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Effect of myoblast fusion during muscle cell differentiation on myosin polysome accumulation and myosin synthesis

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Effect of myoblast fusion during muscle cell differentiation on myosin polysome accumulation and myosin synthesis

by

Ronald B. Young

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

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LIST OF ABBREVIATIONS

ATP = adenosine 5'-triphosphate
ATPase = adenosine triphosphatase
°C = degree celsius
cAMP = 3'-5' cyclic adenosine monophosphate
cGMP = 3'-5' cyclic guanosine monophosphate
Ci = Curie
cm = centimeter
DNA = deoxyribonucleic acid
EDTA = ethylenediaminetetraacetic acid
$g$ = acceleration due to gravity
$g$ = gram
GTP = guanosine 5'-triphosphate
I-DNA = informational DNA
hr = hours
IF = initiation factor
min = minutes
ml = milliliters
nm = nanometer
poly A = polyadenylic acid
poly U = polyuridylic acid
RNA = ribonucleic acid
S = sedimentation coefficient
SDS = sodium dodecyl sulfate
sec  = seconds
TCA  = trichloroacetic acid
tcRNA = translational control RNA
Tris = tris-(hydroxymethyl)-aminomethane
µCi  = microcurie
µM   = micromolar
I. INTRODUCTION

Although most cell types perform various kinds of intracellular movements and some cells are motile in their entirety, the macromolecular organization of the contractile elements in these cells varies widely. For example, the contractile units of skeletal and cardiac muscle exhibit an extremely organized lattice-like array when examined by electron microscopy, and many of the regularly-spaced lattice distances such as those between actin and myosin filaments as well as those among individual actin and myosin molecules within filaments are known very precisely. Hence, arrangement of the eight or nine different proteins that can be isolated from intact skeletal or cardiac muscle myofibrils approaches the upper limit of organizational sophistication observed for biological molecules. Skeletal and cardiac muscle myofibrils are even more remarkable because these highly ordered filamentous arrays are capable of sliding past each other during contraction and relaxation. Although other contractile systems, such as smooth muscle and cytoplasmic contractile systems, lack the very regular and highly ordered organization of functional contractile units that is seen in skeletal and cardiac muscle, use of the energy of ATP hydrolysis and the interaction of actin and myosin
to generate force for movement are generally considered to
be similar, if not identical, chemical processes in these
diverse cell types.

Accretion of myofibrillar proteins into functional
sarcomeres in skeletal muscle must involve at least two
distinct cytoplasmic processes: 1) synthesis of functional
myofibrillar protein molecules; and 2) assembly of these
native molecules into the correct three-dimensional array.
Although there is a distinct possibility that myofibrillar
proteins "self-assemble" in the cytoplasm of muscle cells,
very little evidence has yet been obtained to support or
to refute this idea. On the other hand, synthesis of
myofibrillar proteins has been studied in greater detail,
and the work described in this dissertation focuses on
the protein synthetic aspect of formation of contractile
muscle cells in culture.

The myofibrillar protein, myosin, composes approxi-
mately 55 percent of the total myofibrillar protein frac-
tion, and studies on factors regulating myofibrillar pro-
tein synthesis in cultures of skeletal muscle cells have
involved almost exclusively studies on myosin synthesis.
Two unique properties of the myosin molecule have fostered
this emphasis: 1) myosin is insoluble in 0.03 M KCl, where-
as most other cytoplasmic proteins are completely soluble
at this concentration of potassium chloride; and 2) the two
large, identical, polypeptide subunits of myosin have a molecular weight of 200,000 daltons each—a molecular weight larger than that of nearly all other known proteins—and these large polypeptide chains can be completely separated from the polypeptide chains of other muscle proteins by using SDS-polyacrylamide gel electrophoresis. These two unique properties of myosin have made it possible, therefore, to measure myosin synthesis quantitatively by measuring incorporation of radioactively labeled amino acids into the 200,000-dalton myosin heavy chains because myosin synthesized in cell-free protein synthetic assays can be separated from other muscle proteins by two rapid purification steps based on the two unique properties of myosin.

Use of these two properties of myosin to monitor changes in rate of myosin biosynthesis during differentiation of muscle cells in highly synchronized cultures has shown that the rapid burst of fusion of mononucleated myoblasts into multinucleated myotubes that marks terminal differentiation of embryonic muscle cells is followed by a dramatic increase in rate of synthesis of the two large polypeptide chains of myosin; this increase begins approximately four to eight hours after fusion. Although fusion of mononucleated myoblasts seems to initiate a remarkable increase in rate of myosin synthesis, neither the biochemical stimulus nor the factors that regulate this activation
of gene expression are clearly understood.

The broad goal of this research project, therefore, was to study the regulation of myosin synthesis in synchronized cultures of differentiating embryonic chicken muscle cells. The results of this study, when analyzed together with results of several related studies done by other workers, indicate that rate of myosin synthesis is not regulated simply by the amount of myosin messenger RNA present in the cytoplasm of the cells. Instead, low levels of myosin messenger RNA seem to be present in mononucleated myoblasts before fusion, and this messenger RNA is associated with ribosomes to form myosin-synthesizing polysomes. Furthermore, the results to be described in this dissertation suggest that rate of movement of ribosomes along myosin messenger RNA may be slower in mononucleated myogenic cells than it is in multinucleated myotubes.
II. LITERATURE REVIEW

Before reviewing the available literature on control of synthesis of the major myofibrillar protein, myosin, it is necessary to examine a number of different aspects of muscle differentiation and to discuss what is known about how these different phases of myogenesis interact to form highly differentiated, contractile muscle fibers. This review will be divided into six sections that will discuss the following aspects of muscle differentiation: (1) changes in morphology and ultrastructure; (2) changes in nucleic acid metabolism; (3) changes in energy metabolism; (4) changes in cyclic nucleotide metabolism; (5) changes in structure and function of membranes; and (6) control of myofibrillar protein synthesis. The last section on control of myofibrillar protein synthesis establishes the basis for the work described in this dissertation, and will serve as a prelude to the Results and Discussion.

A. Changes in Morphology and Ultrastructure of Differentiating Muscle Cells

It has been well established that almost all muscle tissue differentiates from mesoderm, the middle of the three primary germ layers of the embryo (Herrmann et al., 1970).
Although Holtzer (1970) and Abbott et al. (1974) have suggested that no uncommitted or undifferentiated cells exist at the molecular level, it is impossible to recognize unequivocally those cells destined to form muscle tissue before they reach the myoblast stage. Cell culture studies have indicated that cells committed to becoming muscle cells but structurally difficult to distinguish conclusively from other cells committed to becoming fibroblasts exist before the myoblast stage. These cells are called presumptive myoblasts and undoubtedly have undergone considerable differentiation from the early mesodermal cells (Abbott et al., 1974).

Myoblasts generally appear in muscle cell cultures as spindle-shaped, mononucleated cells. Because they can be conclusively identified only if they contain myosin and actin filaments, and because they have ceased mitotic activity, myoblasts have been defined as postmitotic cells capable of synthesizing contractile proteins and of fusing with other similar postmitotic cells (Bischoff and Holtzer, 1969; Fischman, 1972; Holtzer and Bischoff, 1970; Ishikawa et al., 1968). The recent results of Stockdale and O’Neill (1972), and O’Neill and Stockdale (1972a; 1972b), however, indicate that some cells may exist that are capable of fusing but that can also be induced by changing environmental conditions to undergo one or more additional mitotic cycles.
The first event that can be detected ultrastructurally that is unique to myogenesis is fusion of myoblasts to form myotubes (Fischman, 1972). Because fusion can be observed ultrastructurally and because it seems to be the committed step in myogenesis, myoblast fusion has been studied extensively; however, the biochemical nature of the events that occur in mononucleated myoblasts to launch the fusion process are completely unknown. Biochemical studies coupled with microscope examination have shown that mitotically dividing cells only fuse in the G1 phase of the cell cycle. Bischoff and Holtzer (1969) and Holtzer and Bischoff (1970) have proposed that the mitosis immediately preceding the G1 period in which fusion occurs is an unusual mitosis (termed a "quantal mitosis") that produces cells irreversibly committed to fusion. O'Neill and Stockdale (1972a; 1972b), on the other hand, have presented evidence that competent myoblasts in G1 are not committed to fusion but may undergo one or more additional mitotic cycles, depending on environmental conditions.

The onset of fusion is initiated by an end-to-end alignment of myoblasts such that surface membranes of adjacent cells approach each other very closely (Fischman, 1972). These surface membranes gradually fuse and then disappear altogether; this disappearance is followed by a gradual confluence of the cytoplasm of the two cells. Fusion
of myoblasts produces elongated, multinucleated cells called myotubes. Structural studies have shown that myoblasts may fuse with other myoblasts, that myoblasts may fuse with myotubes, and that myotubes may fuse with other myotubes (Fischman, 1972).

Almost immediately after fusion, synthesis of contractile proteins increases dramatically, and thick and thin filaments are observed very quickly after myotube formation (Fischman, 1972). Because myosin and actin filaments can also be observed in some mononucleated myoblasts (Konigsberg, 1965), however, it is clear that fusion is not an obligatory requirement for bulk synthesis of contractile proteins. Newly formed thick and thin filaments evidently self-assemble in the cytoplasm of the myotube to form hexagonal myofibril arrays similar to those observed in mature skeletal muscle cells, although somewhat less highly ordered (Fischman, 1967, 1970; Shimada, 1971). The forces responsible for assembly of thick and thin filaments are not known. For example, it is not known whether thick filaments or thin filaments are formed first and serve as nucleation sites for assembly of the remainder of the myofibril. Neither is it known whether Z-disks and M-lines are involved in myofibrillar assembly. It has been observed however, that myofibrils generally form first at the periphery of the cell; this process results in elongated myotubes.
filled with myofibrils at their periphery but having open areas of cytoplasm and nuclei in their center (Fischman, 1970). As myotubes mature, additional myofibrils are formed until the entire interior of the myotube is filled with myofibrils leaving only space for nuclei in the center of the cell (Fischman, 1970; Holtzer et al., 1973). At this stage the nuclei migrate toward the periphery of the cell until they lie immediately under the sarcolemma; this is their location in mature skeletal muscle cells. At this stage, the myotube becomes a myofiber (Fischman, 1970). Neither the physiological significance nor the forces responsible for outward migration of nuclei in skeletal muscle myotubes are known.

B. Changes in Nucleic Acid Metabolism in Differentiating Muscle Cells

The evidence discussed in the preceding section demonstrates that during differentiation myogenic cells cease mitotic proliferation and fuse to form multinucleated myotubes. It would therefore be anticipated that differentiation of myogenic cells is accompanied by drastic alterations in nucleic acid metabolism. The first conclusive demonstration that DNA synthesis subsides during muscle differentiation was made by Stockdale and Holtzer (1961) who added $^{3}$H-thymidine to cultures of embryonic skeletal
muscle cells under three different sets of conditions. In the first series of experiments, cultures of proliferating myogenic cells were pulse-labeled with $^3$H-thymidine for 30 min 18 hr after the cultures were established. These cultures, which consisted of mononucleated cells, were immediately fixed and subjected to autoradiography. Under these experimental conditions, labeled DNA was found only in nuclei of mononucleated cells. None of the nuclei in the small number of multinucleated myotubes were labeled in these young cultures. These results suggested, therefore, that nuclei within myotubes could not synthesize DNA. In the second series of experiments, cultures of proliferating cells were again pulse labeled with $^3$H-thymidine for 30 min 18 hr after the cultures were established. These cultures were also immediately rinsed free of $^3$H-thymidine, but were then incubated for 4 days before they were fixed and subjected to autoradiography. Under these experimental conditions, only a small proportion of the existing mononucleated cells had $^3$H-thymidine-labeled nuclei, but part of the nuclei in the multinucleated myotubes also contained labeled thymidine. Stockdale and Holtzer (1961) concluded from these experiments that nuclei in mononucleated cells undergoing mitosis are later incorporated into multinucleated myotubes but that no further DNA synthesis occurred in these nuclei after they had been incorporated
into the multinucleated myotubes. To confirm this conclusion, a third series of experiments was conducted in which 3 to 5-day myogenic cultures, which consisted largely of multinucleated myotubes, were pulse-labeled with $^3$H-thymidine for 30 min, and the cultures were immediately fixed and subjected to autoradiography. As predicted from the first two series of experiments, nuclei in mononucleated cells synthesized DNA as evidenced by a large number of positive autoradiographic grains, but none of the large number of myotube nuclei contained any radioactivity. These experiments imply that fusion of mononucleated cells into multinucleated myotubes is accompanied by almost complete loss of the ability of nuclei in multinucleated muscle cells to synthesize DNA. These results have since been confirmed by a large number of workers using more direct and quantitative techniques (Okazaki and Holtzer, 1966; O'Neill and Stockdale, 1972b; O'Neill and Strohman, 1969, 1970; Paterson and Strohman, 1972; Stockdale, 1970). Perhaps the most dramatic biochemical demonstration of cessation of DNA synthesis during myoblast fusion was made by O'Neill and Strohman (1970). These workers pulse-labeled cultures of embryonic muscle cells at different stages of differentiation with $^3$H-thymidine and measured the percentage decrease in amount of $^3$H-thymidine incorporated into partly purified DNA per µg of DNA. The
percentage decrease in $^{3}H$-thymidine incorporation was almost identical to the percentage decrease in number of nuclei in mononucleated cells due to fusion of these cells to form multinucleated myotubes. It is not known whether initiation of myoblast fusion is causally related to cessation of DNA synthesis, but the two phenomena are clearly closely related in a temporal sense.

$^{3}H$-thymidine incorporation into DNA is a direct measure of DNA polymerase activity in these cultures, and it has been speculated (O'Neill and Strohman, 1969, 1970; Stockdale, 1970) that the decrease in DNA polymerase activity accompanying myoblast fusion results specifically from reduction in amount of DNA polymerase enzyme present in the nuclei of the differentiating myogenic cells rather than from an inhibition of the activity of existing DNA polymerase. Micha and Stockdale (1972) isolated the DNA polymerases in embryonic chicken muscle and found that at least two DNA polymerases differing in molecular weight and template preference are present in these cells. The smaller of these two DNA polymerases was found predominantly in the nuclear fraction, was most active with native DNA, and lost activity if the native DNA template was denatured or nicked with pancreatic deoxyribonuclease. On the other hand, the larger of the two enzymes was predominantly located in the soluble fraction of the cell and
was most active with a denatured or nicked DNA template. Although Wicha and Stockdale (1972) implied that the nuclear DNA polymerase was possibly the enzyme normally involved in replication, no evidence was given either to support this conclusion or to demonstrate any change in amount or activity of this enzyme during differentiation. Because of the soluble enzyme's preference for nicked or denatured DNA templates, Wicha and Stockdale implied that the cytoplasmic DNA polymerase was involved in repair of nicked DNA in the cell. Again, however, no data was shown to support this postulate.

Although it has been rigorously shown that DNA synthesis is almost totally inhibited in the nuclei of myotubes (O'Neill and Stockdale, 1972b; O'Neill and Strohman, 1969, 1970; Okazaki and Holzer, 1966; Paterson and Strohman, 1972; Stockdale, 1970; Stockdale and Holtzer, 1961), it was unclear for several years whether this decrease in DNA synthesis applied only to the synthetic events required for semiconservative replication or whether both replicative and repair synthesis were inhibited once nuclei had fused into multinucleated myotubes. Stockdale (1971) irradiated muscle cell cultures with ultraviolet light at levels adequate to cause formation of significant levels of pyrimidine dimers and found that myotube cultures retained their ability to excise these dimers and to replace them
with $^3$H-thymidine. Repair DNA synthesis, however, occurred in the myotube nuclei at only half the rate that it did in proliferating myoblasts. Similar results concerning the ability of nonreplicating myotube nuclei to effect repair DNA synthesis have been obtained by using univalent alkylating agents to induce the mutations (Hahn et al., 1971). Together, the results of Hahn et al. (1971) and Stockdale (1970) indicate at least four conclusions concerning DNA synthesis in myotube nuclei: (1) the factors controlling semiconservative DNA replication do not control DNA repair; (2) differentiated skeletal muscle cells can phosphorylate and incorporate $^3$H-thymidine into DNA within myotube nuclei; (3) a polymerizing enzyme must be present in sufficient quantities for this DNA repair synthesis to take place; and (4) the ability to repair chemically altered bases in DNA is partially lost during differentiation. Whether DNA repair synthesis requires reversal of the controls prohibiting replicative synthesis or the maintenance or induction of a separate repair system, and whether the repair enzyme proposed by Wicha and Stockdale (1972) is involved directly in this DNA repair has not been determined.

