approximately 0.5 ug dissolved in 10 ul of Gel Buffer) were mixed with 0.1 volume of a solution

containing glycerol (25%, v/v), bromophenol blue (0.1%, w/v) and xylene cyanol (0.1%, w/v). Electrophoresis was carried out towards the anode at a potential of 5 volts per cm for 90 minutes. The gel was photographed with an MP-4 Polaroid Land camera fitted with a Wratten #42 filter using ultraviolet transillumination.

iii) Nick Translation

The plasmid DNA containing specific sequences was labelled with (alpha-^{32}P)-dCTP (3000 Ci per mmol, Amersham) to obtain greater than 10^8 cpm per ug DNA by nick-translation (Rigby, Dieckman, Rhodes & Berg, 1977). Nick-translation, using kits obtained from Amersham, was performed according to the manufacturer's instructions. The (^{32}P)-labelled DNA was separated from the unincorporated radioactive material by gel filtration on a Sephadex G-50 column.

K) Autoradiography

After washing and drying, the radioactive blot was wrapped in Saran Wrap and exposed to Kodak X-Omat XAR-5 film in the presence of a Cronex Lightning Plus screen (DuPont). Exposure was for a suitable length of time in a light-tight film holder at -70°C. The exposed film was developed for 5 minutes with Kodak GBX developer followed by a brief rinse in water. The film was further treated with Kodak GBX fixer for 5 minutes and rinsed in water for 20 minutes before drying.

Quantitation of specific mRNA on the Northern blot was achieved by integrating the area under the curve obtained from densitometric scans of the autoradiograms. Quantitation was done within the linear range of densitometric

response to the radioactivity on the blot.

L) Miscellaneous

Glassware used for work with RNA and DNA was baked at 190°C overnight before use to eliminate possible traces of RNase. All disposable glassware was siliconized by freatment with a solution containing dimethyldichlorosilane (2%, w/v) in trichloroethane before baking. All plastic-ware used was disposable and was considered to be RNase free. All solutions were passed through a sterile 0.45 micron filter or otherwise autoclaved. Water was glass distilled from demineralized water and autoclaved before use. Other materials such as rubber 'policeman' and polycarbonate ultracentrifuge tubes were treated with a solution containing 1 M NaOH for 1 minute and rinsed with autoclaved water before use.

M) Enzymes

The following enzymes were used in these experiments:

Lysozyme	EC 3.2.1.17	(Boehringer Mannheim)
Protease K	EC 3.[illegible]14	(Sigma Chemicals)
RNase A	EC 3.1.21.4	(Boehringer Mannheim)
Trypsin	EC 3.4.21.4	(Flow Laboratories)

N) Abbreviations

Units used in the text are Standard International (SI) units.

alpha-MEM: Minimum Essential Medium, Eagles (alpha modification)

APT: o-Aminophenylthioether

cpm: radioactive counts per minute

DNA: Deoxyribonucleic acid

DPT: Diazophenylthioether

EDTA: Ethylenediaminetetraacetic acid

Hepes: N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid

HPRI: Human Placental Ribonuclease Inhibitor

i.d.: inside diameter

Kxg: centrifugal force in units of gravity times one thousand

LB: Luria-Bertani

MHC: Myosin Heavy Chain

Mops: Morpholinopropanesulfonic acid

mRNA: Messenger RNA

NBD: 7-Nitrobenz-2-oxa-1,3-diazole

OD_{nnn}: optical density at 'nnn' nanometers wave length

oligo(dT): oligodeoxythymidylic acid (approximately 15 units long)

poly(A): Polyadenylic acid (greater than 200 units long)

PBS: Phosphate Buffered Saline

Pipes: Piperazine-N,N'-bis (2-ethane-sulfonic acid)

PVS: Polyvinylsulfate

RNA: Ribonucleic acid

rp L32: Ribosomal protein L32

SDS: Sodium dodecyl sulfate

SSC: Saline Sodium Citrate Solution

Tris or Tris base: Tris(hydroxymethyl)aminoethane

tRNA: Transfer RNA

v/v: indicates volume by volume ratio

w/v: indicates weight by volume ratio

CHAPTER THREE

Results

A) Cell Fractionation

Extraction conditions employed to separate cells into soluble and cytoskeletal fractions have varied according to the cell type under study (van Venrooij et al., 1981; Lemieux & Beaud, 1982; Zumbe, Stahli & Trachsel, 1982; Fey et al., 1984; Howe & Hershey, 1984 ; Bonneau et al., 1985). The differences in the extraction conditions lie mainly in the choice of salt and Triton X-100 concentrations in addition to the time of extraction. Cytoskeletal preparations of various cell types have been characterized as that fraction containing the bulk of the cellular polysomes. Consequently, using the L6 cell line as the model system, extraction conditions were developed which resulted in polysomes being largely associated with the cytoskeleton. Monolayer cultures of L6 cells were extracted with a lysis buffer containing 0.1% (v/v) Triton X-100 and 10 mM KCl to obtain the soluble fraction. The components associated with the cytoskeletal fraction were then obtained by further treatment with buffer containing $(NH_4)_2SO_4$. The components of the soluble and cytoskeletal fractions were further separated by differential centrifugation to yield ribosomal and post-ribosomal material. As shown in Table 3-1, analysis of ribosomal material obtained from the soluble and cytoskeletal fractions of myoblasts reveals that under these extraction conditions 85% of ribosomal RNA was present in the cytoskeletal fraction.

The distribution of macromolecules between the soluble and cytoskeletal fractions was determined. This was achieved by examining the incorporation of labelled precursors into acid insoluble material obtained from the two

Table 3-1.

Percent Distribution of RNA and Protein in Subcellular Fractions of L6 Myoblasts.

Cells labelled overnight with 5 uCi per ml (^{3}H)uridine (46 Ci per mmol) and 5 uCi per ml (^{35}S)methionine (800 Ci per mmol) were separated into soluble and cytoskeletal fractions. Nuclei were removed and percent acid insoluble counts in post-nuclear supernatant of each fraction was estimated. Estimation of phenol-chloroform extracted ribosomal RNA (absorbance at 260 nm) was performed after separation of soluble and cytoskeletal fractions into ribosomal and post-ribosomal material. ± indicates the range of variation within three independent experiments.

Fraction	Total RNA	Total protein	Ribosomal RNA
Soluble	9± 1	22± 1	15± 1
Cytoskeletal	91± 1	78± 1	85± 1

cytoplasmic fractions. L6 myoblasts were labelled overnight with (^{3}H)uridine and (^{35}S)methionine and processed to obtain soluble and cytoskeletal fractions. The fractions were further centrifuged (2.5Kxg for 5 minutes) to remove nuclei. Aliquots of the post-nuclear supernatants then were examined for incorporation of radioactivity into acid insoluble material. Table 3-1 shows that 22% of cellular protein and 9% of total RNA was present in the soluble fraction. These latter values were lower than that obtained in other systems and suggest that differences in the cytoarchitecture play a role in this type of cell fractionation.

B) Polysome Profiles of Soluble and Cytoskeletal Fractions obtained from L6 Myoblasts

To establish the distribution of ribosomes in the soluble and cytoskeletal fractions, the following experiments were performed. Myoblasts labelled overnight with (^{3}H)uridine were separated into soluble and cytoskeletal compartments. The post-nuclear supernatant (as obtained above) was centrifuged through a 15-40% sucrose density gradient to separate monosomes and polysomes From the radioactivity profiles obtained for the two fractions as illustrated in Figure 3-1, it is evident that the ribosomes in the soluble fraction were primarily present in the form of monosomes while in the cytoskeletal fraction they were present in the form of both monosomes and polysomes. These results support the observations of other investigations that polysomes are primarily present in the cytoskeletal fraction as has been noted in various cell types.

C) Association of Polysomal and Free mRNPs with the Cytoskeletal Framework

The observation that polysomes are largely present in the cytoskeletal

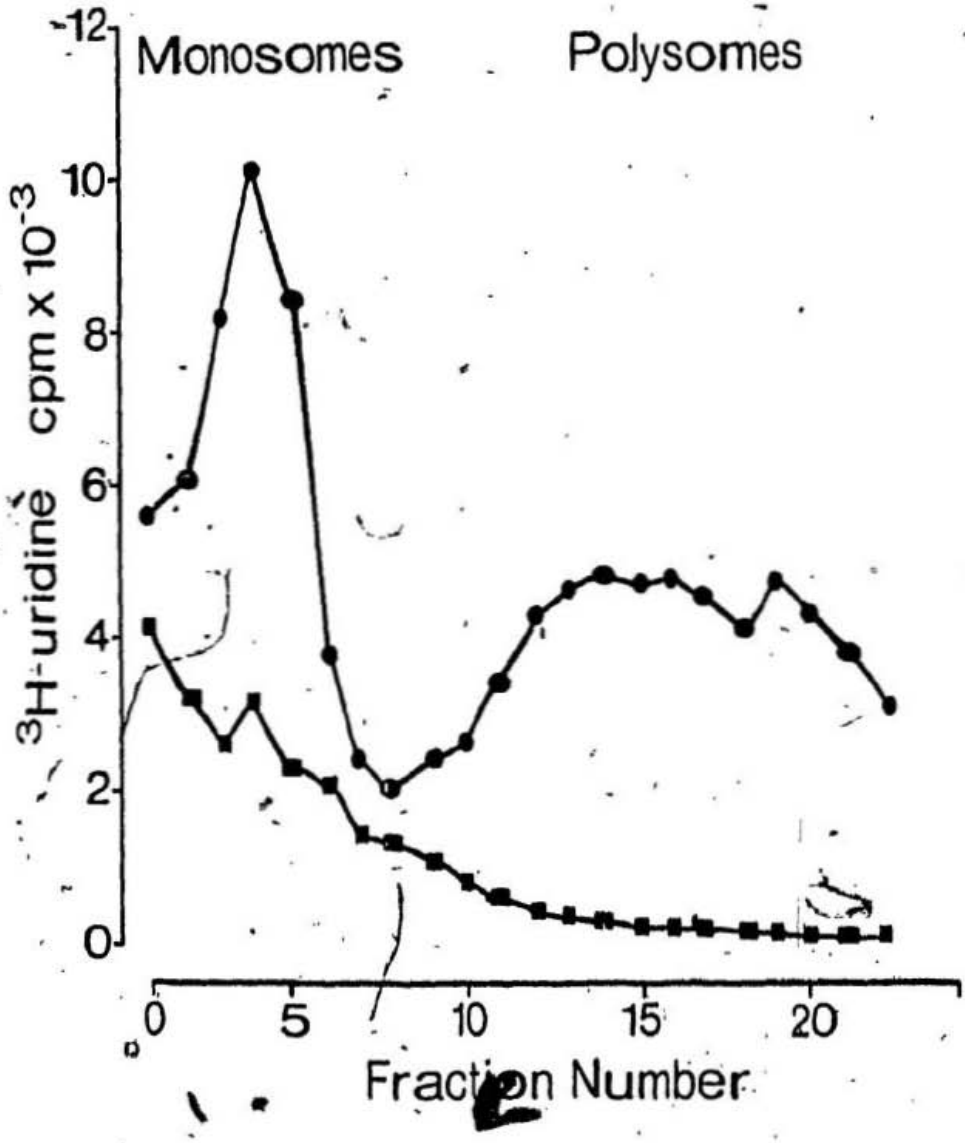
Figure 3-1.

Distribution of Monosomes and Polysomes Isolated from Soluble and Cytoskeletal Fractions of Myoblasts.

Myoblasts were incubated overnight (16 hours) with 5 uCi per ml (^{3}H)uridine (46 Ci per mmol). After extraction of soluble and cytoskeletal fractions, equal volumes of each were applied to a 15-40% linear sucrose gradient and centrifuged at 40K rpm for 120 minutes in a Beckman 'SW 41' rotor at 4°C. The gradients were fractionated and radioactivity in each fraction measured and plotted against fraction number.

■ soluble fraction

● cytoskeletal fraction


Monosomes
Polysomes
^{3}H-uridine cpm x 10^{-3}
12
10
8
6
4
2
0
0
5
10
15
20
Fraction Number

fraction still leaves unanswered the question of the nature of this association. To determine whether association of polysomes with the cytoskeleton was through the mRNA or ribosomes, the cytoskeletal fraction from both myoblasts and myotubes was extracted and separated by differential centrifugation into polysomes and free mRNPs. Poly(A)-containing RNA was isolated from each of these fractions by oligo(dT)-cellulose chromatography and electrophoresed in a 1.2% agarose gel. The RNA was then electroblotted onto DPT-paper and the resulting Northern blot was analysed for the presence of specific mRNAs such as ribosomal protein (rp) L32, actin, histone H4 (A+) and myosin heavy chain (MHC). The results obtained (Figure 3-2) indicated that in addition to being present as polysomal mRNPS, mRNAs for rp L32, actin and histone H4 (A+) were also present as free mRNPs on the cytoskeleton. This observation suggests that the ribosome is not a requisite for the association of mRNA with the cytoskeleton but rather, mRNA (in the form of mRNPs) may itself associate with the cytoskeleton.

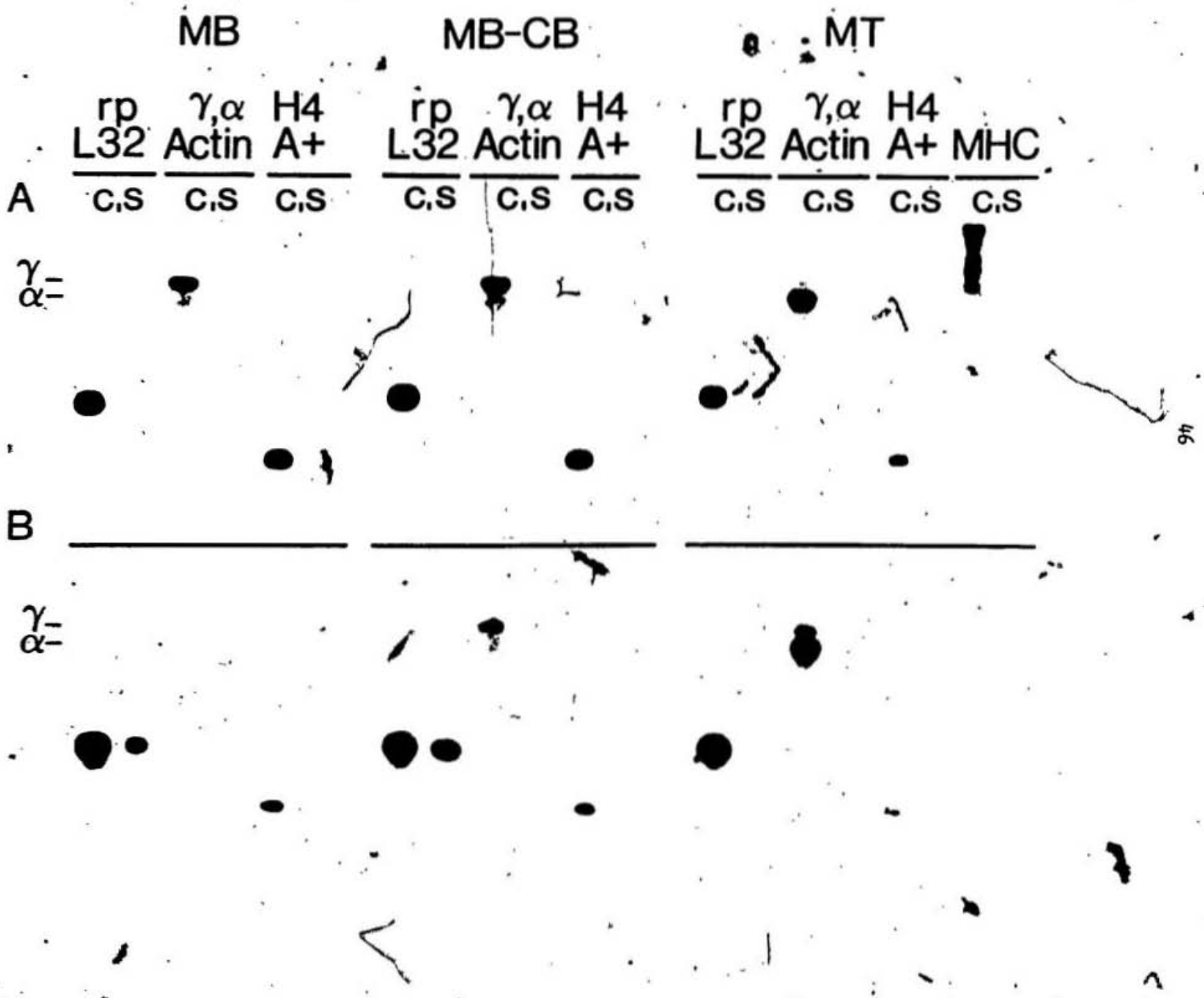
D) Analysis of Poly(A)-containing RNA

Evidence from studies conducted in both HeLa cells and human KB cells suggests that the cytoskeletal framework may have a role in segregating specific mRNAs. The L6 myoblast cell line is a muscle cell line which can be induced to differentiate in vitro. Prior to fusion and differentiation muscle cells synthesize amongst other proteins, nonmuscle beta- and gamma- actins. Upon differentiation, muscle specific genes are expressed resulting in the production of mRNA for alpha-actin and MHC besides mRNAs for other muscle specific proteins (Devlin & Emerson, 1979; Shani, Zevin-Sonkin, Saxel, Carmon, Katcoff, Nudel & Yaffe, 1981 ; Caravatti, Minty, Robert, Montarras, Weydert, Cohen,

Figure 3-2.

Northern Blot Analysis of Poly(A)-containing RNA from (A) Polysomal and (B) Free mRNPs Isolated from the Soluble and Cytoskeletal Fractions.

Poly(A)-containing RNA obtained from various subcellular fractions was size fractionated in a 1.2% agarose gel and electroblotted onto DPT-paper. The Northern blot was hybridized with (^{32}P)-labelled, nick-translated DNA probes for rp L32, actin, histone H4 and MHC respectively and subjected to autoradiography. The results represent multiple reprobings of the same samples. MB -control myoblasts, MB-CB -myoblasts treated with 5 ug per ml cytochalasin B, MT -2 day myotubes, S -soluble, C -cytoskeletal. Amounts of RNA layered in each lane (ug) Polysomal mRNPs: MB/S 1.04, MB/C 5.08, MB-CB/S 0.95, MB-CB/C 5.23, MT/S 0.98, MT/C 5.05. Free mRNPs: MB/S 0.62, MB/C 1.92, MB-CB/S 1.04, MB-CB/C 2.00, MT/S 0.49, MT/C 1.69.


MB
MB-CB
MT
rp L32
γ,α Actin
H4 A+
MHC
c.s
A
B
γ–
α–

Daubas & Buckingham, 1982). In addition, it is known that mRNAs for rp L32 (Jacobs et al., 1985) and histone H4 (A+) (Bird, Jacobs, Stein, Stein & Sells, 1985) are present in both myoblasts and myotubes. To test the hypothesis that the cytoskeletal framework plays a role in segregating mRNAs in differentiating L6 rat myoblasts, poly(A)-containing RNA isolated from the various subcellular fractions of myoblasts and myotubes was analysed for these specific mRNAs.

Poly(A)-containing RNA was prepared from polysomal and free mRNP populations of both cytoplasmic fractions from myoblasts and myotubes. As shown in Table 3-2, approximately 90% of poly(A)-containing RNA was present in the cytoskeletal fractions of both myoblasts and myotubes. The soluble fraction, however, has as low as 5-10% poly(A)-containing RNA most of which was present as polysomal mRNPs in myoblasts and distributed uniformly between polysomal and free mRNPs in myotubes.

Known quantities of poly(A)-containing RNA were subjected to electrophoresis in a 1.2% agarose gel. After transferring the RNA onto DPT-paper, the Northern blot was probed individually with (^{32}P)-labelled nick-translated plasmid DNAs for rp L32, actin, histone H4 and MHC and autoradiographed as shown in Figure 3-2. The autoradiogram obtained with each probe was quantitated by scanning densitometry and the percent distribution of each mRNA species in the various subcellular fractions was determined. Quantitation was done within the linear range of densitometric response to the radioactivity on the blot (Figure 3-3). Amounts of RNA analysed from free mRNPs of the soluble fractions (Figure 3-2) are towards the lower limits of detection by densitometric scanning of autoradiograms (Figure 3-3). This could give rise to discrepancies in calculation of the final percent distribution of specific mRNAs in the various subcellular fractions. This discrepancy can be

Table 3-2.

Percent Distribution of Poly(A)-containing RNA in Subcellular Fractions of Myoblasts and Myotubes.

Soluble and cytoskeletal fractions were obtained from control myoblasts (MB), myoblasts treated with 5 ug per ml of cytochalasin B (MB-CB) and 2-day myotubes (MT). Following removal of nuclei, the post-nuclear supernatant was subjected to differential centrifugation thereby obtaining polysomal and free mRNP material. RNA from these fractions was phenol-chloroform extracted and subjected to oligo(dT)-cellulose chromatography to obtain poly(A)-containing RNA . RNA was estimated by measuring absorbance at 260 nm. Values obtained are from two independent experiments. ± indicates the range of variation within the experiments.