In addition to termination of DNA synthesis described in the preceding paragraphs, muscle differentiation is also accompanied by a large decrease in the amount of nuclear
DNA-dependent RNA polymerase activity (Herrmann et al., 1970; Marchok, 1966; Marchok and Wolff, 1968; Yaffe and Fuchs, 1967). Marchok and Wolff (1968) have shown that this decrease in total RNA polymerase activity is due to a true decrease in the amount of enzyme protein that can be extracted from the nuclei of myotubes and not to an inhibition of activity of existing enzyme molecules. Subsequent to these reports showing that the amount of nuclear DNA-dependent RNA polymerase enzyme decreases during muscle cell differentiation, it was discovered that at least three RNA polymerases different from the nuclear RNA polymerase can be isolated from the cytoplasm of embryonic chicken muscle cells (Bell and Brown, 1972). These cytoplasmic RNA polymerases in differentiating muscle cells could originate from molecules that had just been synthesized on polyribosomes in the cytoplasm and were enroute to the nucleus or from enzymes that had leaked out of nuclei during cell fractionation. In either instance, the presence of this cytoplasmic RNA polymerase would have no functional significance. However, Bell and Brown (1972) demonstrated that the three RNA polymerases present in the cytoplasm of embryonic chicken skeletal muscle had properties which were distinctly different from the properties of DNA-dependent RNA polymerases isolated from nuclei. One of the three cytoplasmic enzymes was shown to add nucleoside triphosphates
terminally to RNA, and the other two enzymes were DNA-dependent and synthesized RNA heteropolymers. One of the latter two enzymes cosedimented with polyribosomes.

In order to explain the significance of DNA-dependent RNA polymerases in the cytoplasm of embryonic chicken muscle cells, Bell and coworkers (Bell, 1969; Bell and Brown, 1972; Bell et al., 1972) have proposed a model in which DNA gene copies, called I-DNA for informational DNA, are synthesized in the nucleus, are transported to the cytoplasm, and there are transcribed into functional mRNA's by DNA-dependent RNA polymerases. The model is based on a large amount of circumstantial evidence obtained by extensive sedimentation-equilibrium characterization of cellular particles in CsCl₂ gradients. After synthesis in the nucleus and before transport to the cytoplasm, the I-DNA aggregates with different amounts of protein to form I-somes of different sizes. These different-sized I-somes all have a buoyant density of 1.57 g/ml, and "grow" in the cytoplasm by accretion of a stabilizing protein which is purported to have a density of 1.3 g/ml. When the I-somes become saturated with protein, they have a buoyant density of 1.43 g/ml and are inactive. The I-somes then begin to polymerize; when they have polymerized to the extent that they sediment at greater than 40S, they become activated in some unknown way. This activation
enables RNA polymerase molecules to attach. This particle, still having a density of 1.43 g/ml, is proposed to be a transcription complex in which the I-DNA serves as a template for synthesis of RNA. It is further suggested that ribosomes attach to the newly synthesized nascent RNA strands resulting in the formation of a transcription-translation complex with a buoyant density of 1.515 g/ml. This model accounts for RNA polymerase activity in the cytoplasm of muscle cells and also suggests that the key events in differentiation may not involve regulation of mRNA transcription, but instead may involve regulation of the production of I-DNA and I-somes. However, until evidence is obtained that I-DNA is required for synthesizing some specific mRNA, the existence and physiological role of I-DNA and I-somes will remain uncertain.

In addition to measuring the amount and activity of RNA polymerase during differentiation of myogenic cells, changes in nucleic acid synthesis during muscle cell differentiation have also been studied by using quantitative RNA-DNA hybridization techniques. Cultures of muscle cells at different stages of differentiation are pulse-labeled with $^3$H-uridine to radioactively label all RNA synthesized during the labeling period. When total RNA is extracted from these cells and hybridized against whole-embryo DNA at saturating levels of RNA, the proportion of a cell's
genome that is being transcribed at any point during muscle differentiation can be determined. Thi Man and Cole (1974) have applied this technique to cultures of both proliferating myoblasts and multinucleated myotubes and have found that 15% of the total genome of proliferating myoblasts is transcribed whereas only 7% of the total genome of the myotubes is transcribed. Therefore, terminal differentiation of muscle cells in culture is accompanied by a drastic decrease in the number of different mRNA's synthesized and, presumably, also by a related decrease in the number of proteins ultimately synthesized in these myotubes.

To obtain additional information on the decrease in number of different RNA species synthesized as myogenic cells differentiate, Thi Man and Cole (1974) also did hybridization studies under conditions where DNA was in excess. Such studies make it possible to distinguish between repeated sequences of DNA and rare or nonrepeated sequences of DNA because formation of RNA-DNA hybrids follows second order kinetics and repeated DNA sequences form hybrids quicker than rare DNA sequences. Analysis of RNA from muscle cell cultures in different stages of differentiation by hybridization with excess DNA showed that 30% of the RNA in replicating myoblasts was synthesized from repeated sequences of DNA and that the remaining 70% of the RNA in these cells was synthesized from nonrepeated
DNA sequences. On the other hand, only 16% of the RNA synthesized in multinucleated myotubes was synthesized from repetitive DNA sequences, and the remaining 84% of the RNA in these cells was synthesized from single-copy DNA sequences (Thi Man and Cole, 1974). Because ribosomal RNA is synthesized from repeated sequences of DNA, Thi Man and Cole (1972) and Clissold and Cole (1973) fractionated RNA from muscle cell cultures by using methylated-albumin-kieselguhr chromatography to separate ribosomal RNA from nonribosomal RNA. These studies showed that 28% of the total RNA in myoblasts was ribosomal RNA, whereas only 20% of the RNA in myotubes was ribosomal RNA. These percentages of ribosomal RNA agreed quite closely with the percentages of repeated-sequence RNA in these two cell types, and Cole and coworkers (Clissold and Cole, 1973; Thi Man and Cole, 1972) concluded that essentially all the repeated sequences of DNA that are being transcribed in proliferating myoblasts and multinucleated myotubes are transcribing for ribosomal RNA. Cole and coworkers (Clissold and Cole, 1973; Thi Man and Cole, 1972) also showed that, during differentiation of myogenic cells, total RNA synthesis per unit of DNA decreases threefold, mRNA synthesis per unit of DNA decreases twofold, and ribosomal RNA per unit of DNA decreases fivefold. During this same period of muscle development when RNA synthesis is declining markedly, the stability of many
species of RNA increases up to fivefold (Buckingham et al., 1974).

The drastic decreases in RNA synthesis and the disappearance of all but repair DNA synthesis during differentiation of myogenic cells are also accompanied by a marked decrease in activity of glucose-6-phosphate dehydrogenase (Love et al., 1969). Glucose-6-phosphate dehydrogenase is the first enzyme in the pentose phosphate pathway and is therefore responsible for initiating synthesis of the ribose and deoxyribose portion of the ribonucleotides and deoxyribonucleotides.

In summary, it is clear that muscle differentiation is accompanied by a vast reorganization of patterns of nucleic acid metabolism. Dramatic decreases in DNA polymerase, RNA polymerase, and glucose-6-phosphate dehydrogenase activities occur, the amount of mRNA synthesized relative to the amount of ribosomal RNA synthesized increases and stability of newly synthesized RNA increases.

C. Changes in Energy Metabolism in Differentiating Muscle Cells

Because the energy required for muscle contraction is derived from hydrolysis of ATP (Taylor, 1972; Tonomura and Osawa, 1972), it would intuitively be expected that
differentiation of myogenic cells would include development of pathways involved in producing this high energy compound. The glycolytic pathway is one of the major metabolic pathways responsible for producing ATP to supply energy for muscle contraction and it has been shown that activities of glycogen phosphorylase (Shainberg et al., 1971; Wahrman et al., 1972a), glycogen synthetase (Wahrman et al., 1973a), aldolase (Turner et al., 1974), and phosphoglycerokinase (Hauschka, 1968) increase several fold during muscle cell differentiation. In addition, much of the energy reserve in muscle is stored in the form of creatine phosphate (Bendall, 1971), which is synthesized from ATP by the enzyme, creatine phosphokinase. Fusion of mononucleated myogenic cells into multinucleated, contractile myofibers is accompanied by at least a 10 to 15-fold increase in the amount of creatine phosphokinase in cytoplasm of myofibers (Keller and Nameroff, 1974; Shainberg et al., 1971; Tarikas and Schubert, 1974; Turner et al., 1974). Shainberg et al. (1971) and Tarikas and Schubert (1974) have shown that the increase in both creatine phosphokinase and adenylate kinase activity observed during muscle cell differentiation is due to an increase in the absolute amount of enzyme per unit of DNA rather than to stimulation of the activity of existing enzyme molecules. Thus, it is evident that myogenic differentiation
includes induction of the required levels of key enzymes needed to provide adequate amounts of ATP for contraction of myofibrils in mature, functional muscle cells.

D. Changes in Cyclic Nucleotide Metabolism in Differentiating Muscle Cells

The mechanism of synthesis and degradation of 3'–5' cyclic adenosine monophosphate (cAMP) and the role of this compound as a second messenger in mediating the effects of various hormones and other biologically active agents on the physiological processes of an organism have been described extensively (Robison et al., 1968; Jost and Rickenberg, 1971). The physiological role of variations in cAMP levels or variations in cyclic guanosine monophosphate (cGMP) levels during different aspects of cell differentiation and morphogenesis, however, is not well understood (McMahon, 1974). Furthermore, studies of cyclic nucleotide metabolism in differentiating muscle are very limited, usually are purely descriptive, and often are conflicting.

Zalin and Montague (1974) reported that a 10 to 15-fold increase in intracellular cAMP concentration occurred approximately 5 to 6 hr before initiation of myoblast fusion. This increase lasted only for approximately one hr. Immediately subsequent to this transient increase,
cAMP concentration returned to a basal level and remained at that level throughout the remainder of muscle differentiation. If cAMP phosphodiesterase was inhibited with 3-isobutyl methyl xanthine so that cAMP levels never decreased to basal levels, however, myoblast fusion was inhibited (Zalin and Montague, 1974). These findings, together with other evidence (Wahrman et al., 1973b; Zalin, 1973) indicating that myoblast fusion is inhibited by theophylline (another cAMP phosphodiesterase inhibitor) or by dibutyryl cAMP (a nonhydrolyzable analog of cAMP) suggests that proper modulation of intracellular cAMP levels is critical for expression of some aspects of muscle differentiation. A transient modification in cAMP level such as that described by Zalin and Montague (1974) is exactly what McMahon's model for cellular differentiation (McMahon, 1974) would predict. McMahon's cell differentiation model proposes that the developmental path of a cell is modulated by temporal sequences of changes in concentrations of cyclic nucleotides and inorganic ions in the developing cell.

In contrast to Zalin and Montague's (1974) results, Reporter (1972) has found that cAMP concentration decreases in differentiating muscle cells immediately prior to fusion. It is possible, however, that Reporter's pre-fusion measurements of cAMP levels were by chance made
during the transient increase in cAMP level described by Zalin and Montague (1974). Such a coincidental event would make it appear that myoblast fusion was accompanied by a decrease in intracellular cAMP levels.

An additional contradiction exists concerning the effects of dibutyryl cAMP on myoblast fusion. Several investigators (Wahrman et al., 1973b,c; Zalin, 1973; Zalin and Montague, 1974) have reported that increasing intracellular cAMP concentration either by adding exogenous cAMP or by adding dibutyryl cAMP, theophylline, or 3-isobutyl methyl xanthine to the culture medium inhibited fusion of mononucleated myoblasts into multinucleated myotubes. On the other hand, Turner et al. (1974) have presented extensive evidence to show that dibutyryl cAMP had no effect on myoblast fusion. Because similar levels of dibutyryl cAMP were used in these studies, the reason for this discrepancy is unknown. Aw et al. (1973) found that dibutyryl cAMP actually stimulated myoblast fusion rather than suppressing it. The results of Aw et al. (1973), however, are impossible to evaluate because the authors did not mention the concentration of dibutyryl cAMP used for their experiments.

Other components of the cAMP system have also been reported to change during muscle differentiation. The amount of adenylate cyclase gradually increases several
fold during muscle cell differentiation (Hommes and Beere, 1971; Zalin and Montague, 1974), protein kinase increases slightly (Zalin and Montague, 1974), and cAMP phosphodiesterase activity decreased (Hommes and Beere, 1971).

In summary, it is clear that the metabolism of cAMP changes during the transition of proliferative myogenic cells into contractile myofibers, but neither the precise nature of these changes nor their relation to withdrawal of myogenic cells from the cell cycle have been determined.

E. Changes in Membrane Structure and Function in Differentiating Muscle Cells

For myofibrils encased within skeletal muscle cells to contract, a nerve impulse must be transmitted from the end plate lying next to a motor neuron on the exterior surface of muscle cells along the muscle cell membrane and into the interior of the cell where the myofibrils are situated. Development of this specialized ability of muscle cells to receive electrical messages and translate them into mechanical motion is an integral part of muscle development, and much work has been done in an attempt to understand the processes that occur during membrane differentiation. It has been known for a number of years that the chemical messenger between motor nerve endings and muscle cells is acetylcholine; however, very little was
known about the development of acetylcholine sensitivity until this process was extensively studied in cultures of skeletal muscle cells.

One of the pioneering investigations on development of acetylcholine sensitivity during myogenesis was by Fambrough and Rash (1971) who studied development of acetylcholine sensitivity in low density cultures of myogenic cells by iontophoretic application of acetylcholine to the surface of individual cells in different developmental stages followed by intracellular recording of electrical changes with a microelectrode. This technique permitted measurement of both the membrane resting potential and the sensitivity of the membranes enclosing these cells to depolarization by acetylcholine. These electrophysiological measurements were then correlated with electron microscope analysis of the structural development of the cells. Three different cell types were studied: (1) "short" bipolar myogenic cells (less than 100 µm in length; (2) "long" bipolar myogenic cells (greater than 100 µm in length); and (3) myotubes containing varying numbers of nuclei. Membrane resting potential gradually increased in proportion to myotube size from approximately -8 millivolts (inside negative) for both "short" and "long" bipolar myogenic cells to about -30 millivolts for the largest myotubes. Fibroblasts also had a membrane potential
of about -30 millivolts. Measurement of acetylcholine sensitivity showed that "short" bipolar cells and fibroblasts were not depolarized by acetylcholine but that "long" bipolar myogenic cells and all sizes of myotubes were depolarized by acetylcholine, with the magnitude of depolarization being proportional to the number of nuclei within the myotube. Several important conclusions can be drawn from these results of Fambrough and Rash (1971), and much of the subsequent work on development of acetylcholine sensitivity of muscle membranes is based on this study. First, "short" bipolar myogenic cells were probably presumptive myoblasts that had not yet become committed to fusion and that, therefore, had also not yet developed the ability to become depolarized by acetylcholine. On the other hand, the "long" bipolar myogenic cells were likely myoblasts committed to terminal differentiation but unable to locate another myoblast or myotube with which to fuse. These results led Fambrough and Rash (1971) and Fambrough et al. (1974b) to suggest that commitment of myogenic cells to differentiate may involve activation of all the genes that code for the special proteins characteristic of fully differentiated muscle cells. Thus, acetylcholine receptor synthesis may begin at the same time as myosin synthesis, for example, even though acetylcholine receptors may have no immediate function in
the morphogenesis of skeletal muscle fibers.

A second conclusion reached by Fambrough and Rash (1971) was that all myotubes exhibit acetylcholine sensitivity. In addition, because acetylcholine sensitivity increased as the size of the myotube increased, it seemed likely that the number of acetylcholine-sensitive sites was also increasing as myotubes become more highly differentiated (Fambrough and Rash, 1972; Fischbach et al., 1971). This conclusion has since been substantiated extensively by histochemical and biochemical studies (Fluck and Strohman, 1973; Paterson and Prives, 1973; Tennyson et al., 1973; Wilson et al., 1973).

Third, development of acetylcholine sensitivity occurred in the complete absence of innervation. This finding was one of the first strong pieces of experimental evidence that, although muscle differentiation may be influenced by neural factors, innervation is not an obligatory requirement for muscle cell differentiation.

Because fusion of myogenic cells obviously involves cell-cell recognition at certain very specific sites on the cell membrane and because acetylcholine sensitive sites seemed to appear very early in myogenesis, Fambrough and Rash (1971) examined the possibility that the specific recognition site for cell fusion was the acetylcholine receptor site. When mononucleated cells were treated with
tubocurarine or with excess acetylcholine to block the acetylcholine receptor sites, no measurable inhibition of myoblast fusion or myotube formation occurred. Therefore, the fourth major conclusion reached by Fambrough and Rash (1971) was that even though acetylcholine receptor sites are required for muscle fibers to function in their normal physiological manner, they are not the specific recognition site for fusion of myogenic cells.

Because all of Fambrough and Rash's (1971) results implied that muscle cell membranes develop the ability to be depolarized in the absence of innervation, a number of investigations have been conducted to compare the distribution of acetylcholine sensitivity on noninnervated muscle fibers grown in culture, on innervated muscle fibers grown in culture, and on mature innervated fibers in the intact animal. Measurement of the sensitivity of noninnervated muscle fibers in culture to localized iontophoretic applications of acetylcholine and measurement of the binding to muscle fibers of $^{125}$I-α-bungarotoxin (a snake venom which binds very tightly to acetylcholine receptors) has revealed that acetylcholine receptors are not uniformly distributed over the surface of noninnervated muscle cell membranes (Fambrough, 1974; Fambrough et al., 1974a; Fischbach and Cohen, 1973). Instead, "hotspots" of acetylcholine sensitivity are found, with most of these
concentrated areas of acetylcholine sensitivity located in the immediate vicinity of obvious muscle nuclei.