Subcellular Fraction		Percent Disribution		
		MB	MB-CB	MT
Polysomal	Soluble	4.7± 0.9	7.6± 0.4	2.7± 0.1
	Cytoskeletal	78.2± 0.9	71.2± 2.0	84.4± 0.6
Free	Soluble	2.5± 0.4	3.3± 0.4	2.9± 0.1
	Cytoskeletal	14.6± 0.5	18.2± 2.7	10.1± 0.6

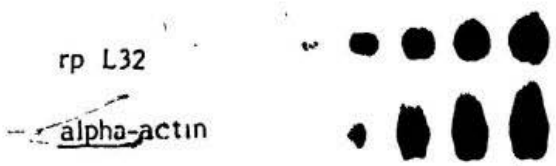
Figure 3-3.

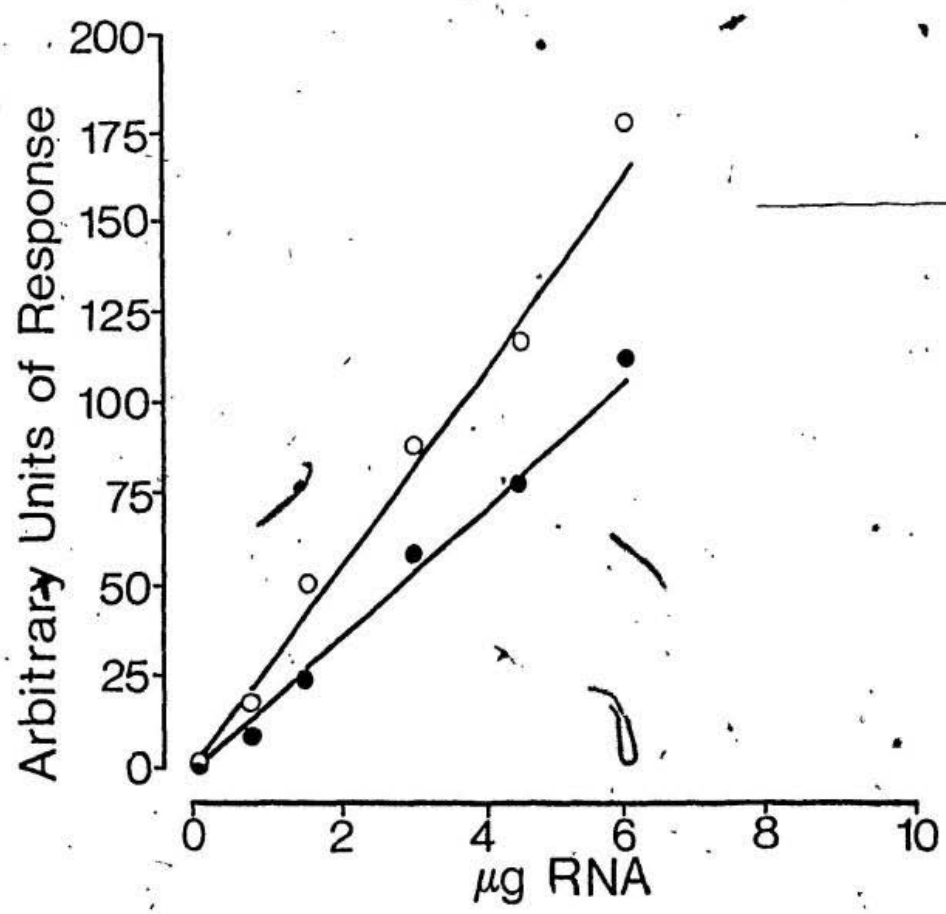
Scanning Densitometry of Northern Blots:

Increasing amounts of poly(A)-containing RNA isolated from myoblasts (for rp L32) and myotubes (for alpha-actin) were electrophoresed in a 1.2% agarose gel. Following electroblotting onto DPT-paper and hybridization with respective (^{32}P)-labelled nick-translated probes, they were subjected to autoradiography. The autoradiograms were then quantitated by scanning densitometry and arbitrary units of response were plotted against amount (ug) of RNA analysed.

○ rp L32

● alpha-actin


rp L32
alpha-actin


Arbitrary Units of Response
200
175
150
125
100
75
50
25
0
0
2
4
6
8
10
μg RNA

overcome, however, by analysing the same set of RNA samples for various specific mRNAs as was done in this investigation. In the light of this argument, values obtained in Table 3-3 give us a comparative estimate of the distribution pattern for each of these mRNA species in the various subcellular fractions.

E) Distribution of Specific mRNAs in Myoblasts

Northern blot analyses (Figure 3-2) of polysomal mRNAs performed for rp L32, actin and histone H4 (A+) reveals that in myoblasts, these specific mRNAs were restricted to the cytoskeletal fraction. Although the soluble fraction contained 15% of ribosomal RNA (Table 3-1) and 5-10% of poly(A)+ containing RNA (Table 3-2), the levels of each of these specific mRNAs was negligible (Table 3-3). Northern blots were analysed also to establish whether the distribution pattern of specific mRNAs was similar between the polysomal and free mRNP populations of both cytoplasmic fractions. It is evident from Figure 3-2 that while mRNAs for rp L32 and histone H4 (A+) were present on the cytoskeletal framework both as polysomal and free mRNPs, that for gamma-actin was present primarily in polysomal arrays. A quantitative analyses of these data presented in Table 3-3 indicates that the levels of the mRNAs specific for rp L32 and histone H4 (A+) as free mRNPs were approximately 10-20% while gamma-actin was not detectable in this population of mRNPs. Interestingly, further examination of the free mRNP population (Figure 3-2, lower panel) reveals that those containing histone H4 (A+) mRNA were present only in the cytoskeletal fraction while rp L32 specific mRNPs were present in both fractions albeit in smaller amounts in the soluble fraction (Table 3-3).

Table 3-3.

Percent Distribution of Specific mRNAs in Subcellular Fractions of Myoblasts and Myotubes.

Poly(A)-containing RNA from various subcellular fractions of control myoblasts (MB), myoblasts treated with 5 ug per ml cytochalasin B (MB-CB) and 2-day myotubes (MT) was electrophoresed in a 1.2 % agarose gel and electroblotted onto DPT-paper. The blot was hybridized with (^{32}P)-labelled nick-translated DNA probes for rp L32, actin, histone H4 and MHC respectively. Following autoradiography (Figure 3-2), the autoradiograms were quantitated by scanning densitometry. Values have been obtained from two independent experiments and the range of variation has been given as $\pm$.

Table 3-3

mRNA	Fraction	Percent Distribution			
		MB	MB-CB		MT
rp	Polysomal Soluble	0.3± 0.3	0.6± 0.6		0.2± 0
L32	Cytosk.	78.0± 9.0	61.0± 9.0		65.0± 3.2
	Free mRNP Soluble	2.1± 1.7	4.8± 3.3		1.7± 0.2
	Cytosk.	19.0± 6.6	34.0± 5.3		33.0± 3.4
H4	Polysomal Soluble	0	0		0
(A+)	Cytosk.	90.0± 1.0	82.0± 0.5		89.0± 2.1
	Free mRNP Soluble	0	0		0
	Cytosk.	10.0± 1.0	18.0± 0.5		11.0± 2.1
MHC	Polysomal Soluble	0	0		0
	Cytosk.	0	0		100± 0
	Free mRNP Soluble	0	0		0
	Cytosk.	0	0		0
Actins		gamma-	gamma-	gamma-	gamma-
	Polysomal Soluble	0.5± 0.5	1.2± 1.2	0	0.2± 0
	Cytosk.	99.0± 1.0	97.0± 1.0	19.0± 3.0	75.0± 0.7
	Free mRNP Soluble	0	0	0	0.1± 0
	Cytosk.	0.5± 0.5	0.6± 0.6	0.5± 0.2	5.1± 0.9

F) Distribution of Specific mRNAs in Myotubes

An examination of the polysomal mRNPs from myotubes reveals that as in myoblasts, polysomes containing mRNAs for rp L32, histone H4 (A+), muscle specific alpha-actin and MHC were found in the cytoskeletal fraction (Figure 3-2 and Table 3-3). The level of polysomes containing gamma-actin specific mRNA, however, dropped in differentiated myotubes. This was accompanied by the appearance of polysomal alpha-actin and MHC mRNA in the cytoskeletal fraction which is in accordance with the events of muscle differentiation in which muscle specific mRNAs for actin and MHC are transcribed and those for non-muscle actin become less prominent.

The distribution of mRNAs for rp L32 and histone H4 (A+) isolated from free mRNPs of either cytoplasmic fractions was similar in both myoblasts and myotubes. mRNPs for gamma- and alpha-actins and MHC, however, behaved differently. mRNA for gamma-actin was not detectable in free mRNPs from myotubes while at least 5% of the mRNA for alpha-actin was present as free mRNPs (Table 3-3). Similarly, MHC mRNA was not detected in free mRNP population of either cytoplasmic fractions of myoblasts and myotubes.

G) Cytoskeletal Element Involved in Anchoring mRNPs and Polysomes.

Analysis of the two cytoplasmic fractions from myoblasts has revealed that both polysomal and free mRNP complexes were associated with the cytoskeletal framework. To determine whether polysomes and/or free mRNPs were associated with the microfilaments, L6 myoblasts were incubated with cytochalasin B to disrupt microfilaments. To ascertain the levels of cytochalasin B necessary to disrupt these filaments, myoblasts were treated with increasing

concentrations of the drug and its effect on microfilament integrity was observed. Cell morphology and integrity were evaluated using both phase contrast (Figure 3-4) and fluorescent microscopy (Figure 3-5) following NBD-phallacidin staining (Barak, Yocum, Nothnagel & Webb, 1980). Control myoblasts appeared as well spread out spindle shaped cells when viewed under phase contrast optics. As the concentration of the drug was increased, greater numbers of cells lost their spindle shaped appearance and began to collapse, losing the rigidity of their stress fibres (Figure 3-4). In control cells (Figure 3-5) the microfilaments were seen as well stacked, intact longitudinal filaments stretched across the length of the cell. Following treatment with 1 ug per ml cytochalasin B, the microfilaments were disrupted and aggregates stained with NBD-phallacidin were seen throughout the cell. Disruption of microfilaments increased progressively, until at 5 ug per ml of cytochalasin B, the cells collapsed completely. Under these conditions (Figure 3-5), the microfilaments were completely dissociated and were not stainable as well defined filaments by NBD-phallacidin.

If polysomes and mRNPs were associated with the microfilaments, disruption of microfilaments might be expected to result in their release into the soluble fraction. To determine whether depolymerization of these filaments resulted in movement of polysomes and specific mRNAs into the soluble fraction, polysomes were isolated from the two cytoplasmic fractions following treatment of myoblasts with cytochalasin B for 30 minutes. Analysis of the ribosomal RNA extracted (Table 3-4) demonstrated that less than a 5% movement of polysomal material from the cytoskeletal to the soluble fraction occured even after incubation with 5 ug per ml cytochalasin B (Figure 3-5).

Northern blot analyses were performed to assess whether specific

Figure 3-4.