Distribution of acetylcholine receptors on the surface of multinucleated myotubes innervated in culture by adding seven-day chicken spinal cord cells has also been measured (Fambrough et al., 1974a; Fischbach and Cohen, 1973). After several days of incubation, some of the neurons formed functional contacts with nearby muscle cells (Fischbach, 1970, 1972), and although "hotspots" of acetylcholine sensitivity developed in the vicinity of the neuromuscular junctions of the myotubes in these mixed cultures, receptor sites continued to be spread over the remainder of the cell surface. Indeed, distribution of acetylcholine sensitivity over the surface of innervated myotubes was barely distinguishable from distribution of this sensitivity over the surface of noninnervated myotubes (Fischbach and Cohen, 1973; Robbins and Yonezawa, 1971). These patterns, however, are completely different from distribution of acetylcholine receptors on the surface of innervated muscle fibers in a mature animal where they are restricted almost totally to the site of the neuromuscular junction.

Events controlling the appearance of acetylcholine receptors in membranes of myogenic cells have been studied by quantitatively measuring the irreversible binding of $^{125}$I-$\alpha$-bungarotoxin to the receptors at different stages
in muscle cell differentiation or after application of different treatments known to affect the course of muscle cell differentiation. Hartzell and Fambrough (1973) and Fambrough (1974) have demonstrated that acetylcholine receptor density increases at the rate of approximately 35 sites/μm²/hr immediately after initiation of fusion. These new sites are functional acetylcholine receptors, and they are evenly distributed over the surface of the myotubes (Fambrough et al., 1974b; Hartzell and Fambrough, 1973). Acetylcholine receptors also continued to appear on the plasma membranes of cultured myogenic cells for several hr after administration of cycloheximide to block protein synthesis (Fambrough, 1974; Hartzell and Fambrough, 1973). Therefore, it seems likely that acetylcholine receptors are assembled inside myotubes, and the completed receptors are then placed intact into the cell membrane. The lag between inhibition of protein synthesis and the decreased rate of appearance of receptor sites in the cell membrane probably reflects the time required for depletion of the supply of assembled receptor sites. That inhibition of ATP synthesis immediately suppresses appearance of new receptor sites suggests that ATP is required for incorporation of the assembled receptor sites into the cell membrane (Hartzell and Fambrough, 1973) and supports the concept that receptor sites are assembled intracellularly before
insertion into the cell membranes. Inhibition of RNA synthesis with actinomycin D (Hartzell and Fambrough, 1973) had no measurable effect on rate of appearance of acetylcholine receptor sites into muscle cell membranes for up to $2^4$ hr after administration. This implies that the mRNA species coding for the receptor proteins are very stable.

In addition to the specialization of plasma membranes of muscle cells to enable them to receive impulses from motor neurons through interaction with acetylcholine and to transmit these electrical impulses along the surface of the cell, specialization of the internal membranes of muscle cells must also occur for the electrical impulse to be transmitted into a contractile response. This highly differentiated internal membrane system in muscle cells is called the sarcoplasmic reticulum, and it responds to the wave of depolarization by releasing Ca$^{++}$ into the intracellular space surrounding the myofibrils (Tonomura and Oosawa, 1972). This Ca$^{++}$ then interacts with the myofibrillar protein, troponin, to initiate a contractile interaction of myosin and actin. After the nerve impulse passes, the sarcoplasmic reticulum begins an ATP-dependent sequestration of the released Ca$^{++}$ (Ebashi, 1974; Ebashi and Endo, 1968; Ebashi et al., 1969). Thus, for a muscle cell to become contractile, its sarcoplasmic reticulum
must be highly differentiated so contraction can be controlled. Lough et al. (1972) have studied both the ATPase of the sarcoplasmic reticulum and its ability to sequester \(\text{Ca}^{++}\) during muscle differentiation in culture and have found that these functions develop gradually over several days in culture. Furthermore, both these sarcoplasmic reticular activities exist at a low but detectable level before initiation of myoblast fusion.

F. Control of Myofibrillar Protein Synthesis

A very large amount of information exists about regulation of myofibrillar protein synthesis at the level of translation; therefore, it may be useful to briefly review what is currently known about the mechanism of messenger RNA-directed synthesis of peptides in eucaryotes. Because the literature on this subject is voluminous, only three comprehensive review articles will be cited (Haselkorn and Rothmon-Denes, 1973; Lucas-Lenard and Lipmann, 1971; Weissbach and Brot, 1974), and no effort will be made to discuss the experimental evidence upon which the mechanism of translation is based.

Eucaryotic ribosomes contain about equal amounts of protein and RNA, have a molecular weight of approximately \(4.5 \times 10^6\) daltons, sediment at 80S, and can be dissociated into two subunits which sediment at 40S and 60S. The 40S
subunit contains approximately 30 proteins and one 18S RNA molecule, and the 60S subunit contains approximately 50 proteins and two RNA molecules of 5S and 28S. Each ribosome is thought to possess two transfer-RNA (tRNA) binding sites: the peptidyl or P site and the aminoacyl or A site. A peptidyl-tRNA bound to the P site is capable of transferring its nascent peptide to an aminoacyl-tRNA bound to the A site, whereas a peptidyl-tRNA bound to the A site cannot transfer its nascent peptide to an aminoacyl-tRNA. The process of translating a polypeptide from its messenger RNA (mRNA) can be divided into initiation, elongation, and termination.

During initiation the 40S subunit interacts with the initiation codon (AUG) of the mRNA in the presence of a protein called initiation factor 3 (IF3) to form a 40S:mRNA:IF3 complex. GTP may be required to stabilize this complex. Methionyl-tRNA (met-tRNA), which has been synthesized by the enzyme methionyl-tRNA synthetase, then binds one molecule of initiation factor 2 (IF2) and one GTP molecule, and the resulting met-tRNA:IF2:GTP reacts with the 40S:mRNA::IF3 complex in the presence of initiation factor 1 (IF1) to form a 40S:mRNA:met-tRNA:IF2:GTP:IF1 initiation complex. IF3 is released during formation of this initiation complex. In the next step, the 60S ribosomal subunit joins the initiation complex, GTP is hydrolyzed and
GDP and inorganic phosphate released, and both IF1 and IF2 dissociate from the initiation complex. The final product of this initiation is an 80S ribosome with met-trNA bound to the P site.

Following formation of the met-trNA:mRNA:80S ribosome complex, the ribosome is ready to move along the mRNA to elongate the peptide. With the exception of the initiator met-trNA, all subsequent amino acids are polymerized into the peptide chain by the same series of reactions. The first step in elongation is binding of an aminoacyl-trNA to the A-site on the ribosome; the specific aminoacyl-trNA bound is determined by the codon on the mRNA and the A site. Each specific aminoacyl-trNA (synthesized by the appropriate specific aminoacyl-trNA synthetase) interacts with a GTP:elongation factor 1 (EF1) complex to form a GTP:EF1:aminoacyl-trNA complex. EF1 is thought to be a lipoprotein containing a number of identical subunits. The GTP:EF1:aminoacyl-trNA complex then reacts with the met-trNA:mRNA:80S ribosome complex, the aminoacyl-trNA is bound to the A site on the ribosome, and GTP is hydrolyzed to yield free EF1:GDP. Formation of a peptide bond between the methionine (bound to the P site as met-trNA) and the second amino acid (bound to the A site as aminoacyl-trNA) is catalyzed by the enzyme, peptidyl transferase. Exact details of the mechanism of peptidyl transferase are not
known. However, the ester linkage between the carboxyl group of methionine and the 3' hydroxyl group of the ribose portion of the 3'-terminal adenosine in met-tRNA is hydrolyzed by peptidyl transferase, and a peptide bond is formed between the carboxyl group of methionine and the α-amino group of the amino acid attached to the tRNA in the A site. Thus, the P site contains a deacylated tRNA, and the A site contains the dipeptidyl-tRNA.

The next step in elongation is called translocation, is mediated by elongation factor 2 (EF2), and requires GTP hydrolysis. In this step, the deacylated tRNA in the P site is removed, the peptidyl-tRNA in the A site is shifted to the P site, and the mRNA is moved by a distance of three nucleotides so that the next codon is situated at the A site. This cycling process continues until all the codons on the mRNA have been translated into their appropriate amino acid in the nascent polypeptide. A number of ribosomes may be undergoing these reactions simultaneously on the same mRNA. The maximum number of ribosomes attached to a given mRNA depends on the size of the mRNA. The macromolecular complexes of ribosomes and a mRNA are called polyribosomes or simply polysomes.

When the polypeptide chain is completed, a termination codon appears at the A site of the ribosome. At this point, a series of unknown reactions occur so that the ester
linkage between the completed peptide and the tRNA in the P site is hydrolyzed, the deacylated tRNA is released from the P site, and the ribosome dissociates from the mRNA either as an 80S particle or as separated 40S and 60S subunits. All of these components presumably can reenter their respective pools and the entire process of poly-peptide synthesis can begin once again.

This brief summary of the complex mechanism of protein synthesis will be used as a basis for a detailed analysis of the literature dealing with purification and properties of some of the specific protein synthetic components in differentiating muscle. In addition, the existing information concerning the mechanism regulating the abrupt burst of myofibrillar protein synthesis that occurs immediately after myoblast fusion will be examined. The remainder of this review, therefore, establishes the foundation for the research described in this dissertation.

Although Florini and Breuer (1965) and Earl and Korner (1965) originally reported that the polysome content of muscle tissue was much less than that of liver, Heywood et al. (1967) demonstrated that recovery of polysomes from embryonic chicken muscle depended critically upon the ionic composition of the medium used to homogenize the cells. Polysome yield from embryonic chicken muscle could be increased to levels comparable with that obtained from liver.
by homogenizing the muscle in a solution containing 0.25 M potassium chloride instead of the more conventionally used 0.05 M potassium chloride. The reason that a higher KCl concentration was needed to obtain satisfactory yields of polysomes from muscle tissue was that muscle contains large amounts of myosin, and myosin aggregates with polysomes. At pH 7, myosin is soluble at an ionic strength near 0.3 (Szent-Gyorgyi, 1960) but is precipitated near an ionic strength of 0.05. Hence, if muscle is homogenized in 0.05 M KCl, the myosin will be insoluble and easily sedimented at low centrifuge speeds (e.g., 11,000 x g for 10 min), and because myosin aggregates with polysomes, the polysomes will largely be sedimented with myosin and the other cellular debris in 0.05 M KCl homogenates. Homogenization in 0.25 M KCl keeps myosin soluble, and both myosin and the polysomes will remain in the supernatant when cellular debris is sedimented at 11,000 x g for 10 min.

Heywood et al. (1967) developed the first successful procedure for isolating polysomes from embryonic muscle. Fourteen-day-old embryonic chicken leg muscle was homogenized with several strokes of a loosely fitting Dounce homogenizer in 0.25 M KCl, 0.01 M MgCl₂, and 0.01 M Tris, pH 7.4, and the homogenate was centrifuged at 11,000 x g for 10 minutes. The supernatant was layered onto a 15-40% linear sucrose gradient, and the gradient was centrifuged
in a Beckman SW 25.1 rotor at 25,000 rpm for 2.0 hours. A reproducible distribution of polysomes on the gradients could be obtained using this procedure. The upper third of the gradient contained a very sharp 75S peak corresponding to monomeric ribosomes, the middle third of the gradient contained a very broad peak, and the lower third of the gradient contained a "hump" off the peak in the middle third of the gradient. A number of factors were shown to affect polysome distribution in Heywood's density gradient purification procedure (Heywood et al., 1967). Homogenizing the embryonic muscle too vigorously caused degradation of the larger polysomes with a resulting increase in the 75S monomer peak, treatment with pancreatic ribonuclease shifted all of the ribosomes to the 75S monomer peak, and as mentioned above, homogenization of the muscle in 0.05 M potassium chloride caused almost complete loss of all sizes of polysomes.

Because native myosin has an intrinsic adenosine triphosphatase (ATPase) activity, Heywood et al. (1967) first attempted to locate the polysomes responsible for synthesis of myosin by measuring ATPase activity of the nascent myosin polypeptides on the polysomes. However, all density gradient fractions exhibited ATPase activity. Treatment of the cell lysate with either 0.5% deoxycholate or ribonuclease shifted all ATPase activity to the top of the gradient; this
indicated that ATPase activity of the density gradient fractions was not associated with ribosomes at all. Heywood et al. (1967) interpreted these results to mean that nascent myosin chains had no intrinsic ATPase activity, and that ATPase activity of the density gradient fractions was due to myosin molecules that were nonspecifically associated with the ribosomes in these fractions. This association was possibly due to the same kind of interactions responsible for coprecipitation of polysomes with myosin described earlier in this section.

The different size classes of polysomes isolated from embryonic chicken muscle were analyzed for their involvement in synthesis of specific myofibrillar proteins by placing fractions from sucrose gradients in an in vitro protein synthetic assay containing all components required for completion of the nascent polypeptide chains on the polysomes. A uniformly labeled $^{14}$C-amino acid mixture was added to the assay so that any polypeptides completed in vitro would be radioactively labeled. Carrier myosin was added to the products of the assay, the myosin was precipitated at low ionic strength (0.03 M), and the resuspended pellet was electrophoresed in 12 M urea on polyacrylamide gels. The gels were frozen, sliced, and distribution of radioactively labeled proteins synthesized by the polysomes from each of the sucrose density gradient
fractions was determined. Polysomes from the lower third of the polysome density gradients incorporated $^{14}$C-amino acids into a polypeptide which migrated in a sharp peak exactly coincident with carrier myosin. Furthermore, polysomes from portions of the gradient capable of incorporating $^{14}$C-amino acids into myosin contained 50-60 ribosomes per polysome when they were negatively stained with uranyl acetate and examined by electron microscopy. Detection of myosin-synthesizing polysomes by their ability to incorporate radioactively labeled amino acids into the 200,000 dalton subunit of myosin (Heywood et al., 1967) forms the foundation for almost all studies that have been made on the control of myosin synthesis and is extremely useful for studying myofibrillar protein synthesis.

Further evidence that polysomes containing 50-60 ribosomes from embryonic muscle are responsible for synthesis of the 200,000-dalton subunit of myosin was obtained in studies involving addition of carrier myosin to the products of cell-free protein synthesis (Heywood and Rich, 1968). The carrier myosin was subjected to a series of purification steps, including repeated precipitation in 0.03 M potassium chloride and fractionation with ammonium sulfate between 40% and 47% saturation. At each step of purification, both the total amount of protein and the total radioactivity was determined, and the specific radioactivity
in cpm/mg of protein was calculated. The specific activity of the myosin decreased during purification, and after five successive purification steps, the specific radioactivity became constant and could not be decreased with additional purification steps. Purification of myosin to a constant specific radioactivity was taken as evidence that nonspecific adsorption of contaminating molecules had been eliminated. That the radioactivity copurified with myosin was additional evidence that the protein being synthesized was indeed myosin.

In addition to identification of myosin-synthesizing polysomes, polysomes which synthesize actin, tropomyosin, and the light chains of myosin have also been identified and partly purified (Heywood and Rich, 1968). Polysomes containing 5 to 9 ribosomes and 15 to 25 ribosomes were removed from density gradients of total embryonic chicken muscle ribosomes and placed in in vitro protein synthetic systems. These polysomes were very active in supporting incorporation of radioactive amino acids into polypeptides that comigrated with carrier tropomyosin and actin, respectively, on polyacrylamide gels in 12 M urea. Because several proteins in muscle could migrate with the same molecular weight as tropomyosin and actin, the products of these two size classes of polysomes were carried through repeated purification steps along with a small amount of the
respective unlabeled carrier protein. As already described for myosin, the specific radioactivity of these proteins decreased during purification and reached a constant level. Although the polysomes containing 5 to 9 ribosomes actively synthesized tropomyosin and the polysomes containing 15 to 25 ribosomes actively synthesized actin, these classes of polysomes synthesized a number of proteins in addition to the specific myofibrillar proteins assayed. In fact, a significant amount of cross-contamination occurred between the actin- and tropomyosin-synthesizing polysomes. Hence, the size of a specific polysome was generally proportional to the molecular weight of the peptide being synthesized; however, the number of ribosomes attached to a specific messenger RNA was quite variable.

Low et al. (1971) and Sarkar and Cooke (1970) have confirmed that myosin heavy-chains are synthesized on polysomes containing about 60 ribosomes and have extended these observations to show that two of the light chains of the myosin molecule are synthesized by a group of polysomes containing 4 to 9 ribosomes. Because a large number of cellular proteins have molecular weights in the same range as the myosin light chains (14,000-20,000 daltons), Low et al. (1971) were careful to demonstrate that myosin light chain synthesis was actually being measured. The radioactivity labeled products of \textit{in vitro} amino acid incorpora-
tion supported by these small polysomes were mixed with native, nonradioactive carrier myosin under conditions which would dissociate the light and heavy chains of myosin. The dissociated light and heavy chains were then reassociated over a period of 16 hours by dialysis against 0.5 M KCl, 0.001 M EDTA, and 0.01 M Tris, pH 7.4. Myosin renatured in this way was precipitated at low ionic strength (0.03 M) and was further purified by DEAE-cellulose chromatography. Final analysis of this purified myosin was accomplished by SDS-polyacrylamide gel electrophoresis to again dissociate the heavy and light chains and to separate them according to size on the gels. Only two peaks of radioactivity were found on these gels, and these two peaks comigrated exactly with the two light chains of the carrier myosin. The only proteins of this size that should survive such a purification procedure are the light chains of myosin.

As mentioned earlier in this section, the number of ribosomes attached to a specific messenger RNA is generally proportional to the molecular weight of the peptide being synthesized. Indeed, Heywood and Rich (1968) used this proportionality to predict that tropomyosin, actin, and myosin heavy chains would be synthesized by polysomes containing 5 to 9 ribosomes, 15 to 35 ribosomes, and 50 to 60 ribosomes, respectively. As discussed previously, this
prediction was tested experimentally and found correct. Hence, Heywood and Rich (1968) concluded that these three proteins were synthesized on monocistronic messenger RNA's. Although it was perhaps not surprising that three different myofibrillar proteins should be synthesized under the direction of three specific messenger RNA's, synthesis of the heavy and light chains of myosin could have been accomplished by any one of three methods (Low et al., 1971): (1) their synthesis could have been directed by a single polycistronic messenger RNA with the proteins coordinately synthesized; (2) they could have been synthesized by a monocistronic messenger RNA as a covalently linked "promyosin" molecule which was then cleaved into separate proteins; or (3) the heavy and light chains could have been synthesized separately from different messenger RNA's on different polysomes. Experiments described by Low et al. (1971) clearly demonstrated that the heavy and light chains of myosin were synthesized from different messenger RNA's on polysomes of 55 to 65 ribosomes and 4 to 9 ribosomes, respectively. Although these results suggest that separate genes are responsible for directing synthesis of the individual polypeptides making up the intact myosin molecule, the possibility that a polycistronic messenger RNA for these polypeptides is synthesized initially from one continuous DNA segment and later separated into smaller,
functional messenger RNA molecules during messenger RNA processing and transport to the cytoplasm has not been ruled out.

The studies of Heywood and Rich (1968), Low et al. (1971), and Sarkar and Cooke (1970) are the only ones reported thus far on the synthesis of tropomyosin and the light chains of myosin. Most investigations on biosynthesis of myofibrillar proteins have dealt with purifying and characterizing myosin messenger RNA, studying its interaction with ribosomes during initiation of protein synthesis, and changes in rate of myosin and myosin messenger RNA synthesis during differentiation in cell cultures.

Evidence that the messenger RNA with 55 to 65 attached ribosomes could actually direct in vitro synthesis of 200,000 dalton polypeptides presumed to be myosin heavy chains was first obtained by Heywood and Nwagwu (1968). RNA used in their experiments was prepared by suspending these large polysomes in 0.02 M sodium acetate, 0.002 M EDTA, 0.5% sodium dodecyl sulfate, 0.04 M Tris, pH 7.8, and extracting this suspension two times with an equal volume of phenol. RNA was then precipitated from the aqueous phase with two volumes of ethanol at -20°C for 10 hours. Monomeric embryonic muscle ribosomes, an S-150 crude enzyme preparation, radioactive amino acids, and other
components required for in vitro protein synthesis were added to the RNA pellet at 37°C, and the radioactively labeled products of cell-free protein synthesis were shown by polyacrylamide gel electrophoresis in 12 M urea and by DEAE-cellulose chromatography to contain significant proportions of labeled polypeptides indistinguishable from myosin heavy chains. Several observations indicated that synthesis of these radioactively labeled myosin heavy chains could not be attributed to nonspecific stimulation of amino acid incorporation: (1) RNA from the large polysomes programmed the synthesis of essentially only one protein; (2) RNA from smaller polysomes programmed the synthesis of a number of radioactive proteins electrophoretically distinct from myosin; and (3) incorporation in the presence of the RNA extracted from free monomeric ribosomes contained no well-defined peaks. Therefore, Heywood and Nwagwu (1968) concluded that the protein whose synthesis was directed by the RNA isolated from these large polysomes was myosin heavy chains.

Heywood and Nwagwu (1968) also used a second approach to establish that the phenol-extracted, ethanol-precipitated RNA associated with large polysomes from embryonic muscle was the mRNA coding for synthesis of myosin heavy chains. Fourteen-day chicken embryos were injected with 32P-labeled phosphoric acid to label RNA as it was synthesized. After
a period of time just sufficient to allow any mRNA synthesized to become associated with ribosomes, the large polysomes containing 55 to 65 ribosomes were separated by the density gradient centrifugation techniques described earlier (Heywood et al., 1967) in this section. These large polysomes were collected on millipore filters and were dissolved in buffer containing 0.5% SDS, 0.005 M EDTA, 0.02 M sodium acetate, and 0.04 M Tris, pH 7.8. This procedure separated protein and RNA, and the dissolved RNA and protein were layered onto 10-30% sucrose gradients in the above buffer and were centrifuged at 95,000 \( \times g_{\text{max}} \) for 18 hrs. Under these conditions, two distinct peaks of RNA were observed with sedimentation coefficients of 18S and 28S. Very little radioactivity was associated with either of these peaks, but a peak of highly labeled \(^{32}\text{P}-\)RNA sedimented at approximately 26S. This highly labeled fraction of RNA directed \textit{in vitro} synthesis of polypeptides which migrated with the 200,000-dalton subunit of myosin on polyacrylamide gels in 12 M urea, cochromatographed with native myosin on DEAE-cellulose chromatography, and precipitated in the presence of antibodies against myosin.

The experiments described in the preceding paragraph provided strong evidence that the 26S RNA was indeed myosin mRNA. It was still possible, however, that the 26S RNA from large embryonic muscle polysomes might only be
nonspecifically stimulating muscle ribosomes in cell-free protein synthesis assays to produce myosin heavy chains. To eliminate this possibility, it was essential to demonstrate that the 26S RNA would direct synthesis of myosin heavy chains in a cell-free system which contained ribosomes from some tissue other than muscle. Heywood (1969) showed that myosin heavy chain synthesis could be directed by 26S RNA in the presence of chicken reticulocyte ribosomes. Thus, synthesis of one of the myofibrillar proteins could be accomplished by ribosomes obtained from a nonmuscle source; this almost eliminated the possibility that the 26S RNA was nonspecifically stimulating myosin heavy-chain synthesis by muscle ribosomes. This already strong evidence that the 26S RNA was indeed myosin mRNA was strengthened when analysis of proteolytic digests of the radioactive cell-free products with two-dimensional paper electrophoresis and with Aminex A-5 ion-exchange chromatography showed that translation of myosin messenger RNA by reticulocyte ribosomes occurred with a high degree of fidelity. The peptide maps of myosin labeled in an in vitro assay were identical to the peptide maps of myosin isolated from chickens injected in vivo with radioactive amino acids. Taken together, results of all these experiments have provided conclusive evidence that myosin messenger RNA has a sedimentation coefficient of 26S and that
it is therefore very similar in its sedimentation characteristics to the 28S RNA component of ribosomes.

Because of the similarity in S-value of 28S ribosomal RNA and 26S myosin messenger RNA, additional steps other than density-gradient centrifugation were required to purify myosin messenger RNA to homogeneity. Two apparently successful procedures for purifying myosin mRNA have been developed by Morris et al. (1972) and Sarkar et al. (1974). These procedures use the differential affinities of ribosomal RNA and mRNA for Sepharose 2B or for cellulose during column chromatography. In the procedure developed by Morris et al. (1972), the mixture of 28S ribosomal RNA and 26S myosin messenger RNA was layered onto a Sepharose 2B column equilibrated with 0.5 M NaCl, 0.02 M Tris, 0.0025 M EDTA, 0.5% SDS, pH 7.5. In this buffer, the 28S ribosomal RNA adhered to the column, and the 26S myosin messenger RNA was eluted in a single peak of approximately 95% purity. In a similar approach, Sarkar et al. (1974) extracted myosin-synthesizing polysomes with phenol in the presence of 0.5% SDS and 0.1 M Tris, pH 9.0, to remove protein. The RNA in the aqueous phase was precipitated by adding ethanol, and the precipitated RNA was collected on millipore filters and was applied to a small cellulose column equilibrated with 0.5 M KCl, 0.0002 M MgCl₂, 0.01 M Tris, pH 7.6. Ribosomal RNA had a very weak affinity for the cellulose under these
conditions and was easily eluted with eluents that allowed myosin mRNA to remain adsorbed to the column. The myosin mRNA was then eluted in a single peak of greater than 95% purity by washing the column with 0.001 M EDTA and 0.001 M Tris, pH 7.0. Purified RNA obtained by the two procedures described above was shown to be myosin mRNA because it directed cell-free synthesis of myosin heavy chains (Morris et al., 1972; Sarkar et al., 1974; Mondal et al., 1974) and because the peptides produced by cyanogen bromide cleavage of in vitro labeled myosin matched those produced by cyanogen bromide cleavage of in vivo labeled myosin (Sarkar et al., 1974).

The ability to purify myosin mRNA by the two procedures described in the preceding paragraph permitted investigation of some of the physical characteristics of the myosin in mRNA molecule. Based on the assumption that the molecular weight of myosin heavy chains is 200,000 daltons, the minimum molecular weight of myosin mRNA necessary to provide three nucleotides for every amino acid had to be approximately $1.9 \times 10^6$ daltons (Mondal et al., 1974; Morris et al., 1972). Mondal et al. (1974) found, however, that myosin mRNA migrated with a molecular weight of $2.23 \times 10^6$ daltons (approximately 6,500 nucleotides) on formamide-polyacrylamide gel electrophoresis. Because it seemed possible that the polyadenylic acid sequence of nucleotides
found on the 3' end of most messenger RNA's might account for some of the difference between the required minimal molecular weight and the actual molecular weight of myosin mRNA, Mondal et al. (1974) attempted to measure the size of the polyadenylic acid sequence on myosin mRNA. The purification scheme used by Mondal et al. (1974) included a step in which the poly A component of myosin mRNA was bound to a cellulose column in 0.5 M KCl, 0.0002 M MgCl₂, and 0.01 M Tris, pH 7.6. The specific binding of poly A to cellulose under these conditions was used to determine the size of the poly A sequence and whether this sequence was located at the 5' or the 3' end of the molecule. Myosin mRNA was treated with NaIO₄ to oxidize the 2' and 3' hydroxyl groups at the 3' terminal nucleotide, and the aldehyde groups formed by this reaction were reduced with ³H-NaBH₄. When this labeled mRNA was digested with pancreatic ribonuclease, and the digest was submitted to the myosin mRNA purification procedure described in the preceding paragraphs, a uniform peak of poly A was eluted from the cellulose column. This poly A had a molecular weight of approximately 0.06 x 10⁶ daltons (about 170 adenylic acid residues) as determined by polyacrylamide gel electrophoresis in formamide, and because the poly A was ³H-labeled, Mondal et al. (1974) concluded that the poly A was located at the 3' end of the mRNA.
Even when allowing for a poly A segment of $0.06 \times 10^6$ daltons, however, the experimental molecular weight of $2.23 \times 10^6$ daltons for myosin mRNA is still $0.27 \times 10^6$ daltons larger than the $1.9 \times 10^6$ daltons minimal molecular weight required to direct synthesis of a 200,000 dalton polypeptide chain ($\left(2.23 \times 10^6 - 0.06 \times 10^6\right) - 1.9 \times 10^6 = 0.27 \times 10^6$ daltons). Therefore, Mondal et al. (1974) concluded that this excess mRNA, which amounts to approximately 10% of the total RNA molecule, is not translated during myosin synthesis. Neither the function of this nontranslated portion nor its location within the myosin mRNA molecule is known.

As indicated several times in the preceding paragraphs, the criteria most often used to verify that the 26S RNA isolated from embryonic chicken muscle is actually myosin mRNA was to measure its capability for directing incorporation of radioactive amino acids into the 200,000-dalton subunit of myosin when placed in a cell-free protein synthesizing assay. Heywood and Nwagwu (1969) found that ribosomes actively supported synthesis of myosin heavy chains when the ribosomes and myosin mRNA were both isolated from the same embryonic chicken muscle (i.e., in a homologous system). When this same assay was performed using a heterologous system (ribosomes isolated from chicken reticulocytes and myosin mRNA isolated from embryonic
chicken muscle), however, only a very low level of myosin synthesis occurred (Heywood, 1969). Hence, ribosomes from a nonmuscle source apparently were very inefficient relative to muscle ribosomes in supporting synthesis of myosin heavy chains directed by presumptive myosin mRNA. While attempting to learn why reticulocyte ribosomes were so inefficient in supporting myosin heavy chain synthesis, Heywood (1969) discovered that reticulocyte ribosomes could be stimulated to synthesize myosin heavy chains at a rate about one-half that of embryonic muscle ribosomes by adding protein factors extracted from muscle ribosomes with 1.0 M KCl. Both reticulocyte ribosomes and embryonic muscle ribosomes were extracted at 2°C with 1.0 M KCl, 0.001 M MgCl₂, 0.02 M Tris, pH 7.6, and the proteins solubilized from chicken reticulocyte ribosomes by this extraction (about 0.2 mg protein/mg ribosomes) were replaced with an equal amount of 1.0 M KCl-soluble proteins from embryonic chicken muscle ribosomes. Reticulocyte ribosomes containing 1.0 M KCl-soluble factors from muscle ribosomes were about one-half as active as ribosomes from embryonic chicken muscle ribosomes in supporting synthesis of myosin heavy chains. Furthermore, when unwashed or 1.0 M KCl-washed muscle or reticulocyte ribosomes were placed on nitrocellulose filters, and 1.0 M KCl-soluble proteins from either muscle or reticulocyte ribosomes were tested for their effect on
binding of myosin mRNA to the filters, three combinations were found effective in binding myosin mRNA: (1) muscle ribosomes that had not been washed with 1.0 M KCl; (2) 1.0 M KCl-washed muscle ribosomes that had been recombined with the 1.0 M KCl-soluble proteins from muscle ribosomes; and (3) 1.0 M KCl-washed reticulocyte ribosomes that had been recombined with the 1.0 M KCl-soluble proteins from muscle ribosomes. Heywood (1969) interpreted this effect of factors extracted from muscle ribosomes to indicate that highly specialized and nondividing cell types, such as muscle and reticulocytes, may contain cell-specific message recognition factors that are required for initiation of synthesis of certain cell-specific proteins.

Because cell-specific mRNA recognition could have an important role in regulation of the expression of unique characteristics of highly differentiated cells, such as muscle cells, at the translational level, an intensive effort was made by Heywood and coworkers to fractionate and study the 1.0 M KCl-soluble components responsible for conferring specificity to translation of myosin heavy-chain mRNA. The 1.0 M KCl-soluble proteins from muscle ribosomes were demonstrated to actually contain initiation factors (Heywood, 1970a; Heywood and Thompson, 1971). Testing of various protein synthetic components for their effect on binding of $^{32}$P-labeled myosin mRNA to ribosomes showed that
the KCl-soluble proteins were required for formation of the 40S:mRNA: presumably IF3 complex, and that both GTP and tRNA were required in addition to the KCl-soluble proteins for formation of the met-tRNA:80S:mRNA complex. These results are consistent with the idea that the 1.0 M KCl wash of muscle ribosomes contains IF1, IF2, and IF3 and that initiation of protein synthesis in muscle occurs by the mechanism presented earlier in this review. Furthermore, addition of muscle initiation factors to reticulocyte ribosomes was required for formation of a met-tRNA:reticulocyte 80S:myosin mRNA initiation complex.

Because these experiments (Heywood, 1969, 1970a) indicating that muscle initiation factors were required for synthesis of myosin on heterologous ribosomes and for specific binding of myosin mRNA to nitrocellulose filters could not eliminate the possibility that the factors removed from reticulocyte ribosomes by the 1.0 M KCl wash were inactivated by the isolation procedure, Heywood (1970b) devised a cell-free amino acid incorporating system using muscle ribosomes to synthesize hemoglobin from globin mRNA. Use of this system showed that synthesis of hemoglobin required the combination of initiation factors from reticulocyte ribosomes with 1.0 M KCl-extracted muscle ribosomes. Consequently, Heywood (1970b) suggested that messenger-specific initiation factors, such as those ostensibly needed
for synthesis of myosin heavy chains and hemoglobin, could be a possible general mechanism for fine control of synthesis of cell-specific proteins.