Phase Contrast Microscopy of L6 Myoblasts.

Cells grown on tissue culture chamber slides were treated with different concentrations of cytochalasin B and viewed under phase contrast optics. Slides were scored for number of cells that were:

- ● intact and well spread out
- ■ cells partially collapsed
- ▲ cells completely collapsed

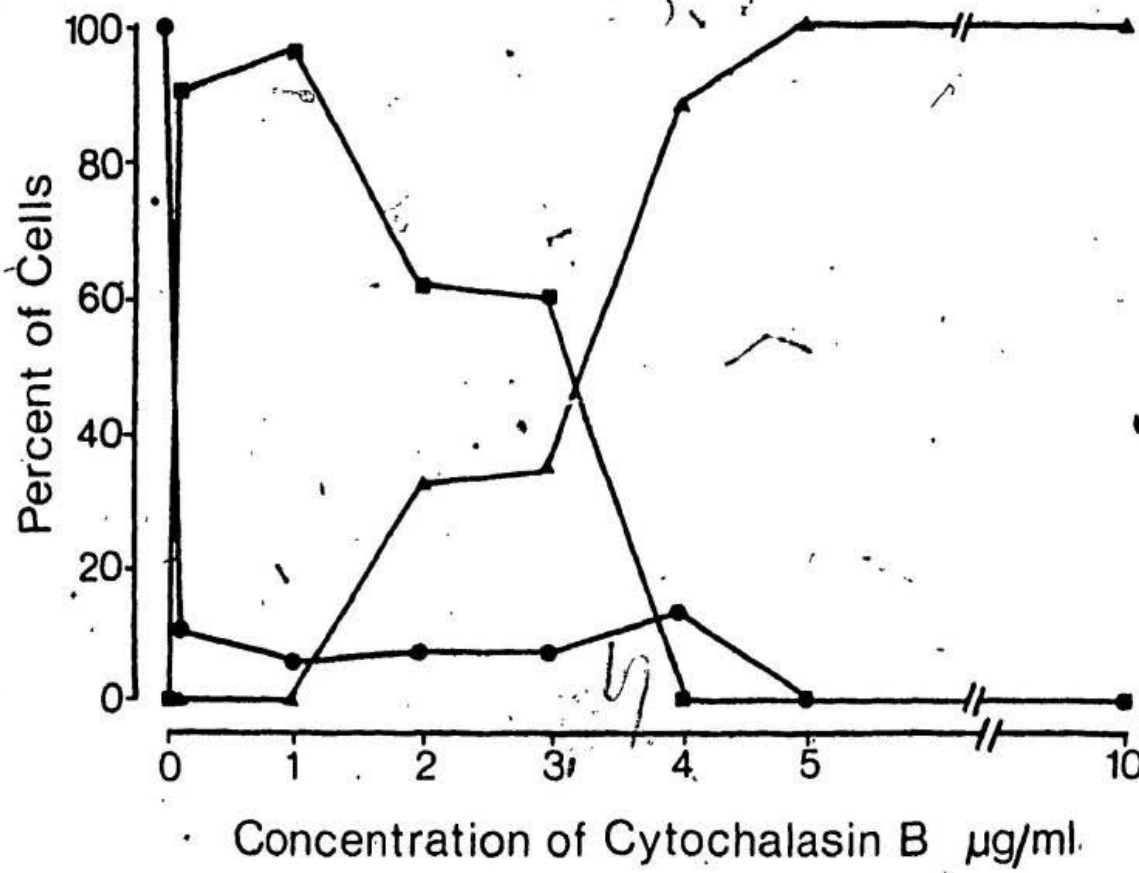

100
80
60
40
20
0
Percent of Cells
0
1
2
3
4
5
10
Concentration of Cytochalasin B µg/ml

Figure 3-5.

Direct Fluorescent Staining of L6 Myoblasts with NBD-phallacidin.

Myoblasts grown on tissue culture chamber slides were treated with different concentrations of cytochalasin B and stained with NBD-phallacidin. (x800)

a- no cytochalasin B

b- myoblasts treated with 1 ug per ml cytochalasin B for 30 minutes

c- myoblasts treated with 5 ug per ml cytochalasin B for 30 minutes

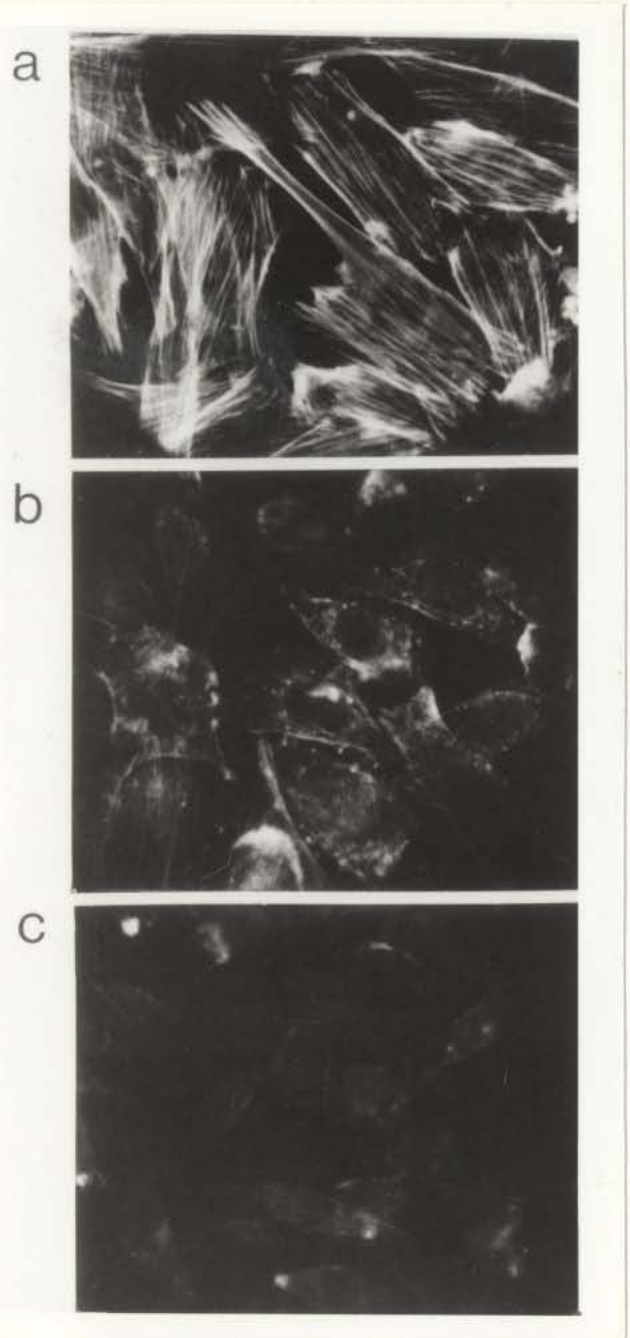

a
b
c

Table 3-4.

Effect of Cytochalasin B on Distribution of Ribosomal RNA in the two Cytoplasmic Fractions from Myoblasts.

Myoblasts were treated with different concentrations of cytochalasin B for 30 minutes and separated into soluble and cytoskeletal fractions. RNA was extracted from polysomal material obtained after differential centrifugation of the two cytoplasmic fractions and absorbance measured at 260 nm. Values have been obtained from three independent experiments and the range of variation has been given as ±.

Cytochalasin B (ug per ml)	Percent RNA in soluble fraction
0	14.84± 0.12
1	15.94± 4.45
3	17.78± 4.04
5	20.23± 4.84

poly(A)-containing mRNAs (rp L32, gamma-actin and histone H4 (A+)) isolated from polysomal or free mRNPs were associated with microfilaments. Cytochalasin B treatment of myoblasts failed to release these mRNAs into the soluble compartment (Figure 3-2 and Table 3-3). These results suggest that in L6 myoblasts neither polysomes nor free mRNPs were associated with the microfilaments.

H) Effect of Cytochalasin B on Macromolecular Syntheses

Results in the previous section demonstrated that cytochalasin B did not alter the cytoplasmic distribution of ribosomes or mRNPs. Experiments were designed to determine, however, whether disruption of microfilaments by this drug affected either RNA or protein synthesis. Myoblasts were incubated with 5 uCi per ml (^{3}H)uridine or 10 uCi per ml (^{3}H)lysine and treated with 5 ug per ml cytochalasin B for various time periods. Examination of radioactivity in acid insoluble material demonstrated (Figure 3-6) that at 5 ug per ml cytochalasin B, incorporation of labelled uridine into RNA was inhibited by approximately 70%. To assess whether this inhibition resulted from a block in RNA synthesis or from reduced uptake of (^{3}H)uridine, the level of precursor uridine in acid soluble pool was measured. The results obtained demonstrated that cytochalasin B treated cells contained only 30% of the labelled uridine found in control cells suggesting inhibition was not at the level of RNA synthesis or processing but rather at the level of precursor uptake. Parallel experiments measuring the effect of cytochalasin B on protein synthesis (Figure 3-7) demonstrated that the incorporation of (^{3}H)lysine into protein is inhibited to only 18%. Uptake of (^{3}H)lysine, however, was not affected by cytochalasin B treatment.

To establish whether the effect of cytochalasin B on the uptake of

Figure 3-6.

Effect of 5 ug per ml Cytochalasin B on Incorporation of Labelled Uridine into Acid Soluble and Insoluble Material.

Myoblasts were incubated with 5 uCi per ml (^{3}H)uridine (46 Ci per mmol) with or without cytochalasin B. After various times of incubation, duplicate samples were analysed for radioactivity incorporated into acid soluble and insoluble material. All points represent mean of two experiments.