In an attempt to identify and purify the components that exist in crude initiation factor preparations and that confer mRNA specificity, 1.0 M KCl extracts containing these initiation factors from muscle ribosomes were subjected to column chromatography under several conditions (Heywood, 1970b). Fractions collected from these columns were assayed for their ability to promote specific binding of \(^{32}\text{P}\)-labeled myosin mRNA to ribosomes in the nitrocellulose filter assay (Heywood, 1969). These chromatographic experiments showed that the factors responsible for mRNA were excluded from Sephadex G-200 and therefore eluted as a single, sharp peak in the void volume of such columns. The proteins in this void volume peak were then applied to a DEAE-cellulose column and were eluted with a linear 0.05-0.5 M KCl gradient. The elution profiles from this column were identical for extracts from muscle and reticulocyte ribosomes. Three of the peaks in this elution profile were identified as the initiation factors, IF1, IF2, and IF3, which eluted at 0.05 M, 0.3 M, and 0.22 M KCl, respectively. Although IF2 and IF3 were not totally separated by DEAE-cellulose chromatography, a series of experiments testing the effect on initiation complex formation of different combinations of
IF1, IF2, and IF3 from either reticulocyte or muscle ribosomes in the presence of globin or myosin mRNA established that IF3 was responsible for the specificity of initiation. Therefore, in an in vitro protein synthesizing assay containing 1.0 M KCl-extracted reticulocyte ribosomes and myosin mRNA, significant levels of radioactivity could be incorporated into a mRNA:4OS:IF3 complex only when muscle IF3 was present. Analogously, in an assay containing 1.0 M KCl-extracted muscle ribosomes and globin mRNA, formation of significant amounts of an mRNA:4OS:IF3 complex occurred only when reticulocyte IF3 was present in the assay. Furthermore, Rourke and Heywood (1972) demonstrated that myosin heavy chains were freely synthesized in an assay mixture in which 1.0 M KCl-washed muscle ribosomes, muscle IF1, and muscle IF2 were replaced with 1.0 M KCl-washed reticulocyte ribosomes, reticulocyte IF1, and reticulocyte IF2 while leaving muscle IF3 in the assay. If muscle IF3 was replaced with reticulocyte IF3, however, myosin heavy-chain synthesis was inhibited by approximately 95%. One interpretation of these data is that highly differentiated cells such as reticulocytes and muscle contain multiple, messenger-specific IF3's (Rourke and Heywood, 1972).

In an effort to more closely define the limits of mRNA selectivity in embryonic muscle, Thompson et al. (1973) investigated the translation of myoglobin mRNA obtained from
red muscle of 19-day embryonic chicken thigh muscle in a cell-free protein-synthesizing system that contained ribosomes and ribosomal factors prepared from either red (thigh) or white (breast) muscle of 19-day chicken embryos. Myoglobin, an oxygen-binding protein of about 17,000 daltons, is present in red muscle cells but is absent or present in only very low quantities in white muscle cells. Myosin, on the other hand, is present in both red and white muscle cells. IF3 fractions prepared from both red and white muscle ribosomes promoted synthesis of myosin heavy chains by reticulocyte ribosomes, but only the IF3 fraction from red muscle would promote myoglobin synthesis. Thompson et al. (1973) interpreted these results to indicate that a specific IF3 required for translation of myosin mRNA was present in both red and white muscle, but that a different, specific IF3 lacking in white muscle and present in red muscle was required for translation of myoglobin mRNA. This interpretation was supported by the observation that a protein from one of the fractions eluted from phosphocellulose chromatography of the red muscle IF3 fraction stimulated synthesis of myoglobin about two-fold with no effect on myosin synthesis when it was added to a reticulocyte cell-free system containing both myosin and myoglobin mRNAs (Heywood et al., 1974). Moreover, addition of protein from a separate phosphocellulose fraction to this same assay
system caused a two-fold stimulation of myosin heavy chain synthesis with very little effect on myoglobin synthesis. The corresponding experiments using initiation factors from white muscle were not described in these reports (Heywood et al., 1974; Thompson et al., 1973), but in total, these data strongly support the idea that a factor necessary for translation of myosin mRNA is present in both red and white muscle but a similar factor necessary for translation of myoglobin mRNA is lacking in white muscle and present in red muscle. A number of other workers have also suggested that specific ribosomal factors are involved in mRNA selection in eucaryotes (Nudel, et al., 1973; Wigle, 1973; Lebleu et al., 1972; Fuhr and Natta, 1972).

Selection of specific messenger RNA's during initiation of protein synthesis as described in the preceding paragraphs could provide a powerful post-transcriptional regulation of gene expression in differentiating cells. Such a mechanism could not only regulate the onset of synthesis of specific proteins but could also influence the amount of specific proteins synthesized. Thompson et al. (1973) and Heywood et al. (1974) have argued that it is unlikely that translation of each mRNA present in specialized cells is regulated by a specific factor, but that post-transcriptional control of gene expression could entail a relatively small number of specific factors, each of which may recognize
different sets of mRNA's whose translation products are functionally related, such as the myofibrillar proteins. In this scheme, terminal differentiation of muscle could involve not only the appearance of myofibrillar protein mRNA's in the cytoplasm but also the appearance of an IF3 specific for enhancing translation of these myofibrillar protein mRNA's. It is important to note, however, that this model for control of specific protein synthesis at the level of initiation has been proposed strictly on the basis of studies on embryonic muscle and that it has not been tested in synchronized cultures of differentiating muscle cells.

It has recently been shown that one of the fractions separated by DEAE-cellulose chromatography of the IF3 fraction from muscle or reticulocyte ribosomes contains a small RNA molecule in addition to the three initiation factors (Heywood et al., 1974). This RNA molecule has a molecular weight of approximately 6,500 daltons and has been proposed to be involved in translational control of protein synthesis (Heywood et al., 1974; Kennedy et al., 1974; Bogdanovsky et al., 1973; Fuhr and Natta, 1972). Although details of the mechanism by which this RNA (termed translational control RNA or tcRNA by Heywood et al., 1974) functions are unclear at this time, Heywood et al. (1974) have shown that tcRNA isolated from muscle IF3 inhibits
globin synthesis by approximately 30% in a reticulocyte assay but has no effect or slightly enhances myosin and myoglobin synthesis. In converse experiments, the tcRNA from reticulocyte IF3 had no measurable effect on globin synthesis but abolished myosin and myoglobin synthesis. Kennedy et al. (1974) have argued that the best explanation for these results is that tcRNA is a tissue specific molecule which recognizes a portion of the respective heterologous mRNA and forms a double-stranded region at or near the ribosomal binding site of this heterologous RNA to produce an abortive initiation complex. This possibility deserves some consideration because double stranded RNA complexes are known to inhibit translation (Ehrenfeld and Hunt, 1971; Kaempfer and Kaufman, 1973); however, it is currently unknown whether such a mechanism is specifically involved in regulation of muscle protein synthesis. Alternate explanations for the observations of Heywood and coworkers (Heywood et al., 1974; Kennedy et al., 1974) would be that tcRNA is involved in blocking utilization of foreign mRNA's (such as those of viral origin) or possibly that tcRNA consists of short fragments of partially degraded RNA that happen to contain AUG initiation codons (Heywood et al., 1974; Kennedy et al., 1974).

The studies described thus far on control of myofibrillar protein synthesis have involved purification and
properties of a number of the individual protein synthetic components that can be isolated from embryonic muscle. Although a number of interesting ideas about how myofibrillar protein synthesis could be regulated have emerged from these studies, most of these ideas cannot be tested in embryonic muscle because such muscle contains myogenic cells at all stages of differentiation ranging from presumptive myoblasts to multinucleated myotubes and myofibers. Although it should be advantageous technically to study control of myofibrillar protein synthesis in synchronized cultures of differentiating muscle cells, no such studies have been reported, possibly because of the large number of cultures that would be required for such experiments. Consequently, with the exception of a few studies described in the following paragraphs on changes in rate of myosin synthesis during muscle cell differentiation, the literature on regulation of myofibrillar protein synthesis during myogenesis is sketchy and is often contradictory.

One widely accepted aspect of myogenesis that has been substantiated by biochemical and immunological techniques is that bulk synthesis of myosin begins within a matter of hours after the onset of myoblast fusion (Coleman and Coleman, 1968; Morris et al., 1972; Paterson and Strohman, 1972; Stockdale and O'Neill, 1972; Yaffe and Dym, 1972). Different workers disagree, however, about whether myosin
synthesis also occurs before initiation of myoblast fusion. Coleman and Coleman (1968) and Yaffe and Dym (1972) detected no myosin synthesis in prefusion muscle cultures, and, although Paterson and Strohman (1972) found low levels of myosin synthesis in prefusion cultures, they attributed this synthesis exclusively to the small number of myotubes which contaminate proliferating mononucleated muscle cell cultures. On the other hand, Morris et al. (1972), Stockdale and O'Neill (1972), and Rubenstein et al. (1974) have all reported low levels of myosin synthesis in cultures of mononucleated myoblasts, and Rubenstein et al. (1974) have even reported that fibroblasts and chondroblasts synthesize actin and myosin. The work described in the Results and Discussion of this dissertation also support the view that mononucleated myoblasts synthesize significant levels of myosin.

The few studies done on appearance of the mRNA coding for synthesis of the heavy chains of myosin during muscle differentiation have also been conflicting. Yaffe and Dym (1972) have studied the rate of myosin synthesis in muscle cell cultures to which the RNA polymerase inhibitor, actinomycin D, had been administered at different times during myogenesis. This antibiotic inhibits RNA synthesis, and all proteins synthesized in actinomycin D treated cells, therefore, are translated by mRNA's existing in the cells
before addition of the drug to the culture medium. When myogenic cells were treated with actinomycin D several hours before the onset of extensive fusion, the rate of myosin heavy-chain synthesis and myoblast fusion proceeded normally for a number of hours before the cells were overcome by the toxicity of the antibiotic (Yaffe and Dym, 1972). That myosin heavy-chain synthesis occurred after fusion in cells treated with actinomycin D before fusion, however, suggested that some myosin mRNA is actually present in mononucleated myoblasts for 6-10 hrs before the normal burst in rate of myosin synthesis occurs. Subsequently, Buckingham et al. (1974) identified in cultures of mononucleated fetal calf muscle cells a 26S, poly A-containing RNA that hybridized to DNA complementary to myosin mRNA with kinetics identical to those of the original myosin mRNA. The capacity of this 26S RNA to direct synthesis of myosin heavy chains in a cell-free protein-synthesizing assay was not demonstrated, however. The results of the two studies mentioned above suggest that prefusion myogenic cultures contain myosin mRNA. In contrast to these results, however, other workers have tried unsuccessfully to isolate from extracts of mononucleated myogenic cells a mRNA that would direct synthesis of myosin heavy chains in a reticulocyte protein-synthesizing assay (Pryzbyla et al., 1973; Pryzbyla and Strohman, 1974; Strohman et al., 1974).
The contradictory results described in the preceding paragraphs prompted the present investigation into the effect of myoblast fusion on myosin synthesis and myosin polysome accumulation. Rather than attempting indirect studies, such as those reported by Buckingham et al. (1974), and in view of the uncertainties involving ability to quantitatively initiate protein synthesis on myosin mRNA's (Heywood and Nwagwu, 1969) or to assay purified myosin mRNA in a cell-free assay as attempted by Strohman and coworkers (Pryzbyla et al., 1973; Pryzbyla and Strohman, 1974; Strohman et al., 1974), a more direct approach was taken. If prefusion muscle cells synthesize myosin, then these cells must contain myosin polysomes. Under proper conditions, the number of nascent myosin heavy chains completed in an in vitro protein synthesizing assay containing $^{3}$H-leucine should reflect the amount of myosin polysomes contained by the cells at the time they were sampled. Therefore, this approach was used in the present study to learn more about the relationship between myoblast fusion, myosin synthesis, and myosin-synthesizing polysome accumulation.
III. MATERIALS AND METHODS

Unless otherwise indicated, all preparations were done at 0 to 4°C and used precooled solutions. Double-deionized, distilled water that had been redistilled in glass and stored in polyethylene containers was used for preparation of all solutions. All reagents were the highest purity available.

Fertilized eggs used as a source of embryonic muscle for all cell culture experiments were obtained from Hyline International of Dallas Center, Iowa, and were incubated at 37°C and 95% humidity until immediately before use in preparing muscle cell cultures, embryo extract, polysomes, and aminoacyl tRNA synthetases.

A. Preparative Procedures

1. Muscle cell cultures

After removal of skin and bones, muscle from legs of 12-day chicken embryos was placed in 10 ml of complete medium. Complete medium contained 85% Eagle's Minimum Essential Medium (Eagle, 1959), 10% horse serum, 5% chicken embryo extract prepared as described below, 50 units/ml of penicillin, 50 µg/ml of streptomycin, and 2.5 µg/ml of Fungizone. The embryonic muscle tissue was dissociated with a vortex mixer at maximum speed for 20 sec, and the resulting suspension of single cells, cell clumps, and debris was
filtered through two sterile Swinny filters. The first filter contained a single layer of 200 x 200 mesh nylon cloth to retain large aggregates, and the second filter contained a double layer of lens paper to retain small clumps of cells. The filtrate was centrifuged at 700 x $g_{max}$ for 5 min at room temperature, and the cell pellet was resuspended into complete medium by aspiration with a disposable pipet. Cells were counted with a hemocytometer and were plated at a constant cell density of $1.4 \times 10^5$ cells/cm$^2$ in Falcon tissue culture dishes that had been coated with 1.75 µg/cm$^2$ of collagen and air-dried for at least 48 hr. Cells were incubated in 15 ml of complete medium at 37°C in an atmosphere of 95% air and 5% carbon dioxide. Every 24 hr, medium was removed from the dishes and was replaced with 15 ml of fresh medium that had been prewarmed to 37°C.

2. Chicken embryo extract

Twelve-day chicken embryos were decapitated, were expelled through a 50 ml disposable syringe, and an equal volume of buffered saline solution ($0.137$ M NaCl, $0.0027$ M KCl, $0.001$ M CaCl$_2$, $0.001$ M MgCl$_2$, $0.00015$ M NaH$_2$PO$_4$, $0.00136$ M Na$_2$HPO$_4$, $0.006$ M NaHCO$_3$, $0.0055$ M glucose, pH 7.4) was added. This mixture was left at room temperature for 0.5 hr and was then centrifuged at $2,000 \times g_{max}$ for 20 min
at 2°C. The supernatant from this centrifugation was kept at -20°C for up to six months. When embryo extract was thawed, it was again centrifuged at 2,000 x g_{max} for 20 min to remove insoluble material before use in preparation of complete medium.

3. Collagen

Collagen (Worthington Biochemical Corp.) was extracted in 1.0% acetic acid at 2°C with stirring for 48 hr and was diluted to 0.2 mg/ml with 0.1% acetic acid prior to storage at -20°C for up to six months.

4. Aminoacyl transfer RNA synthetases

Leg muscle from 14-day chicken embryos was placed in one-half volume (w/v) of incubation buffer (0.15 M KCl, 5 mM MgCl₂, 10% glycerol, Tris, pH 7.6) and was homogenized with five strokes of a Dounce homogenizer (A pestle). This homogenate was centrifuged at 150,000 x g_{max} for 2 hr. The lower two-thirds of the supernatant from this centrifugation was dialyzed against two changes of incubation buffer for 24 hr each and against one change of incubation buffer containing 50% glycerol for 24 hr. Concentration of this protein solution was normally about 0.4 mg/ml as determined by the method of Ehresman et al. (1973), and the aminoacyl tRNA synthetases retained their activity for up to three months when stored at -20°C (Heywood and Nwagwu, 1969). Immediately
before use, the enzyme preparation was diluted to 10 mg/ml with incubation buffer without glycerol.

5. **Transfer RNA**

Transfer RNA (tRNA) was prepared from whole 12-day chicken embryos by the phenol extraction, ethanol precipitation method of von Ehrenstein (1968). The tRNA from the final ethanol precipitation was resuspended in incubation buffer and was stored at -20°C for up to six months without loss of activity.

6. **Embryonic muscle ribosomes**

Ribosomes were prepared from leg muscle of 14-day embryonic chicken leg muscle according to the procedure of Heywood and Nwagwu (1969). Muscle was homogenized in one-half volume of incubation buffer without glycerol with five strokes of a Dounce homogenizer (B pestle). The homogenate was made 0.015 M NaF, was incubated at 37°C for 20 min, and was centrifuged at 30,000 x $g_{max}$ for 30 min. The supernatant (1-6 ml) was layered onto 2.0 ml of incubation buffer containing 50% glycerol, and the ribosomes were pelleted by centrifuging at 225,000 x $g_{max}$ for 2.0 hr. The ribosome pellet was resuspended in incubation buffer containing 10% glycerol over a period of approximately two hr by frequent gentle aspiration with a Pasteur pipet. Resuspension was followed by gentle homogenization with five strokes of a
Dounce homogenizer (B pestle). The ribosomes were clarified at 2,000 x $g_{\text{max}}$ for 15 min and diluted to 5.0 mg/ml before use. The 260:280 nm absorbance ratio of ribosomes prepared in this way was between 1.75 and 1.85.

7. Rabbit reticulocyte ribosomes

Rabbits were made anemic by subcutaneous injection of 1.0 ml of 2.5% phenylhydrazine in water, pH 7.0, on six successive days followed by no injection on the seventh day (Shafritz et al., 1972). On the eighth day, rabbits were anesthetized by intravenous injection of 2.0 ml of pentobarbital (60 mg/ml), and blood was withdrawn by cardiac puncture with a 100 ml syringe containing 1.5 ml of heparin (1,000 units/ml). Cells were pelleted at 10,000 x $g_{\text{max}}$ for 10 min and were resuspended twice in an equal volume of incubation buffer without glycerol followed each time by centrifugation at 10,000 x $g_{\text{max}}$ for 10 min. The reticulocytes were resuspended finally in an equal volume of incubation buffer without glycerol and were homogenized with five strokes of a Dounce homogenizer (B pestle). The homogenate was made 0.015 M NaF, incubated at 37°C for 20 min, and centrifuged at 30,000 x $g_{\text{max}}$ for 30 min. The supernatant (1-6 ml) was layered onto 2.0 ml of incubation buffer containing 50% glycerol, and the ribosomes were pelleted by centrifuging at 225,000 x $g_{\text{max}}$ for 2.0 hr. The
ribosome pellet was resuspended in incubation buffer containing 10% glycerol over a period of two hr by frequent gentle aspiration with a Pasteur pipet. Resuspension was followed by gentle homogenization with five strokes of a Dounce homogenizer (B pestle). The ribosomes were clarified at 2,000 x g_{max} for 15 min and diluted to 5.0 mg/ml before use.

8. KCl-washed ribosomes

Embryonic chicken muscle ribosomes prepared and pelleted as described above were resuspended by homogenization with five strokes of a Dounce homogenizer (B pestle) in a solution containing 1.0 M KCl, 0.1 mM MgCl₂, 20 mM Tris, pH 7.4. These conditions solubilized about 0.2 mg protein per mg of ribosomes. These 1.0 M KCl-soluble proteins have been shown to be a crude preparation of initiation factors (Heywood and Nwagwu, 1969). The KCl-washed ribosomes were sedimented at 225,000 x g_{max} for 2.0 hr and resuspended in incubation buffer containing 10% glycerol as described under preparation of embryonic chicken muscle ribosomes.

9. Crude initiation factors

The supernatant remaining after sedimenting the KCl-washed ribosomes as described in the preceding paragraph was dialyzed against two changes of incubation buffer containing 10% glycerol over a period of 48 hr. Protein
concentration of the dialyzed solution was determined by the method of Ehresman et al. (1973) and these 1.0 M KCl-soluble proteins were used as a preparation of crude initiation factors (Heywood and Nwagwu, 1969).

10. **Total polysomes from embryonic chicken muscle**

Legs from 14-day chicken embryos were removed and quickly placed into a petri dish containing 10 ml of cold (2°C) isolation buffer (0.25 M KCl, 10 mM MgCl$_2$, 10 mM Tris, pH 7.4). Skin and bones were removed, and the muscle was homogenized with five strokes of a Dounce homogenizer (B pestle). The homogenate was centrifuged at 12,000 x $g_{\text{max}}$ for 10 min, and the volume of supernatant was adjusted so that the volume in ml was equal to the number of 14-day embryos used in preparing the homogenate. One-half to 2.0 ml aliquots of this homogenate were layered onto 2.0 ml of a solution containing 1.5 M sucrose, 0.25 M KCl, 10 mM MgCl$_2$, 10 mM Tris, pH 7.4, and centrifuged at 225,000 x $g_{\text{max}}$ for 2.0 hr. The supernatant was removed and the pellet was rinsed carefully one time with 2.0 ml of incubation buffer. Pellets were dissolved as described under assay of myosin-synthesizing polysomes.

11. **Total polysomes from muscle cell cultures**

Three to four hr before polysome isolation, cultures were fed 15 ml of complete medium which had been prewarmed
to 37°C. Cultures were removed from the incubator, and the medium was poured off and very quickly was replaced with approximately 25 ml of ice-cold isolation buffer to chill the cultures and to rinse out remaining complete medium. All subsequent steps were performed at 2°C. Cells were scraped from the surface of the dishes into isolation buffer (0.4 ml per 15 cm culture dish) containing 0.5% Triton X-100 and were lysed either by pulling the suspension in and out of a Pasteur pipet 20 times as described by Morse et al. (1971) or by a mild homogenization with five strokes of a Dounce homogenizer (A pestle). The supernatant (1-6 ml) remaining after centrifugation of the lysate at 12,000 x g_max for 10 min was layered onto 2.0 ml of a solution containing 1.5 M sucrose, 0.25 M KCl, 10 mM MgCl_2, 10 mM Tris, pH 7.4, 10% glycerol, and the polysomes were sedimented at 225,000 x g_max for 2.0 hr. Polysome pellets were dissolved as described under assay of myosin-synthesizing polysomes.

12. Sucrose density-gradient fractionation of polysomes

Polysomes were fractionated according to size by layering 5.0 ml of the 12,000 x g_max-supernatant from embryonic chicken muscle or muscle cell cultures (see two preceding paragraphs) onto a 60 ml 15-40% linear sucrose gradient in isolation buffer and centrifuging at 105,000 x g_max in a Beckman SW 25.2 rotor for 2.0 hr. In some instances, 0.5 ml of the 12,000 x g_max-supernatant was layered onto a 13
ml, 15-40% linear sucrose gradient in isolation buffer and were centrifuged at 150,000 \times g_{\text{max}} for 1.5 hr. In either procedure, gradients were removed by puncturing the bottom of the tube and by pumping 1.5 M sucrose up through the tube with a Harvard infusion pump. Distribution of polysomes on the gradient was monitored continuously at 260 nm with a flow cell in a Beckman DB-GT recording spectrophotometer, which had been blanked against isolation buffer containing 40% sucrose. Polysomes in fractions taken from these gradients were pelleted either through 1.5 M sucrose in isolation buffer at 250,000 \times g_{\text{max}} for 2.0 hr or through 2.0 M sucrose in isolation buffer at 105,000 \times g_{\text{max}} for 16 hr. Pellets were resuspended and analyzed as described in assay of myosin-synthesizing polysomes.

B. Analytical Procedures

1. Counting of nuclei

To prepare muscle cell cultures for counting of nuclei, cultures were rinsed two times with buffered saline solution at 37°C, were fixed in absolute methanol for 5 min with occasional mixing, and were stained with Giemsa stain for 20 min at room temperature. At least 1,000 nuclei were counted in randomly chosen fields, and the percentage of total nuclei within multinucleated myotubes was calculated and used as an index of the extent of differentiation of
muscle cell cultures. Because both area of the petri dishes and diameter of the field of vision of the inverted phase microscope used for counting were known, the absolute number of nuclei in each petri dish was also calculated.

2. Pulse-labeling of cultures

Rate of myosin heavy-chain synthesis in muscle cell cultures at various stages of differentiation was determined by pulse-labeling the cells with $^3$H-leucine in a manner similar to that of Paterson and Strohman (1972). Cultures in 10-cm plastic petri dishes were labeled at $37^\circ$C for 4.0 hr in 4.0 ml of complete medium containing 1 $\mu$Ci of $^3$H-leucine/ml (specific radioactivity 5 Ci/m mole). At the end of the labeling period, the dishes were rinsed two times with cold ($2^\circ$C) 0.25 M KCl, 20 mM Tris, pH 7.4, and the cells were scraped from the surface of the dishes with a plastic spatula into 1.0 ml of the 0.25 M KCl, 20 mM Tris, pH 7.4, buffer. Cells were homogenized with 30 strokes of a 7-ml Dounce homogenizer (B pestle), and the homogenate was centrifuged at 12,000 x $g_{\text{max}}$ for 10 min. Enough cold ($2^\circ$C) water was added to lower the KCl concentration to 0.025 M, the tubes were left at $2^\circ$C for at least two hr, and myosin-containing material was pelleted at 12,000 x $g_{\text{max}}$ for 20 min. The pellet was dissolved and the precipitated protein was analyzed by sodium dodecyl sulfate (SDS)-poly-
acrylamide gel electrophoresis as described in the next paragraph.

3. SDS-polyacrylamide gel electrophoresis of proteins

Myosin-containing pellets were dissolved by heating at 100°C for 10 min in 0.075 ml of a solution containing 4.6 M 2-mercaptoethanol, 0.3% bromphenol blue, 5.6% SDS, 0.065 M sodium phosphate, pH 7.0, 20% glycerol. The dissolved samples were loaded quantitatively onto 8-cm, 7.5% polyacrylamide gels and were electrophoresed according to the procedure of Weber and Osborn (1969). Gels were stained with 0.1% coomassie blue and destained electrophoretically in a H$_2$O: glacial acetic acid: methanol mixture (87.5:7.5:5 v/v).

4. Measurement of radioactivity in SDS-polyacrylamide gels

Destained SDS-polyacrylamide gels were frozen in dry ice, and a series of 0.8 mm slices was taken through the region of the gels containing the 200,000-dalton subunit of myosin. Slices were dissolved in 0.2 ml of 30% H$_2$O$_2$ by heating at 50°C for 3 hr in polyethylene minivials (Nuclear Associates, Inc.), 4.5 ml of Aquasol (New England Nuclear) was added to each vial, and after allowing the samples to cool to 2°C for at least 24 hr, radioactivity was counted in a Model 3320 Packard Liquid Scintillation Spectrometer. Counts per min were converted into disintegrations per min (dpm's) using the automatic external standardization
method with either chloroform or pyridine as the quenching agent.

5. Assay of myosin-synthesizing polysomes

Polysomes that had been pelleted by any of the procedures described in the preceding paragraphs were resuspended over a period of at least two hr at 2°C by periodic gentle aspiration with a Pasteur pipet in 0.25 ml of incubation buffer (0.15 M KCl, 5 mM MgCl₂, 10% glycerol, 10 mM Tris, pH 7.6). Assay of the ability of myosin-synthesizing polysomes to incorporate amino acids into myosin heavy chains was performed in a volume of 0.55 ml at 37°C in the presence of 4.0 mM ATP, 1 mM GTP, 6 mM 2-mercaptoethanol, 10 μM each of 19 unlabeled amino acids, 10 μM ³H-leucine (5 Ci/m Mole), 2.6 mg/ml phosphocreatine, 0.2 mg/ml creatine phosphokinase, 0.1 mg/ml tRNA prepared from embryonic chicken muscle as described above, 5 mg/ml aminoacyl tRNA synthetases prepared as described in preparation of aminoacyl tRNA synthetases, 0.15 M KCl, 5 mM MgCl₂, 10% glycerol, 10 mM Tris, pH 7.6. Aliquots were then removed at different times and were placed into enough cold (2°C) water to lower the KCl concentration to 0.025 M. After thorough mixing, tubes were left at 2°C for 2 to 4 hr, and the myosin-containing material was pelleted at 12,000 x g_max for 20 min. The pellet was resuspended, electrophoresed on 8 cm, 7.5%
SDS-polyacrylamide gels, and analyzed for incorporation of radioactivity into myosin heavy chains as described in the preceding paragraphs on SDS-polyacrylamide gel electrophoresis of myosin-containing material and analysis of radioactivity in polyacrylamide gels.

6. Assay for $^{14}$C-polyphenylalanine synthesis

Ribosomes prepared from embryonic chicken muscle or from rabbit reticulocytes were assayed for their ability to incorporate $^{14}$C-phenylalanine into $^{14}$C-polyphenylalanine in an assay system containing the synthetic messenger RNA, polyuridylic acid. Ribosomes were incubated at 0.2 mg/ml for different amounts of time in a volume of 0.5 ml at 37°C in the presence of 4 mM ATP, 1 mM GTP, 6 mM 2-mercaptoethanol, 2.6 mg/ml phosphocreatine, 0.2 mg/ml creatine phosphokinase, 0.1 mg/ml RNA, 0.8 mg/ml crude aminoacyl tRNA synthetases, 0.050 M KCl, 15 mM MgCl$_2$, 10% glycerol, 10 mM Tris, pH 7.6. Concentration of polyuridylic acid was varied as indicated in the appropriate figure legends. Time-points were taken by removing aliquots into 5 ml of 10% TCA at 2°C, and $^{14}$C-phe-tRNA was hydrolyzed by heating the samples at 100°C for 15 min. The insoluble material containing polyphenylalanine was collected by suction on 0.45 μ pore-size Millipore filters, the filters were washed three times with 5 ml of 10% TCA each time, and the filters
were dried at 50°C for 2 hr. After placing the dried filters in polyethylene scintillation vials, 4.5 ml of Aquasol was added to each vial. Alternatively, the TCA-insoluble material was sedimented at 2,000 x \( g_{\text{max}} \) for 15 min. The pellet was resuspended and washed three times with 5 ml of 10% TCA each time. After the final centrifugation, the pellet was dissolved in Aquasol. Radioactivity in the TCA-insoluble material was measured by liquid scintillation spectrometry.

7. Measurement of protein concentration

Protein concentration was determined by the spectrophotometric method of Ehresman et al. (1973) using bovine serum albumin as a standard.

8. Measurement of ribosome and polysome concentration

Concentration of ribosomes and polysomes was determined by measuring absorbance at 260 nm and using an extinction coefficient of 11.2 absorbance units/mg protein/ml (Arlinghaus and Ascione, 1972).

C. Electron Microscopy

Three different procedures were used to examine polysomes from cultures of muscle cells at different stages of differentiation. In the first procedure, polysomes sedimenting near the bottom of sucrose density gradients were
collected and were pelleted at 225,000 \( \times g_{\text{max}} \) for 2.0 hr. Polysomes in this pellet were fixed for electron microscopy in 4% formaldehyde, 10 mM MgCl\(_2\), 5 mM Tris, pH 6.8, and were positively stained with 2% uranyl acetate on carbon-coated grids. Polysomes prepared by this method of resuspending polysome pellets were highly fragmented, and very few polysomes containing more than 20 to 25 ribosomes could be detected. In the second method, an Amicon Model 10 concentrator with an exclusion limit of 300,000 daltons was used to concentrate the polysomes and to remove excess sucrose. After polysomes had been concentrated about tenfold at 50 pounds of nitrogen pressure per square inch, the polysome solution was fixed in 1.25% glutaraldehyde, 10 mM MgCl\(_2\), 10 mM Tris, pH 7.4, at 2°C for one hr. A drop of this polysome suspension was placed on carbon coated grids, and the polysomes were stained for 1.0 min with 2% uranyl acetate. Although polysome integrity was quite good under these conditions, polysomes were extremely sparse on the grids. The final method was designed so that polysome integrity was retained and polysomes were more concentrated on the grid. In this method, polysomes isolated from near the bottom of sucrose density gradients were fixed directly in 1.25% glutaraldehyde, 10 mM MgCl\(_2\), 10 mM Tris, pH 7.4, at 2°C for one hr, and the polysomes were sedimented at 150,000 \( \times g_{\text{max}} \) for 1.5 hr in a Beckman SW 41 rotor onto
carbon-coated grids in the bottom of the tubes. Polysomes on the grid were stained for one min with 2% uranyl acetate.
As indicated in the Introduction of this dissertation, the goal of this research project was to study the effect of myoblast fusion on myosin polysome accumulation and myosin synthesis in cultures of embryonic chicken muscle cells. Such studies demand that the extent of differentiation in these cultures be known as accurately as possible. Moreover, because the studies described in this dissertation were the first to use the Muscle Biology Group's cell culture facilities at Iowa State University, a number of preliminary experiments were required to demonstrate that muscle cell cultures prepared by the procedure described in Materials and Methods were comparable to those used by other workers.

The amount of time between establishment of muscle cell cultures and initiation of myoblast fusion can be varied greatly by plating the cells at different initial densities (Konigsberg, 1971). For example, under the conditions used in this study, percentage of nuclei within multinucleated myotubes after 30 hr in culture could be increased from 5% to 35% by increasing the initial plating density from $1.4 \times 10^5$ cells/cm$^2$ to $9.1 \times 10^5$ cells/cm$^2$. It was important, therefore, that initial plating of cells be performed with sufficient precision to allow valid
comparisons between experiments. To determine the degree of precision obtainable with the cell culture techniques described in Materials and Methods, data from five separate experiments in which total number of nuclei per culture was determined at various times after plating in cultures at an initial density of $1.4 \times 10^5$ cells/cm$^2$ were combined (Figure 1). In addition, percentage of total nuclei in myotubes at different times after plating was calculated for these same cultures by using the procedure described in Materials and Methods (Figure 2). That the combined data from these different experiments give very reproducible patterns of cell proliferation (Figure 1) and fusion (Figure 2) demonstrates that initial plating of cells was performed with sufficiently high precision to allow comparisons among experiments and that counting of cells with a hemocytometer was therefore a sufficiently precise method of estimating myogenic cell number before plating.