■ acid soluble

O acid insoluble

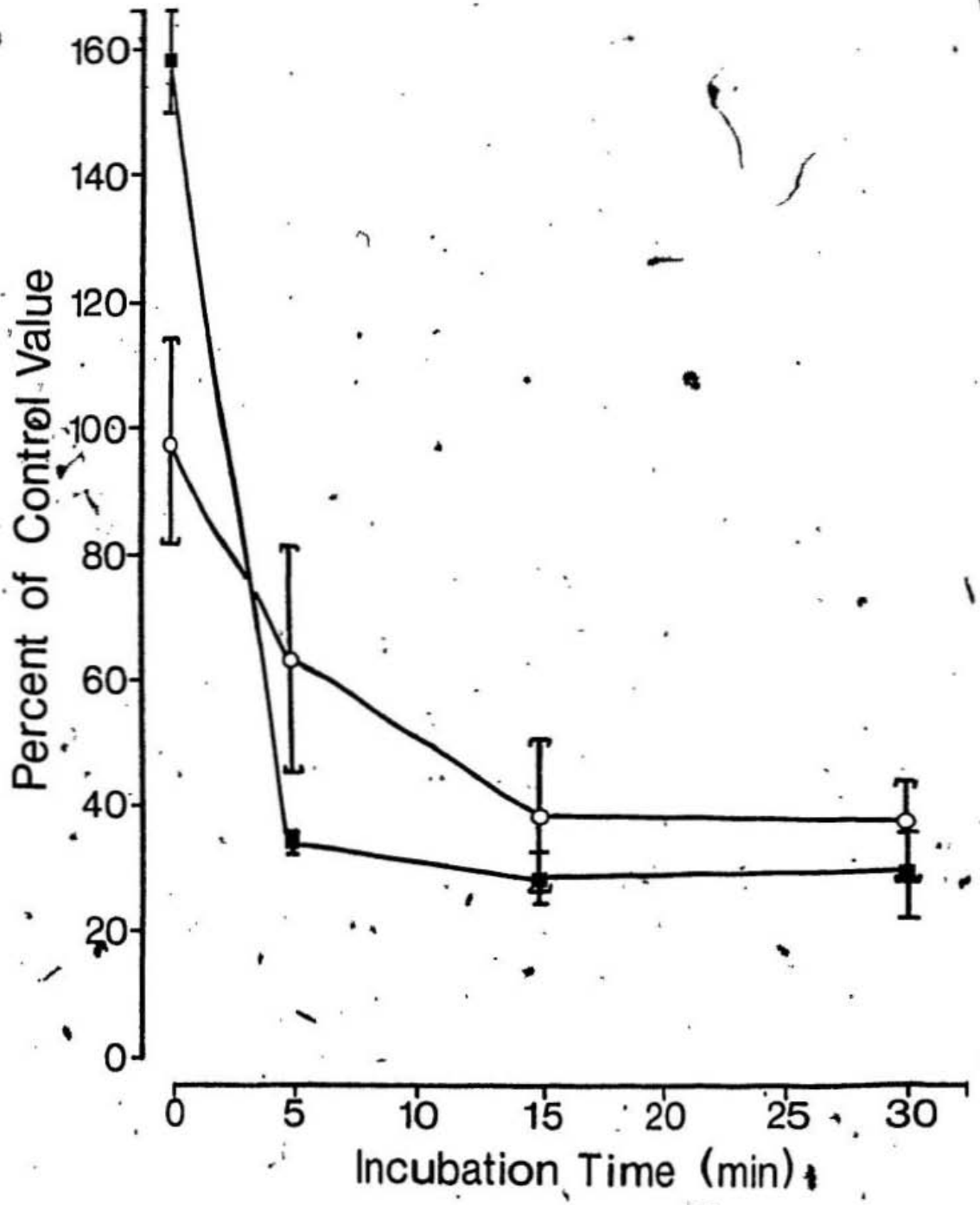

Percent of Control Value
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Incubation Time (min)

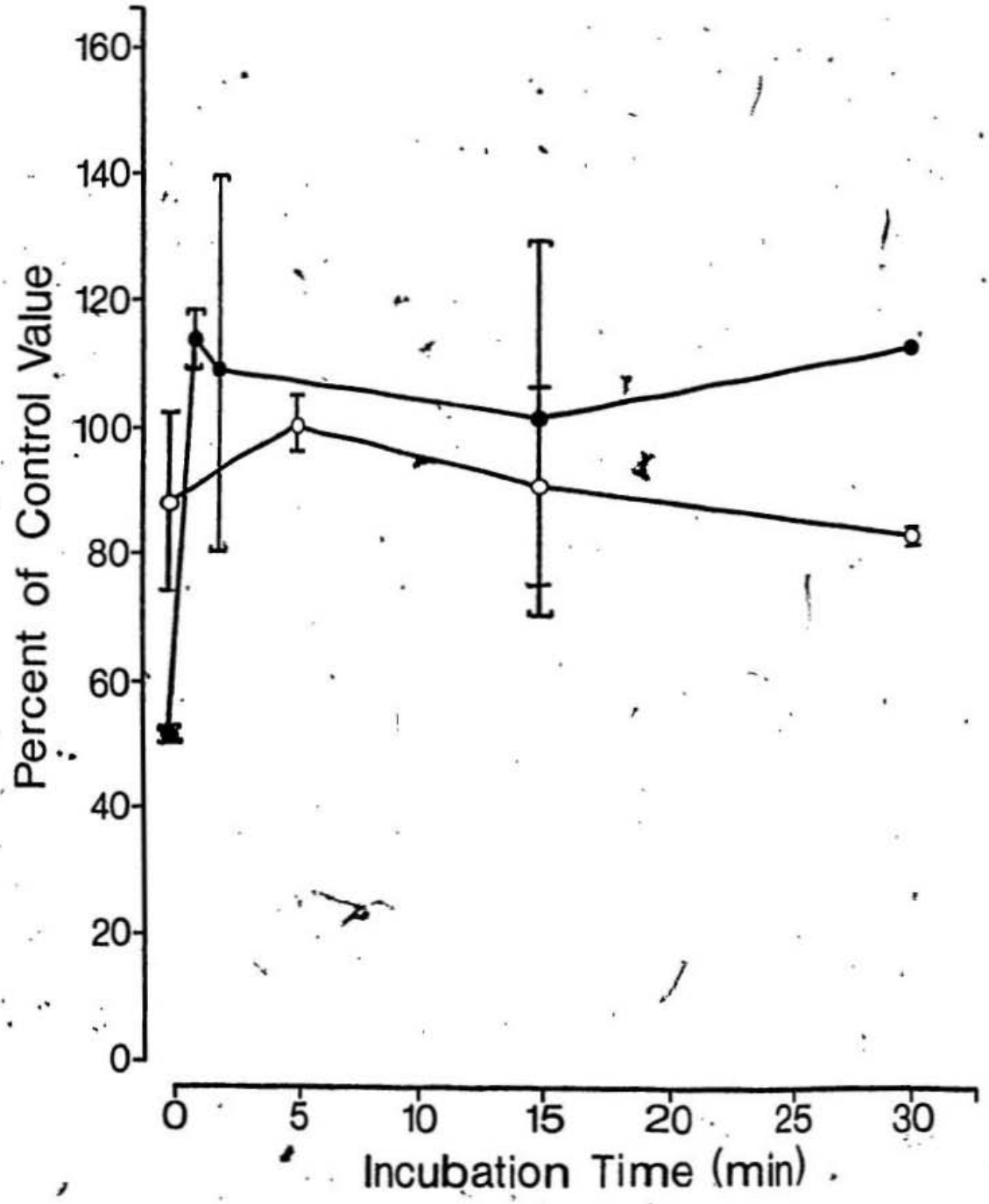
Figure 3-7.

Effect of 5 ug per ml Cytochalasin B on Incorporation of Labelled Lysine into Acid Soluble and Insoluble Material.

Myoblasts were incubated with 10 uCi per ml (^{3}H)lysine (100 Ci per mmol) with or without cytochalasin B. After various times of incubation, duplicate samples were analysed for radioactivity incorporated into acid soluble and insoluble material. All points represent mean of two experiments.

● acid soluble

○ acid insoluble


160
140
120
100
80
60
40
20
0
Percent of Control Value
0
5
10
15
20
25
30
Incubation Time (min)

precursor was reversible, myoblasts were treated with 5 ug per ml of cytochalasin B for 30 minutes and allowed to recover in Growth Medium for varying lengths of time following which uptake of labelled precursor was measured. Figure 3-8 demonstrates that the ability to transport uridine to levels comparable with control cells was achieved within 15 minutes. The observation that the effects of cytochalasin B were rapidly reversible lends credence to the use of this drug in such studies.

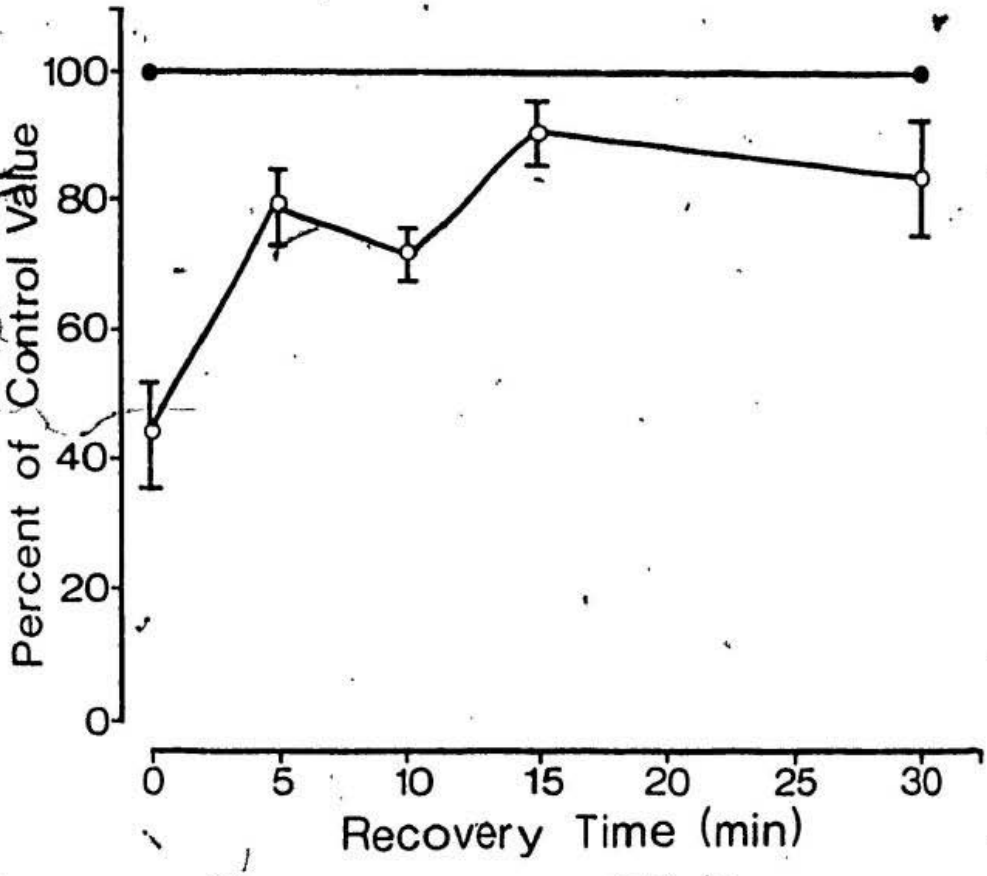
Figure 3-8.

Recovery of L6 Myoblasts from the Effects of Cytochalasin B.

Myoblasts treated with 5 ug per ml cytochalasin B for 30 minutes were rinsed twice in Growth Medium and allowed to recover in Growth Medium for varying periods of time. They were then labelled with 5 uCi per ml (^{3}H)uridine (46 Ci per mmol) for 10 minutes and radioactivity in acid soluble material was measured. Each point represents duplicate samples of two independent experiments.