Muscle cells grown in culture under the conditions outlined in Materials and Methods replicate mitotically for approximately 30 hr with a doubling time of 20-25 hr (doubling time is the number of hr required for the total number of nuclei in a culture dish to double). Beginning at approximately 30 hr, however, some cells in these cultures stop dividing mitotically and fuse to form multinucleated myotubes; the proportion of cells ceasing mitotic
Figure 1. Growth kinetics of embryonic chicken muscle cells in culture. Number of nuclei in stained cultures was determined at various times after plating by using an inverted phase microscope as described in Materials and Methods. Each point is the mean of duplicate determinations on two different cultures plated at the same time.
Figure 2. Kinetics of fusion and myosin synthesis in myogenic cell cultures. Percent fusion and rate of myosin heavy chain synthesis were determined as described in Materials and Methods. Points denoting percent fusion are means of duplicate determinations made on the same sample. Points denoting myosin heavy-chain synthesis are means of single determinations made on each of duplicate samples.
% FUSION

3 H-LEU dpm's IN MYOSIN/10^10 NUCLEI (o-o)

CULTURE AGE (hrs)
division and fusing increases so that by 55 hr in culture, approximately half the nuclei are within multinucleated myotubes (Figure 2). Although the main burst of fusion is completed by 55 hr, the percentage of nuclei in myotubes continues to slowly increase until at least 80 hr (Figure 2). After 80 hr in culture, however, the percentage of nuclei in myotubes decreases because approximately 30% of the cells in the cultures are fibroblasts that continue to proliferate after muscle cell fusion is completed. All subsequent experiments described in this dissertation used cultures plated at an initial density of $1.4 \times 10^5$ cells/cm$^2$. This level was chosen because investigations could conveniently be carried out on three successive days on cultures at three different stages of differentiation: (1) 24-hr cultures that have not yet started massive fusion; (2) 48-hr cultures that are fusing very rapidly; and (3) 72-hr cultures that have completed the main burst of fusion and that are rapidly synthesizing myosin heavy chains as will be discussed below. The growth rates and fusion kinetics shown in Figures 1 and 2 agree very closely with those published by other workers (Coleman and Coleman, 1968; Morris et al., 1972; Paterson and Strohman, 1972; Shainberg et al., 1971; Stockdale and O'Neill, 1972; Yaffe and Dym, 1972). This indicates that the embryonic muscle cell preparations and culture conditions used in
this study are very similar to those used by other workers in this area.

In addition to rate of cell fusion, rate of synthesis of the 200,000-dalton subunit of myosin at different times after plating was used as a criterion of differentiation of myogenic cells in culture. Myosin is the major myofibrillar protein, and its synthesis by differentiating muscle cultures is a prerequisite for myofibril assembly and development of contractile properties. Rate of myosin heavy chain synthesis was determined in cultures of different ages by pulse labeling the cells with $^{3}H$-leucine for a standard length of time (usually 4.0 hr) followed by SDS-polyacrylamide gel electrophoresis of 0.025 M KCl-insoluble material as described in Materials and Methods. Expressing these results in terms of rate of myosin heavy-chain synthesis per $10^{10}$ nuclei (Figure 2) showed that rate of myosin heavy-chain synthesis per unit of DNA in these cultures increased at least sevenfold between 24 and 72 hr in culture. Therefore, several hr after initiation of fusion, cultures prepared by the techniques described in Materials and Methods begin bulk synthesis of myosin heavy chains at a rate generally comparable to that observed in similar myogenic cultures by a number of workers (Coleman and Coleman, 1968; Morris et al., 1972; Paterson and Strohman, 1972; Stockdale and O'Neill, 1972;
Yaffe and Dym, 1972). Whether low levels of myosin synthesis occur in cultures of embryonic chicken muscle cells several hr before onset of obvious morphological differentiation has been a controversial issue and, as was explained in the Literature Review, was one of the uncertainties that prompted the work in this dissertation.

Live muscle cultures can be observed for at least several weeks by using an inverted phase microscope. Spontaneous contractions begin in such cultures after approximately 4 to 5 days in culture, and characteristic cross-striations of myofibrils can be clearly seen after 6 to 8 days in culture. After approximately 10 days in culture, some of the cells contract continuously and will often contract vigorously enough to detach one end of the myofiber from the surface of the culture dish. Although myofiber lengths and nuclei content were not systematically measured in these highly differentiated cells, it was generally observed that some of these myofibers stretched for 7 to 8 mm over the surface of the petri dish and contained at least 100 nuclei per myofiber.

An extensive study of the accumulation of ribosomes and polysomes in cultured muscle cells during differentiation has shown that total ribosomes per unit of DNA increases approximately twofold during the period of extensive
fusion and myotube formation and then plateaus at a constant level that is maintained until approximately 100 hr in culture (Hosick and Strohman, 1971). In order to compare the amounts of ribosomes in muscle cell cultures at different stages of differentiation in the present study, polysomes were extracted from muscle cultures and were displayed on sucrose density gradients as described in Materials and Methods. Area under the 260 nm absorbance curves of total polysomes (including monomers) was determined and was used as a measure of the total amount of ribosomes on the gradient. Calculating total ribosomal absorbance per $10^5$ nuclei and then plotting total ribosomal absorbance versus cultures age showed (Figure 3) that the change in total ribosomal content of cultures used in this study was similar to that described by Hosick and Strohman (1971).

These preliminary experiments demonstrated that differentiation patterns of muscle cell cultures used in this study were indistinguishable from those reported by a large number of other workers when compared on the basis of cell growth rates, fusion kinetics, myosin synthesis rates, polysome accumulation, and ability to form contractile myofibers as observed by phase microscopy.

Cultures of embryonic chicken muscle cells have been widely used to study the relationship of myosin synthesis
Figure 3. Total polysome content of myogenic cell cultures at different stages of differentiation. Total polysomes were extracted from cells and displayed on a linear 15-40% sucrose density gradient in isolation buffer after centrifugation at 105,000 \( x_{\text{gmax}} \) for 2.0 hrs as described in Materials and Methods. Area under the absorbance profile at 260 nm was measured and was used as a relative measure of total polysomes in these cells. The number of nuclei in cultures was determined as described in Materials and Methods. Points denoting total polysomes are means of two to four determinations on different samples.
to fusion of mononucleated myoblasts into multinucleated myotubes (Coleman and Coleman, 1968; Morris et al., 1972; Paterson and Strohman, 1972; Stockdale and O'Neill, 1972; Yaffe and Dym, 1972). Under the culture conditions described in Materials and Methods, the percentage of nuclei within myotubes increases from less than 5% after 30 hr in culture to a plateau of 60 to 70% after 55 hr (Figure 2). Immediately after this burst of fusion, approximately a sevenfold increase in the rate of myosin heavy-chain synthesis per unit of DNA occurs (Figure 2). Heywood et al. (1967) have demonstrated that polysomes containing 50 to 60 ribosomes in embryonic chicken muscle are responsible for synthesis of the 200,000-dalton heavy chain of myosin. Because rate of myosin synthesis per unit of DNA increases sevenfold soon after the onset of fusion (Figure 2), cultures of multinucleated myotubes might be expected to contain a similar increase in amount of 50 to 60-ribosome polysomes when compared with cultures of mononucleated myoblasts on sucrose density gradients. Total polysomes were isolated from 24-hr and 72-hr cultures of cells that contained similar number of nuclei, and an optical density tracing at 260 nm of the density gradient separation of the polysome fractions from these 24-hr and 72-hr cultures is shown in Figure 4. As expected (Hosick and Strohman, 1971), the 72-hr cultures contained more total polysomes per
Figure 4. Density-gradient centrifugation of polysomes from 24-hr and 72-hr myogenic cell cultures. Polysomes were isolated from 24-hr and 72-hr myogenic cell cultures as described in Materials and Methods and were displayed on a linear 15-40% sucrose gradient in isolation buffer after centrifugation at 105,000 x g_{max} for 2.0 hrs. Direction of sedimentation is from right to left. Bracket denotes the part of the gradient from which polysomes were isolated for electron microscopy.
nucleus than the 24-hr cultures, but distribution of polysomes from cultures at these two stages of differentiation was qualitatively similar (Figure 4). The similarity in proportion of polysomes in the lower third of the gradient (at the left side of Figure 4) is especially interesting because this is the region of the gradient from which myosin-synthesizing polysomes have been isolated (Heywood et al., 1967). Polysomes from the region of the gradient designated by the bracket in Figure 4 were isolated and prepared for electron microscopy as described in Materials and Methods. Figure 5 shows that polysomes in this region of the density gradients from both fused and nonfused cultures were large enough to synthesize a peptide chain as large as myosin heavy chains.

The presence of polysomes large enough to support synthesis of the 200,000-dalton subunit of myosin in mononucleated muscle cells prompted an investigation into whether these polysomes would incorporate $^{3}H$-leucine into polypeptides that would migrate with the heavy chains of myosin in SDS-polyacrylamide gel electrophoresis. Before attempting to measure myosin synthesis in mononucleated myogenic cell cultures that may or may not be synthesizing myosin, it was necessary to demonstrate that the assay described in Materials and Methods would accurately measure synthesis of myosin heavy chains. Hence, total polysomes
Figure 5. Electron micrographs of large polysomes isolated from 24-hr and 72-hr embryonic chicken muscle cell cultures. Polysomes from the region of the gradient designated by the bracket in Figure 4 were isolated and were stained with uranyl acetate as described in Materials and Methods. Polysomes from 24-hr and 72-hr muscle cell cultures appear similar in the electron micrograph and both contain over 50 ribosomes per polysome. x98,470.
were isolated from leg muscles of 14-day embryonic chickens where myosin synthesis was unequivocally occurring and were assayed for ability to incorporate amino acids into protein. Protein in the in vitro protein-synthesizing system was separated by SDS-polyacrylamide gel electrophoresis, and after staining, the SDS-polyacrylamide gels were sliced, and the slices were assayed for radioactivity. Radioactivity in gel slices through the myosin band was highest at the distance of migration corresponding to a molecular weight of 200,000 daltons (Figure 6). The sum of all radioactivity in the slices making up this peak (indicated by the bracket in Figure 6) was used as the total $^3$H-leucine that had been incorporated into myosin heavy chains. Plotting this sum versus assay time (Figure 7) shows that completion of myosin heavy chains occurred very rapidly during the first 5 min of incubation of total embryonic chicken muscle polysomes at 37°C, occurred less rapidly between 5 and 10 min, and was finished by approximately 10 min. Because the assay contained no ribosomes other than those attached to mRVA's in the form of polysomes, only chain-completion synthesis of myosin occurred in this system. The lower curve in Figure 7 is endogenous incorporation into myosin heavy chains that occurs when polysomes are omitted from the assay. Such a control was run with each experiment, and this control value was always
Figure 6. Synthesis of myosin heavy chains by total embryonic chicken leg muscle polysomes. Polysomes isolated from 14-day embryonic chicken leg muscle were incubated for 0 or 20 min in the assay mixture described in Materials and Methods. Reactions were stopped by adding to enough cold (2°C) water to lower the KCl concentration to 25 mM and the precipitated myosin was sedimented 12,000 x_{max} for 20 min. The sedimented pellets were dissolved in SDS and analyzed by SDS-polyacrylamide gel electrophoresis. After staining, the gels were sliced and radioactivity in the slices was measured. The peak of radioactivity migrated with a molecular weight of 200,000 daltons. Slices included under the bracket were used to calculate the 20 min time-points in Figure 7. Bottom line is incorporation after 0 min of incubation and the upper line is incorporation after 20 min of incubation.
Figure 7. Time-course of myosin heavy-chain synthesis by embryonic chicken muscle polysomes. Polysomes isolated from 14-day embryonic chicken leg muscle were incubated in the cell-free assay described in Materials and Methods. Total myosin heavy-chain synthesis was determined as the sum of radioactivity measured in gel slices through the region of the myosin heavy-chain band on SDS-polyacrylamide gels of the protein synthesized in this cell-free assay (see Figure 6). The lower curve is endogenous incorporation into myosin that occurs when polysomes are omitted from the assay medium. Such a control was run with each experiment, and this endogenous incorporation was subtracted from total myosin synthesis by polysomes from fused or from nonfused muscle cell cultures.
subtracted from total myosin-synthesizing ability of the polysomes being analyzed. These results are compatible with reports that approximately 4 to 8 min are required for movement of ribosomes along the entire length of a myosin mRNA (Coleman and Coleman, 1968; Herrmann et al., 1970; Morris et al., 1972).

When total polysomes isolated from 14-day embryonic chicken leg muscle as described above were subjected to sucrose density gradient analysis after they had been incubated 20 min at 37°C in the in vitro protein synthesis system (i.e., after accumulation of completed polypeptides had subsided), essentially all ribosomes sedimented as 80S monomers or as subunits (Figure 8) and no rapidly sedimenting polysomes remained. This finding indicates that virtually none of the ribosomes in the total polysome fraction remained bound to mRNA's after 20 min of incubation and suggests that polysomes prepared according to the procedures used in this study were capable of actively moving along the mRNA's and completing the synthesis of polypeptides.

Figure 6 shows only the distribution of radioactivity incorporated into protein migrating in the region of SDS-polyacrylamide gels corresponding to approximately 200,000 daltons. Because precipitation of myosin heavy chains in 0.025 M KCl is not a rigorous purification step, other
Figure 8. Distribution of embryonic chicken muscle polysomes on a 15-40% sucrose gradient after these polysomes had been incubated for 0 or 20 min in the assay mixture described in Materials and Methods. Gradients were centrifuged at 150,000 xg_{max} for 1.5 hrs in a Beckman SW 41 rotor. Direction of sedimentation is from right to left.
proteins (including several of the myofibrillar proteins) also precipitate under these conditions. Figure 9 shows the distribution of radioactivity incorporated during the \textit{in vitro} protein-synthesizing assay into all the proteins that precipitate in 0.025 M KCl after separation of these proteins by SDS-polyacrylamide gel electrophoresis. It is obvious that a peak of radioactivity corresponding to myosin heavy chains (designated by the bracket in Figure 9) can be identified clearly enough for the radioactivity in this peak to be quantitatively measured separate from radioactivity in neighboring peaks. The nature of the other proteins containing radioactivity in these gels was not determined.

The assumption that centrifuging the \textit{in vitro} protein synthesis assay systems at 12,000 x $g_{\text{max}}$ for 20 min after diluting them to 0.025 M KCl quantitatively sediments all newly synthesized myosin heavy chains was evaluated by adding TCA at a final concentration of 5% to the supernatant remaining after the 12,000 x $g_{\text{max}}$ centrifugation. The TCA-treated supernatant was centrifuged at 1,000 x $g_{\text{max}}$ for 15 min, the tube was drained and dried, and the pellet was dissolved for SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Even after very heavy loading, gels resulting from this treatment contained no detectable band near the top of the gels and corresponding
Figure 9. Incorporation of $^3$H-leucine into 25 mM KCl-insoluble proteins by total polysomes isolated from 14-day embryonic chicken muscle after incubation for 0 or 20 min in the assay described in Materials and Methods. Protein precipitated from the assay mixtures in 25 mM KCl was dissolved in SDS and analyzed by SDS-polyacrylamide gel electrophoresis. After staining, the gels were sliced and radioactivity in slices was measured. The bracket indicates the portion of the gel that corresponds to a molecular weight of 200,000 daltons. The lower curve is the distribution of radioactivity that occurs after 0 min of incubation.
to myosin heavy chains (Figure 10). The sum of all radioactivity in those slices which correspond to myosin heavy chains (designated by the bracket in Figure 10) was only approximately 15% of the amount of total radioactivity found in the corresponding region of gels of the 0.025 M KCl pellet. Although myosin heavy chains are not quantitatively recovered by precipitation in 0.025 M KCl, an 85% recovery was judged to be acceptable.