● control cells

○ cells recovered from the effects of cytochalasin B


Percent of Control Value
100
80
60
40
20
0
0
5
10
15
20
25
30
Recovery Time (min)

CHAPTER FOUR

Discussion

Concepts of cellular organization have evolved over the years from the cytoplasm viewed as a formless medium to one which is highly organized at all levels. Improved biochemical and ultrastructural methods have helped in resolving the cytoplasm as a complex organization of macromolecules. Evidence suggests that elements as diverse as water (Parsegian & Rau, 1984), organic molecules (Mastro & Keith, 1984), proteins (Masters, 1984 ; Paine, 1984) and organelles (Satir, 1984) are highly organized within the cytoplasm. Furthermore, as part of this spatial and functional organization within the cytoplasm, is also the demonstration of a specific association of polysomes and mRNA with the cytoplasmic matrix or cytoskeletal framework.

The present investigation was undertaken with a view to examine the role of the cytoskeletal framework in protein synthesis, in differentiating L6 rat myoblasts. Myoblasts are mononucleated, spindle shaped cells and undergo cell proliferation. Upon differentiation, they stop proliferating and fuse to form syncytial myotubes. Accompanying the onset of differentiation is the expression of a set of muscle specific genes. Such a system offers an opportunity to investigate the metabolism of specific mRNAs in two different metabolic and physiological stages of the same cell line.

Based on ultrastructural analyses of several cell types it is believed that polysomes are largely associated with the cytoskeletal framework (Lenk et al., 1977; Wolosewick & Porter, 1979 ; van Venrooij et al., 1981). In extrapolating such observations to in vitro studies involving the cytoskeletal framework, extraction conditions have been developed which result in the polysomes being

attached to the cytoskeletal framework. Employing appropriate extraction conditions for the L6 cell line also results in a preparation in which the ribosomes are largely present in the cytoskeletal fraction where they exist primarily as polysomes (Figure 3-1). The ribosomes in the soluble fraction on the other hand are primarily monomers and constitute approximately 15% of total cellular ribosomal material (Table 3-1). The soluble fraction contains 22% of the cellular protein and 9% of the total RNA (Table 3-1). The protein content of the soluble fraction from L6 myoblasts is much lower than that observed for other systems such as HeLa cells and African green monkey kidney cells where more than 60% of the cellular protein is solubilized. This difference could reflect differences in the internal organization of the cytoplasm in different cell types.

Several hypotheses have been presented regarding the molecular mechanisms linking polysomes to the cytoskeleton. Although attachment to the cytoskeletal framework is not fully understood, evidence from other reports as well as the present findings suggest that polysomes are associated with the framework through the mRNA and not through the ribosomes. This conclusion was drawn from the results (Figure 3-2 and Table 3-3) which demonstrated that free mRNPs containing specific mRNAs were associated with the cytoskeleton.

Given the observation that free mRNPs were found in both cytoplasmic fractions, mRNPs might not associate with the cytoskeletal framework by themselves but rather through the transient interactions between the translational factors and the cytoskeletal framework. Translational factors such as eIF-2, eIF-3, eIF-4A, eIF-4B and cap binding proteins are known to function in the formation of the 40S intermediary complex (Woldave, 1985). Howe & Hershey (1984) demonstrated that there is a selective enrichment of

translational factors such as eIF-2, eIF-3, eIF-4A and eIF-4B in the cytoskeletal fraction of HeLa cells. Furthermore, their results suggest that ribosomes associate with the cytoskeleton only after having initiated protein synthesis. It is therefore quite possible that, mRNP complexes on the cytoskeleton may represent either initiation complexes or 'run off' mRNAs from polysomal translational complexes.

In a differentiating system such as the L6 cell line, by using various inhibitors of protein synthesis, the events leading to the association of specific mRNAs and ribosomes with the cytoskeleton, and the steps involved in dissociation of specific mRNAs from the cytoskeleton could be analysed. In addition, the specificity of interaction of protein synthesis initiation factors during these processes can be delineated. An analysis of the protein components of mRNP complexes in both soluble and cytoskeletal fractions could provide some insight into the nature of these interactions.

Differentiation of mononucleated myoblasts into syncytial myotubes involves the expression of a set of muscle specific genes leading to the synthesis of new mRNAs and their corresponding proteins. An association of translatable mRNAs (ie in polysomes) with the cytoskeletal framework has been demonstrated in both HeLa cells (Cervera et al., 1981) and human KB cells (van Venrooij et al., 1981) where upon viral infection, host mRNAs are released from the cytoskeletal framework followed by the attachment of viral specific mRNAs to this framework. As a corollary, newly synthesized muscle specific mRNAs involved in protein synthesis would be expected to appear on the cytoskeletal framework of myotubes. Furthermore, mRNA species which are myoblast specific should be associated with the cytoskeletal framework only in the myoblasts. Northern blot analyses of specific mRNAs from the two cytoplasmic fractions

has revealed that mRNAs for rp L32 and histone H4 (A+) are largely associated with polysomes on the cytoskeleton in both myoblasts and myotubes. In the case of mRNA for actin, however, the distribution is different. Whereas non-muscle gamma-actin mRNA was found in the cytoskeleton of myoblasts, it was found at reduced levels, accompanied with the appearance of mRNA for alpha-actin in the cytoskeleton of myotubes. Similarly, mRNA for MHC was found only in the cytoskeleton of myotubes and not myoblasts.

Northern blot analyses of the above mRNAs also revealed differences in the compartmentalization of these mRNAs. While mRNAs for rp L32 and histone H4 (A+) were present in both polysomal and free mRNP compartments in myoblasts and myotubes, the distribution of actin mRNAs and MHC mRNA was quite different. mRNA for gamma-actin was present largely as polysomal mRNP in myoblasts (Figure 3-2 and Table 3-3). In contrast, mRNA for muscle specific alpha-actin was present in both polysomal and free mRNPs in myotubes. Furthermore, mRNA for MHC which was absent from myoblasts was present only as polysomal mRNP in myotubes. Finally, of the messages examined, only mRNA for rp L32 was found as free mRNPs in the soluble fraction albeit in very small amounts (Table 3-3). It can be concluded from these observations that the high demand for muscle specific proteins following differentiation is reflected in the presence of these specific mRNAs in polysomes attached to the cytoskeleton.

Possible candidates for the mRNP binding constituents of the cytoskeleton are microfilaments, microtubules and intermediate filaments. Investigations in different cell types have revealed this association to be specific to the cell type under study. In HeLa cells, disruption of microfilaments with cytochalasin B resulted in the movement of mRNA, polysomes and translational factors into the soluble fraction (Lenk et al., 1977 ; Howe &

Hershey, 1984) suggesting their association with the microfilament. In contrast, the cap binding protein which is involved in initiation complex formation was localized on the intermediate filament in baby hamster kidney cells (Zumbe et al., 1982). In the case of Ascidian eggs, Jeffery (1984) has reported that association of actin and histone specific mRNA with the cytoskeleton was independent of the integrity of microfilaments. Thus there appears to be lack of uniformity among various cell types with regard to the cytoskeletal component responsible for mRNP attachment. Currently, the number of mRNAs and their compartmentalization related to the cytoskeleton, that have been studied, is limited. Further studies with a larger variety of messages is required before firm conclusions can be made in this area.

Results obtained in the present investigation revealed that disruption of microfilaments with cytochalasin B did not result in release of ribosomes (Table 3-4), poly(A)-containing RNA (Table 3-2) or specific mRNAs (Figure 3-2 and Table 3-3) into the soluble fraction. In addition, the results from this investigation suggest that mRNAs being translated are associated with polysomes on the cytoskeleton. Thus, if attachment of mRNAs and polysomes to the cytoskeleton is required for translation to occur, dislodging these components from the cytoskeleton should affect protein synthesis. An examination of the effects of the microfilament disrupting drug, cytochalasin B revealed that although it affects the uptake of uridine (Figure 3-6), it had no effect on protein synthesis (Figure 3-7). From these results it is therefore assumed that the translational machinery including mRNA and polysomes are not present on the microfilaments in L6 myoblasts. In the light of these observations and reports in the literature, it may be reasonable to assume that participation of microfilaments in cytoskeletal association of mRNAs and polysomes depends

on the type of cell line under investigation.

Conclusions

This work was performed to test the hypothesis that mRNA attachment to the cytoskeleton is independent of the ribosome. Results obtained indicate that this is indeed the case and suggests that the attachment may be through a component of the mRNP complex. Investigations into the cytoskeletal component involved in anchoring mRNPs and ribosomes revealed that disruption of microfilaments did not release these components from the cytoskeletal fraction to the soluble fraction. This indicated that in L6 myoblasts, microfilaments were not involved in attachment of the translational machinery with the cytoskeleton. An analysis of specific mRNAs in the various subcellular fractions of myoblasts and myotubes revealed that following differentiation, mRNAs for muscle specific proteins were found in polysomal arrays associated with the cytoskeleton. Furthermore, results also indicated differences in the distribution pattern of these specific mRNAs. An analysis of a wider range of specific mRNAs might give more information on sequences in the mRNA that could be responsible for its interaction with the cytoskeleton.

BIBLIOGRAPHY AND REFERENCES CITED

Al-Atia, G.R., Fruscoloni, P. & Jacobs-Lorena, M. (1985). Translational regulation of mRNAs for ribosomal proteins during early Drosophila development. **Biochemistry 24**, 5798-5803.

Albrecht-Buehler, G. & Goldman, R.D. (1976). Microspike-mediated particle transport towards the cell body during early spreading of 3T3 cells. **Experimental Cell Research 97**, 329-339.

Albrecht-Buehler, G. (1977). Phagokinetic tracks of 3T3 cells: Parallels between the orientation of track segments and of cellular structures which contain actin or tubulin. **Cell 12**, 333-339.

Bag, J. & Sells, B.H. (1979). The presence of protein kinase activity and acceptors of phosphate groups in nonpolysomal cytoplasmic messenger ribonucleoprotein complexes of embryonic chicken muscle. **Journal of Biological Chemistry 254**, 3137-3140.

Bag, J., Hubley, M. & Sells, B.H. (1980). A cytoplasmic ribonucleo-protein complex containing a small RNA inhibitor of protein synthesis. **Journal of Biological Chemistry 255**, 7055-7058.

Bag, J. & Sells, B.H. (1981). Cytoplasmic nonpolysomal ribonucleo-protein complexes and translational control. **Molecular and Cellular Biochemistry 40**, 129-141.

Baker, C.C. & Ziff, E.B. (1981). Promoters and heterogenous 5' termini of the messenger RNAs of Adenovirus serotype-2. **Journal of Molecular Biology 149**, 189-221.

Bantle, J.A., Maxwell, I.H. & Hahn, W.E. (1976). Specificity of oligo(dT)-cellulose chromatography in the isolation of polyadenylated RNA. **Analytical Biochemistry 72**, 413-427.

Barak, L.S., Yocum, R.R., Nothnagel, E.A. & Webb, W.W. (1980). Fluorescence staining of the actin cytoskeleton in living cells with 7-nitrobenz-2-oxa-1,3-diazole-phallacidin. **Proceedings of the National Academy of Sciences of the U. S. A. 77**, 980-984.

Beltz, G.A. & Flint, S.J. (1979). Inhibition of HeLa cell protein synthesis during Adenovirus infection. Restriction of cellular messenger RNA sequences to the nucleus. **Journal of Molecular Biology 131**, 353-373.

Ben-Ze'ev, A., Horowitz, M., Skolnik, H., Abulafia, R., Lanb, O. & Aloni, Y. (1981). The metabolism of SV 40 RNA is associated with the cytoskeletal framework. **Virology 111**, 475-487.

Bester, A.J., Kennedy, D.S. & Heywood, S.M. (1975). Two classes of translational control RNA: Their role in the regulation of protein

synthesis. Proceedings of the National Academy of Sciences of the U. S. A. 72, 1523-1527.

Bird, R.C., Jacob, F.A., Stein, G., Stein, J. & Sells, B.H. (1985). A unique subspecies of histone H4 mRNA from rat myoblasts contains poly(A). Proceedings of the National Academy of Sciences of the U. S. A. 82, 6760-6764.

Birnboim, H.C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research 7, 1513-1523.

Bonneau, A-M., Darveau, A. & Sonenberg, N. (1985). Effect of viral infection on host protein synthesis and mRNA association with the cytoplasmic cytoskeletal structure. Journal of Cell Biology 100, 1209-1218.

Brinkley, B.R., Fuller, G.M. & Highfield, D.P. (1975). Cytoplasmic microtubules in normal and transformed cells in culture: Analysis by tubulin antibody immunofluorescence. Proceedings of the National Academy of Sciences of the U. S. A. 72, 4981-4985.

Brown, D.D. & Gurdon, J.B. (1977). High-fidelity transcription of 5s DNA injected into Xenopus oocytes. Proceedings of the National Academy of Sciences of the U. S. A. 74, 2064-2068.

Cervera, M., Dreyfuss, G. & Penman, S. (1981). Messenger RNA is translated when associated with the cytoskeletal framework in normal and VSV-infected HeLa cells. Cell 23, 113-120.

Ciliberto, G., Castagnoli, L., Melton, D.A. & Cortese, R. (1982). Promoter of a eukaryotic $tRNA^{Pro}$ gene is composed of three noncontiguous regions. Proceedings of the National Academy of Sciences of the U. S. A. 79, 1195-1199.

Civelli, O., Vincent, A., Buri, J-F. & Scherrer, K. (1976). Evidence for a translational inhibitor linked to globin mRNA in untranslated free cytoplasmic messenger ribonucleoprotein complexes. FEBS Letters 72, 71-76.

Civelli, O., Vincent, A., Maundrell, K., Buri, J-F. & Scherrer, K. (1980). The translational repression of globin mRNA in free cytoplasmic ribonucleoprotein complexes. European Journal of Biochemistry 107, 577-585.

Davidson, E.H. (1976). Gene activity in early development. Academic Press, New York. pp. 87-118.

Feinberg, B., McLaughlin, C.S. & Moldave, K. (1982). Analysis of temperature-sensitive mutant ts 187 of Saccharomyces cerevisiae altered in a component required for the initiation of protein synthesis. Journal of Biological Chemistry 257, 10846-10851.

Fertuck, H.C. & Salpeter, M.M. (1976). Quantitation of junctional and extrajunctional acetylcholine receptors by electronmicroscope autoradiography after ^{125}I-alpha-bungarotoxin binding at mouse neuromuscular junctions. **Journal of Cell Biology 69**, 144-158.

Fey, E.G., Wan, K.M. & Penman, S. (1984). Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: Three-dimensional organization and protein composition. **Journal of Cell Biology 98**, 1973-1984.

Fitzgerald, M. & Shenk, T. (1981). The sequence 5'-AAUAAA-3' forms part of the recognition site for polyadenylation of late SV 40 mRNAs. **Cell 24**, 251-260.

Fraser, N.W., Nevins, J.R., Ziff, E. & Darnell, J.E. (1979). The major late Adenovirus type-2 transcription unit: Termination is downstream from the last poly(A) site. **Journal of Molecular Biology 129**, 643-656.

Freed, J.J. & Lebowitz, M.M. (1970). The association of a class of saltatory movements with microtubules in cultured cells. **Journal of Cell Biology 45**, 334-354.

Fulton, A.B., Wan, K.M. & Penman, S. (1980). The spatial distribution of polyribosomes in 3T3 cells and the associated assembly of proteins into the skeletal framework. **Cell 20**, 849-857.

Fulton, A.B. (1984). "The cytoskeleton: Cellular architecture and choreography." Chapman & Hall, New York.

Gaedigk, R., Oehler, S., Kohler, K. & Setyono, B. (1985). In vitro reconstitution of messenger ribonucleoprotein particles from globin messenger RNA and cytosol proteins. **FEBS Letters 179**, 201-207.

Galli, G., Hofstetter, H. & Birnstiel, M.L. (1981). Two conserved sequence blocks within eukaryotic tRNA genes are major promoter elements. **Nature 294**, 626-631.

Geoghegan, T., Cereghini, S. & Brawerman, G. (1979). Inactive mRNA-protein complexes from mouse sarcoma-180 ascites cells. **Proceedings of the National Academy of Sciences of the U. S. A. 76**, 5587-5591.

Goumans, H., Thomas, A., Verhoeven, A., Voorma, H.O. & Benne, R. (1980). The role of eIF-4C in protein synthesis initiation complex formation. **Biochimica et Biophysica Acta 608**, 39-46.

Greenberg, J.R. (1979). Ultraviolet light-induced cross-linking of mRNA to proteins. **Nucleic Acids Research 6**, 715-732.

Greenberg, J.R. (1980). Proteins cross-linked to messenger RNA by irradiating polyribosomes with ultraviolet light. **Nucleic Acids Research 8**, 5685-5701.

Greenberg, J.R. & Carroll, E. (1985). Reconstitution of functional mRNA-protein complexes in a rabbit reticulocyte cell-free translation system. **Molecular and Cellular Biology 5**, 342-351.

Hauschka, P.V. (1973). Analysis of nucleotide pools in animal cells. **Methods in Cell Biology 7**, 361-462.

Heggeness, M.H., Wang, K. & Singer, S.J. (1977). Intracellular distributions of mechanochemical proteins in cultured fibroblasts. **Proceedings of the National Academy of Sciences of the U. S. A. 74**, 3883-3887.

Helentjaris, T. & Ehrenfeld, E. (1978). Control of protein synthesis in extracts from Poliovirus-infected cells. I. mRNA discrimination by crude initiation factors. **Journal of Virology 26**, 510-521.

Hentschel, C.C. & Birnstiel, M.L. (1981). The organization and expression of histone gene families. **Cell 25**, 301-313.

Holmgren, A. (1981). Regulation of ribonucleotide reductase. **Current Topics in Cellular Regulation 19**, 47-76.

Howe, J.G. & Hershey, J.W.B. (1984). Translational initiation factor and ribosome association with the cytoskeletal framework fraction from HeLa cells. **Cell 37**, 85-93.

Inamine, G., Nash, B., Weissbach, H. & Brot, N. (1985). Light regulation of the synthesis of the large subunit of ribulose-1,5-biphosphate carboxylase in peas: Evidence for translational control. **Proceedings of the National Academy of Sciences of the U. S. A. 82**, 5690-5694.

Jacobs, F.A., Bird, R.C. & Sells, B.H. (1985). Differentiation of rat myoblasts. Regulation of turnover of ribosomal proteins and their mRNAs. **European Journal of Biochemistry 150**, 255-263.

Jeffery, W.R., Tomlinson, C.R. & Brodeur, R.D. (1983). Localization of actin messenger RNA during early Ascidian development. **Developmental Biology 99**, 408-417.

Jeffery, W.R. (1984). Spatial distribution of messenger RNA in the cytoskeletal framework of Ascidian eggs. **Developmental Biology 103**, 482-492.

Jorgensen, A.O., Subrahmanyan, L., Turnbull, C. & Kalnins, V.I. (1976). Localization of the neurofilament protein in neuroblastoma cells by immunofluorescence staining. **Proceedings of the National Academy of Sciences of the U. S. A. 73**, 3192-3196.

Kennedy, D.S., Siegel, E. & Heywood, S.M. (1978). Purification of myosin mRNP translational control RNA and its inhibition of myosin and globin messenger translation. **FEBS Letters 90**, 209-214.

Kirk, M.M. & Kirk, D.L. (1985). Translational control of protein synthesis, in

response to light, at a critical stage of Volvox development. Cell 41, 419-428.

Kozak, M. (1983). Comparison of initiation of protein synthesis in procaryotes, eucaryotes and organelles. Microbiological Reviews 47, 1-45.

Kozak, M. & Shatkin, A.J. (1977). Sequences of two 5'-terminal ribosome-protected fragments from Reovirus messenger RNAs. Journal of Molecular Biology 112, 75-96.

Lazarides, E. (1976). Actin, alpha-actinin, and tropomyosin interaction in the structural organization of actin filaments in nonmuscle cells. Journal of Cell Biology 68, 202-219.

Lazarides, E. (1980). Intermediate filaments as mechanical integrators of cellular space. Nature 283, 249-256.

Lemieux, R. & Beaud, G. (1982). Expression of Vaccinia virus early mRNA in Ehrlich Ascites tumor cells. European Journal of Biochemistry 129, 273-279.

Lenk, R., Ransom, L., Kaufmann, Y. & Penman, S. (1977). A cytoskeletal structure with associated polyribosomes obtained from HeLa cells. Cell 10, 67-78.

Liautard, J.P. & Egly, J.M. (1980). In vitro translational studies of the cytoplasmic nonpolysomal particles containing messenger RNA. Nucleic Acids Research 8, 1793-1804.

Li-Weber, M. & Schweiger, H-G. (1985). Evidence for and mechanism of translational control during cell differentiation in Acetabularia. European Journal of Cell Biology 38, 73-78.

Lodish, H.F. & Rose, J.K. (1977). Relative importance of 7-methylguanosine in ribosome binding and translation of Vesicular Stomatitis virus mRNA in wheat germ and reticulocyte cell-free systems. Journal of Biological Chemistry 252, 1181-1188.

Luby, K.J. & Porter, K.R. (1980). The control of pigment migration in isolated melanophores of Holocentrus ascensionis (Osbeck). I. Energy requirements. Cell 21, 13-23.

Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982). "Molecular Cloning: A Laboratory Manual". Cold Spring Harbour Laboratory Press, New York.

Masters, C. (1984). Interactions between glycolytic enzymes and components of the cytomatrix. Journal of Cell Biology 99, 222s-225s.

Mastro, A.M. & Keith, A.D. (1984). Diffusion in the aqueous compartment. Journal of Cell Biology 99, 180s-187s.

Merlie, J.P. & Sanes, J.R. (1985). Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibres. **Nature 317**, 66-68.

Miesfeld, R. & Arnheim, N. (1984). Species-specific rDNA transcription is due to promoter-specific binding factors. **Molecular and Cellular Biology 4**, 221-227.

Mishima, Y., Financsek, I., Kominami, R. & Muramatsu, M. (1982). Fractionation and reconstitution of factors required for accurate transcription of mammalian ribosomal RNA genes: Identification of a species dependent initiation factor. **Nucleic Acids Research 10**, 6659-6670.

Mitchison, T. & Kirschner, M. (1984). Microtubule assembly nucleated by isolated centrosomes. **Nature 312**, 232-237.

Moldave, K. (1985). Eukaryotic protein synthesis. **Annual Review of Biochemistry 54**, 1109-1149.

Nevins, J.R. & Darnell, J.E. (1978). Steps in the processing of Ad2 mRNA: Poly(A)+ nuclear sequences are conserved and poly(A) addition precedes splicing. **Cell 15**, 1477-1493.

Nevins, J.R. (1979). Processing of late Adenovirus nuclear RNA to mRNA. Kinetics of formation of intermediates and demonstration that all events are nuclear. **Journal of Molecular Biology 130**, 493-506.

Nielsen, P., Goelz, S. & Trachsel, H. (1983). The role of the cytoskeleton in eucaryotic protein synthesis. **Cell Biology International Reports 7**, 245-254.

Noronha, J.M., Sheys, G.H. & Buchanan, J.M. (1972). Induction of reductive pathway for deoxyribonucleotide synthesis during early embryogenesis of the sea urchin. **Proceedings of the National Academy of Sciences of the U. S. A. 69**, 2006-2010.

Orr, T.S.C., Hall, D.E. & Allison, A.C. (1972). Role of contractile microfilaments in the release of histamine from mast cells. **Nature 236**, 350-351.

Osborn, M. & Weber, K. (1976). Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structure towards the plasma membrane. **Proceedings of the National Academy of Sciences of the U. S. A. 73**, 867-871.

Osborn, M., Franke, W.W. & Weber, K. (1977). Visualization of a system of filaments 7-10 nm thick in cultured cells of an epithelioid (PtK2) by immunofluorescence microscopy. **Proceedings of the National Academy of Sciences of the U. S. A. 74**, 2490-2494.

Paine, P.L. (1984). Diffusive and nondiffusive proteins in vivo. **Journal of Cell Biology 99**, 188s-195s.

Parsegian, V.A. & Rau, D.C. (1984). Water near intracellular surfaces. Journal of Cell Biology 99, 196s-200s.

Paterson, B.M. & Rosenberg, M. (1979). Efficient translation of prokaryotic mRNAs in eukaryotic cell-free system requires addition of a cap structure. Nature 279, 692-696.

Pederson, T. (1983). Nuclear RNA-protein interactions and messenger RNA processing. Journal of Cell Biology 97, 1321-1326.

Peterson, D.T., Safer, B. & Merrick, W.C. (1979). Role of eukaryotic initiation factor 5 in the formation of 80s initiation complexes. Journal of Biological Chemistry 254, 7730-7735.

Petryshyn, R., Levin, D.H. & London, I.M. (1982). Regulation of double-stranded RNA-activated eukaryotic initiation factor 2-alpha kinase by type 2 protein phosphatase in reticulocyte lysates. Proceedings of the National Academy of Sciences of the U. S. A. 79, 6512-6516.

Pickett-Heaps, J.D., Tippit, D.H. & Porter, K.R. (1982). Rethinking mitosis. Cell 29, 729-744.

Piper, P.W. (1979). Polyoma virus transcription early during productive infection of mouse 3T6 cells. Journal of Molecular Biology 131, 399-407.

Preobrazhensky, A.A. & Spirin, A.S. (1978). Informosomes and their protein components: The present state of knowledge. Progress in Nucleic Acids Research and Molecular Biology 21, 1-38.

Ranu, R.S. & London, I.M. (1976). Regulation of protein synthesis in rabbit reticulocyte lysates: Purification and initial characterization of the cyclic 3':5'-AMP independent protein kinase of the heme-regulated translational inhibitor. Proceedings of the National Academy of Sciences of the U. S. A. 73, 4349-4353.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. & Berg, P. (1977). Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. Journal of Molecular Biology 113, 237-251.

Ruzdijic, S., Bag, J. & Sells, B.H. (1984). Cross-linked proteins associated with a specific mRNA in the cytoplasm of HeLa cells. European Journal of Biochemistry 142, 239-245.

Safer, B., Peterson, D. & Merrick, W.C. (1977). "Translation of natural and synthetic polynucleotides." (A. B. Legocki, Ed.). Poznan, University of Agriculture, Poznan.

Safer, B. (1983). 2B or not 2B: Regulation of the catalytic utilization of eIF-2. Cell 33, 7-8.

Sakonju, S., Bogenhagen, D.F. [illegible] D.D. (1980). A control region in the

centre of the 5s RNA gene directs specific initiation of transcription: I. The 5' border of the region. **Cell 19**, 13-25.

Salpeter, M.M. & Harris, R. (1983). Distribution and turnover rate of acetylcholine receptors throughout the junction folds at a vertebrate neuromuscular junction. **Journal of Cell Biology 96**, 1781-1785.

Sarkar, S. (1984). Translational control involving a novel cytoplasmic RNA and ribonucleoprotein. **Progress in Nucleic Acids Research and Molecular Biology 31**, 267-293.

Satir, P. (1984). Cytoplasmic matrix: Old and new questions. **Journal of Cell Biology 99**, 235s-238s.

Schliwa, M. & van Blerkom, J. (1981). Structural interaction of cytoskeletal components. **Journal of Cell Biology 90**, 222-235.

Schmid, H-P., Kohler, K. & Setyono, B. (1982). Possible involvement of messenger RNA-associated proteins in protein synthesis. **Journal of Cell Biology 93**, 893-898.

Seal, S.N., Schmidt, A. & Marcus, A. (1983). Fractionation and partial characterization of the protein synthesis system of wheat germ. II. Initiation factors D1 (eucaryotic initiation factor 3), D2c (eucaryotic initiation factor 5), and D2d (eucaryotic initiation factor 4C). **Journal of Biological Chemistry 258**, 866-871.

Seed, B. (1982). Diazotizable arylamine cellulose papers for the coupling and hybridization of nucleic acids. **Nucleic Acids Research 10**, 1799-1810.

Soeda, E., Arrand, J.R., Smolar, N., Walsh, J.E. & Griffin, B.E. (1980). Coding potential and regulatory signals of the Polyoma virus genome. **Nature 283**, 445-453.

Spirin, A.S. & Ajtkhozhin, M.A. (1985). Informosomes and polyribosome-associated proteins in eukaryotes. **Trends in Biochemical Sciences 10**, 162-165.

Standart, N.M, Bray, S.J., George, E.L., Hunt, T. & Ruderman, J.V. (1985). The small subunit of ribonucleotide reductase is encoded by one of the most abundant translationally regulated maternal RNA's in clam and sea urchin eggs. **Journal of Cell Biology 100**, 1968-1976.

Thelander, L. & Reichard, P. (1979). Reduction of ribonucleotides. **Annual Review of Biochemistry 48**, 133-158.

Thompson, H.A., Sadnik, I., Steinbuks, J. & Moldave, K. (1977). Studies on ribosomal subunits from rat liver. Purification and characterization of a ribosome dissociation factor. **Biochemistry 16**, 2221-2230.

Tinourian, H. & Uno, J. (1967). Protein synthesis requirements for the first two

cleavages in sea urchin eggs. **Experimental Cell Research 48**, 173-176.

van Venrooij, W.J., Sillekens, P.T.G., van Eekelen, C.A.G. & Reinders, R.J. (1981). On the association of mRNA with the cytoskeleton in uninfected and Adenovirus-infected human KB cells. **Experimental Cell Research 135**, 79-91.

Vincent, A., Goldenberg, S. & Scherrer, K. (1981). Comparisons of proteins associated with duck-globin mRNA and its polyadenylated segment. Polyribosomal and repressed free messenger ribonucleoprotein complexes. **European Journal of Biochemistry 114**, 179-193.

Wagenaar, M.B. (1983). The timing of synthesis of proteins required for mitosis in the cell cycle of the sea urchin embryo. **Experimental Cell Research 144**, 393-403.

Wang, E., Cross, K.R. & Choppin, P.W. (1979). Involvement of microtubules and 10 nm filaments in the movement and positioning of nuclei in syncytia. **Journal of Cell Biology 83**, 320-337.

Weber, K. & Groeschel-Stewart, U. (1974). Antibody to myosin: The specific visualization of myosin-containing filaments in nonmuscle cells. **Proceedings of the National Academy of Sciences of the U. S. A. 71**, 4561-4564.

Welch, W.J. & Feramisco, J.R. (1985). Disruption of the three cytoskeletal networks in mammalian cells does not affect transcription, translation, or protein translocation changes induced by heat shock. **Molecular and Cellular Biology 5**, 1571-1581.

Wilt, F.H., Sakai, H. & Mazia, D. (1967). Old and new protein in the formation of the mitotic apparatus in cleaving sea urchin eggs. **Journal of Molecular Biology 27**, 1-7.

Wodnar-Filipowicz, A., Szczesna, E., Zan-Kowalczewska, M., Muthukrishnan, S., Szybiak, U., Legocki, A.B. & Filipowicz, W. (1978). 5'-Terminal 7-methylguanosine and mRNA function. The effect of enzymatic decapping and of cap analogs on translation of Tobacco-Mosaic-Virus RNA and globin mRNA in vitro. **European Journal of Biochemistry 92**, 69-80.

Wolosewick, J.J. & Porter, K.R. (1979). Microtrabecular lattice of the cytoplasmic ground substance. Artifact or reality. **Journal of Cell Biology 82**, 114-139.

Young, C.W., Hendler, F.J. & Kanofsky, D.A. (1969). Synthesis of protein for DNA replication and cleavage events in the sand dollar embryo. **Experimental Cell Research 58**, 15-26.

Ziff, E.B. & Evans, R.M. (1978). Coincidence of the promoter and capped 5' terminus of RNA from the Adenovirus 2 major late transcription unit.

Cell 15, 1463-1475.

Zumbe, A., Stahli, C. & Trachsel, H. (1982). Association of a M_r 50,000 cap-binding protein with the cytoskeleton in baby hamster kidney cells. **Proceedings of the National Academy of Sciences of the U. S. A. 79**, 2927-2931.