Up to this point, all experiments had involved development and testing of optimal conditions and techniques for incorporating $^3$H-leucine into myosin heavy chains and for detection of these myosin heavy-chain polypeptides in SDS-polyacrylamide gels. To use the assay for myosin-synthesizing polysomes to quantitatively measure relative amounts of these polysomes in muscle cell cultures, it is necessary, or at least simplifying, for the amount of radioactivity incorporated into myosin to depend linearly on the amount of myosin-synthesizing polysomes in the assay. Therefore, experiments were conducted to measure total incorporation of $^3$H-leucine into myosin heavy chains after 20 min of incubation in the presence of different levels of added polysomes. Different volumes of the supernatant remaining after centrifuging homogenized 14-day embryonic chicken leg muscle at 12,000 x $g_{max}$ for 10 min as described in Materials and Methods were used as the source
Figure 10. Incorporation of $^3$H-leucine into proteins insoluble in 5% TCA by total polysomes isolated from 14-day embryonic chicken muscle after incubation for 0 or 20 min in the protein-synthesizing assay described in Materials and Methods. The proteins were precipitated with 5% TCA, dissolved in SDS, and analyzed by SDS-polyacrylamide gel electrophoresis. After staining, the gels were sliced and the radioactivity in the slices was measured. The bracket indicates the distance of migration corresponding to 200,000 daltons. The lower curve is the distribution of radioactivity that occurs after 0 min of incubation.
of different levels of polysomes. These total polysomes were pelleted through 1.5 M sucrose at 150,000 x g_max for 2 hr and were assayed for their ability to direct synthesis of myosin heavy chains. Radioactivity migrating as myosin in each of the assays done at different polysome levels was plotted versus the amount of polysomes in the assay (Figure 11). Incorporation of ^3H-leucine into myosin did not increase linearly with the increase in amount of polysomes in the assay. This nonlinearity could be due to either one of two causes: (1) amount of some component or components other than polysomes in the incorporation mixture could be limiting incorporation at high added polysome levels; or (2) the greater amount of mechanical agitation required to dissolve the larger polysome pellets resulting from sedimentation of the higher levels of polysomes used in the assays could result in addition of fewer biologically active polysomes than calculated at the higher polysome levels. This extra agitation could cause either stripping of the ribosomes from the mRNA or actual breaking of the mRNA. Therefore, experiments were designed to distinguish between these two alternatives. If the limitation was due to limiting amounts of some component other than polysomes, then incorporation into myosin should increase linearly when the volume of the assay (with all components in the same concentrations) was increased in the
Figure 11. Effect of different levels of polysomes isolated from 14-day embryonic chicken muscle on incorporation of $^3$H-leucine into myosin heavy chains. Polysomes from different volumes of 12,000 x $E_{\text{max}}$-supernatant of homogenized muscle were sedimented and assayed for ability to incorporate $^3$H-leucine into myosin heavy chains as described in Materials and Methods.
1,600
1,200
800
400
0

0.5
1.0
1.5
2.0

MILLILITERS OF 12,000xg_{max} SUPERNATANT

3H-LEU dpm's IN MYOSIN HEAVY CHAINS
same proportion that the amount of polysomes being assayed is increased. If the relatively lower counts at higher polysome concentrations were due to breakage of the mRNA or stripping of the ribosomes from the mRNA, an assay with a constant proportion of polysomes to total assay volume should likewise level off at higher polysome levels similar to the curve in Figure 11. Therefore, instead of assaying the polysomes from 1.0 ml and 1.5 ml of 12,000 × g\text{max}-supernatant (see Figure 11; this is the level of polysomes where incorporation started to depart from linearity) in the standard 0.55 ml assay described in Materials and Methods, polysomes from 1.0 ml of polysome-containing 12,000 × g\text{max}-supernatant were assayed in a volume of 1.10 ml, and polysomes from 1.50 ml of polysome-containing solution were assayed in a volume of 1.65 ml. Aliquots were removed at 0 and 20 min from assay tubes containing polysomes pelleted from 0.5 ml, 1.0 ml, and 1.5 ml of 12,000 × g\text{max} supernatant, and the 0.025 M KCl-insoluble proteins were prepared for electrophoresis as already described. The results of such experiments showed that incorporation into myosin heavy chains increased linearly as amount of total polysomes added to the in vitro assay increased (Figure 12), and suggested that the non-linearity of myosin heavy-chain synthesis versus polysome concentration observed in Figure 11 occurred because of a
Figure 12. Effect of different levels of polysomes isolated from 14-day embryonic chicken muscle on incorporation of $^3$H-leucine into myosin heavy chains when the polysomes were added to the assay medium at a constant ratio of polysomes to volume of assay medium. Polysomes sedimented from 0.5 ml, 1.0 ml, and 1.5 ml of 12,000 x_g_max-supernatant were assayed in a final volume of 0.55 ml, 1.10 ml, and 1.65 ml, respectively. Myosin heavy chain synthesis was determined as described in Materials and Methods.
3H-LEU dpm's in myosin heavy chains

MILLITERS OF 12,000 x g_{max} SUPERNATANT
limiting amount of some component(s) other than polysomes in the incorporation mixture.

The inhibition of run-off incorporation observed at higher levels of polysomes in Figure 11 must originate from such a severe depletion or destruction of one of the components of the assay mixture that movement of the ribosome along the mRNA is totally inhibited, because the only way for myosin heavy-chain synthesis to be detected is for the ribosome to move all the way to the termination codon of the mRNA. Therefore, the only way for myosin heavy-chain synthesis not to increase linearly with an increase in total polysome concentration in an assay system measuring principally peptide chain completion on already initiated ribosomes would be for some of the ribosomes to fail to move all the way to the termination end of the mRNA. Possible causes for failure of ribosomes to move all the way to the termination codon of the mRNA in in vitro protein synthesis mixtures can be separated into three general categories: (1) the enzyme system (i.e., aminoacyl tRNA synthetases, elongation factors, and other required enzymes); (2) the energy generating system (i.e., ATP, GTP, creatine phosphate, and creatine kinase); and (3) the amino acids (i.e., 19 unlabeled amino acids and $^3$H-leucine).

The data in Figure 13 and Figure 14 show that activity of the enzyme system is not limiting incorporation of amino
Figure 13. Effect of different levels of poly U on incorporation of $^{14}$C-phenylalanine into $^{14}$C-polyphenylalanine by 14-day embryonic chicken muscle ribosomes after 20 min of incubation in the assay system described in Materials and Methods. $^{14}$C-polyphenylalanine synthesis was measured by counting the radioactivity insoluble in 10% TCA as described in Materials and Methods. Sixty μg of poly U was used as a saturating level to measure the time-course of $^{14}$C-phenylalanine into $^{14}$C-polyphenylalanine (see Figure 14).
dpm's incorporated into $^{14}$C-polyphenylalanine

Micrograms of polyuridylic acid per assay
Figure 14. Time-course of incorporation of $^{14}$C-phenylalanine into $^{14}$C-polyphenylalanine by 14-day embryonic muscle ribosomes in the presence of saturating levels of poly U (Figure 13). Incorporation was done in the cell-free assay system described in Materials and Methods. $^{14}$C-polyphenylalanine synthesis was measured by counting the radioactivity insoluble in 10% TCA as described in Materials and Methods.
dpm's INCORPORATED INTO $^{14}$C-POLYPHENYLALANINE

Graph with axes labeled:
- X-axis: Reaction Time (min)
- Y-axis: DPM's

The graph shows a linear decrease in DPM's as the reaction time increases.
acids into myosin heavy chains at the high levels of myosin polysomes used in Figure 11. Ribosomes prepared by NaF treatment as described in Materials and Methods are active in translating artificial mRNA's such as poly U in cell-free protein-synthesizing assays when Mg\(^{++}\) concentration is 15 mM. When excess poly U is added to such an assay so that ribosomes are rate-limiting and poly U is saturating (Figure 13), the enzyme fraction used in this study will support the synthesis of \(^14\)C-polyphenylalanine at a linear kinetic rate (Figure 14) for at least 20 min under conditions identical to those used in the runoff assays shown in Figures 11 and 12.

The same arguments used to eliminate the enzyme system as a factor limiting incorporation of radioactivity into myosin heavy chains can be used to argue that the energy generating system is not limiting \textit{in vitro} incorporation because this same energy system supports \(^14\)C-polyphenylalanine synthesis at a linear kinetic rate for 20 min (Figure 14). In addition, if the limitation in incorporation of \(^3\)H-leucine into myosin heavy chains was due to depletion of the energy supply, rate of myosin heavy-chain synthesis would be expected to decrease much earlier in the incubation period when higher levels of polysomes were present and more energy would be required than when low levels of polysomes were present. However, the time-
course of \(^3\)H-leucine incorporation into myosin heavy chains is identical regardless of the amount of polysomes in the assay (Figure 15). Completion of nascent myosin heavy chains is about 60 to 70% complete after 5 min and 100% complete after 10 min (Figure 15).

The only other group of components in the in vitro protein-synthesis assay is the amino acids. Although the data in Figures 13, 14, and 15 also clearly show that the amount of amino acids available for incorporation is not limiting, unlabeled intracellular leucine adheres to the polysomes when they are pelleted through 1.5 M sucrose. This unlabeled leucine could decrease the specific radioactivity (specific radioactivity = µCi/µ mole) of the \(^3\)H-leucine in the assay system. Because the amount of unlabeled leucine contaminating the assay mixture would increase as the size of the polysome pellet increases, specific radioactivity of leucine in the assay system would be progressively decreased as larger amounts of polysomes were added. A decrease in specific radioactivity of the leucine in the assay system would cause nonlinear synthesis of myosin heavy chains at high polysome concentrations, just as was observed in Figure 11. The data in Figures 12-15 indicate that a decrease in specific radioactivity of leucine in the assay system is a more logical explanation for the decrease in myosin heavy-chain synthesis at high
Figure 15. Time-course of myosin heavy-chain synthesis by 14-day embryonic chicken muscle polysomes prepared either from 0.5 ml of muscle homogenate (o—o) or from 2.0 ml of muscle homogenate(e—e). Incorporation of $^3$H-leucine into myosin heavy chains was measured as detailed in Materials and Methods.
polysome concentration than explanations involving cessation of ribosome movement along the mRNA in an assay where both the enzymes and energy generating systems are functioning adequately.

To estimate the magnitude of the decrease in specific radioactivity of $^3$H-leucine caused by addition of large amounts of polysomes, different sizes of polysome pellets were prepared by sedimenting carefully measured aliquots of a homogenate of a known amount of 14-day chicken embryo leg muscle to which a very small amount of $^3$H-leucine of very high specific radioactivity (50,000 $\mu$Ci/$\mu$ mole) had been added before homogenization. By knowing all volumes, and by counting the radioactivity in the polysome pellets and assuming the tissue concentration of leucine to be 1 mM (Lewis and D'Mello, 1967), the decrease in specific radioactivity of leucine due to contamination by leucine sedimented with the polysome pellets could be estimated. The results indicated that specific radioactivity was reduced approximately 4% when 0.5 ml of muscle homogenate was used for isolation of total polysomes and approximately 15% when 2.0 ml of muscle homogenate was used for isolation of total polysomes. Dilution of this magnitude is sufficient to account for most of the seeming decrease in myosin heavy-chain synthesis observed when high levels of polysomes were added to the in vitro protein synthesis assay (Figure 11).
The important potential source of error revealed by these experiments with specific radioactivity of leucine had to be considered in evaluating all measurements on activity of polysomes isolated from muscle cell cultures. Clearly, assay of myosin polysome activity would be easier and more amenable to varying experimental conditions if widely differing levels of polysomes could be used in these assays. Because of the decrease in specific radioactivity of leucine and the resulting spurious decrease in myosin heavy-chain synthesis when large amounts of polysomes were added to the in vitro protein synthesis assay used in this study, however, the amount of polysomes added to each assay was adjusted so that approximately 600 to 1,000 dpm's were incorporated into myosin heavy chains during the assay period. In this way it was possible to minimize, although not completely eliminate, errors in measuring nascent myosin heavy-chain synthesis due to the observed nonlinearity in rate of myosin synthesis at different levels of added polysomes. It was impossible to add equivalent amounts of polysomes in some experiments, and the results of these experiments are therefore subject to this error. In virtually all instances, however, results of the experiments described in this dissertation are clearcut, and small errors of the magnitude encountered in the experiments on specific radioactivity of leucine would have little or no
consequence on interpretation of the experimental results obtained.

The experiments described in the preceding paragraphs show that it is possible to determine the amount of myosin-synthesizing polysomes in cultures of differentiating muscle cells by using an assay system in which completion of nascent myosin heavy chains is measured. It is obvious, however, that sensitivity of this assay system would be increased severalfold if ribosomes were initiating synthesis of new peptides at a rate equal to the rate of movement of ribosomes along the mRNA. If ribosomes could initiate synthesis of new peptide chains in the assay system used in this study, a linear rate of incorporation of \(^{3}\text{H}\)-leucine into myosin heavy chains would continue for much longer periods of time than the approximately 5 min obtained when measuring only runoff synthesis (Figure 15). Consequently, initiation of new peptide chains would make it possible to use lower concentrations of polysomes from fewer muscle cell cultures in assays of myosin heavy-chain synthesis and would therefore enable assay of myosin-synthesizing polysomes in cells that contain only very low quantities of such polysomes. Therefore, two approaches were used in an attempt to stimulate initiation of myosin synthesis by adding exogenous ribosomes to the assay.

In the first approach, an effort was made to stimulate
initiation of ribosomes on the myosin mRNA by adding both ribosomes and crude initiation factor preparations to the \textit{in vitro} assay. Heywood (1969) has shown that 1.0 M KCl extraction of ribosomes releases approximately 200 \( \mu \)g of protein per mg of ribosomes and that this protein fraction contains initiation factors. Equal amounts of total poly­somes from 14-day embryonic chicken leg muscle were assayed for ability to incorporate \(^3\text{H}-\text{leucine}\) into myosin heavy chains in both the presence and absence of 0.5 mg of 1.0 M KCl-washed ribosomes and 0.4 mg of crude initiation factors prepared as described in Materials and Methods. The results of these experiments (Figure 16) showed that four times the normal level of initiation factors do not stimulate initiation by exogenous ribosomes. Indeed, incorporation into myosin in the presence of crude initiation factors was always slightly less than in their absence (Figure 16). Because it was possible that some part of the preparation procedure was inactivating the 1.0 M KCl-washed ribosomes irreversibly and making them incapable of either initiation or elongation, the capability of these ribosomes for translation was measured in an \textit{in vitro} assay containing poly U as a synthetic mRNA and \(^{14}\text{C}-\text{phenylalanine}\) as the labeled substrate. The amount of \(^{14}\text{C}-\text{polyphenylaline}\) synthesis by these ribosomes was dependent on the amount of poly U present in the assay (Figure 13), and these ribosomes
Figure 16. Time-course of myosin heavy-chain synthesis by 14-day embryonic chicken muscle polysomes in either the presence (0—0) or absence (•—•) of 0.5 mg of 1.0 M KCl-washed ribosomes from 14-day embryonic chicken muscle and 0.4 mg of crude initiation factors solubilized from 14-day embryonic chicken muscle ribosomes by 1.0 M KCl, 0.0001 M MgCl₂, and 0.02 M Tris, pH 7.4. Ribosomes, initiation factors, and polysomes were prepared, and myosin heavy chain synthesis was measured by the procedures described in Materials and Methods.
synthesized $^{14}$C-polyphenylalanine at a linear kinetic rate for at least 20 min (Figure 14). These experiments demonstrate that the ribosomes used in these experiments have the capacity for translation, but they apparently are incapable of initiation.

A very high rate of endogenous incorporation into myosin heavy chains occurred in the assays of polysomes for myosin synthesis in the presence of KCl-washed ribosomes and crude initiation factors (Figure 16). For example, in the experiment shown in Figure 16, the endogenous control was about 30% of the total dpm's incorporated into myosin heavy chains in the presence of polysomes (for comparison see Figure 7). This high rate of endogenous incorporation was evidently due to myosin mRNA adhering to the muscle ribosomes during preparation (Heywood and Nwagwu, 1969). To decrease this high endogenous incorporation and to attempt simultaneously to stimulate initiation, the muscle ribosomes in these assays were replaced with rabbit reticulocyte ribosomes prepared as described in Materials and Methods. Reticulocytes synthesize very little myosin compared to embryonic muscle, and ribosomes isolated from reticulocytes, therefore, have very low endogenous incorporation into myosin. Incubation of reticulocyte ribosomes in an assay mixture containing $^{14}$C-phenylalanine and saturating levels of poly U (Figure 17) in the presence of
Figure 17. Effect of different amounts of poly U on incorporation of $^{14}$C-phenylalanine into $^{14}$C-polyphenylalanine by 20 min incubation with rabbit reticulocyte ribosomes. $^{14}$C-polyphenylalanine synthesis was measured by counting the radioactivity insoluble in 10% TCA, and rabbit reticulocyte ribosomes were prepared as described in Materials and Methods. Sixty micrograms of poly U were used as a saturating level of poly U to measure the time-course of $^{14}$C-phenylalanine incorporation (see Figure 18).
15 mM Mg\textsuperscript{++} results in a linear rate of \textsuperscript{14}C-polyphenylalanine synthesis for at least 30 min (Figure 18). Fifteen mM Mg\textsuperscript{++} causes nonspecific initiation or initiation factor-independent initiation (Heywood, 1969; Rourke and Heywood, 1972) in protein synthesizing assays. Hence, it seemed possible that addition of 15 mM Mg\textsuperscript{++} to the assays of myosin synthesis in the presence of reticulocyte ribosomes might cause enough nonspecific initiation by the reticulocyte ribosomes that synthesis of myosin heavy chains would occur at a linear kinetic rate for extended periods of time. Addition of 15 mM Mg\textsuperscript{++} to assays of embryonic muscle polysomes, however, did not stimulate myosin synthesis but actually inhibited it when compared to run-off controls containing no reticulocyte ribosomes and 5 mM Mg\textsuperscript{++} (Figure 19). Thus, in poly U-directed synthesis of TCA-precipitable \textsuperscript{14}C-polyphenylalanine, the presence of 15 mM Mg\textsuperscript{++} results in nonspecific initiation continuing for greater than 30 min (Figure 18), but in assays of myosin synthesis by embryonic muscle polysomes, the presence of 15 mM Mg\textsuperscript{++} results in nonspecific dissociation of ribosomes from the myosin mRNA at such a rate that only about 20% of the nascent myosin heavy chains are completed, and no additional, newly initiated myosin molecules are synthesized (Figure 19).

Although Heywood and coworkers have presented extensive
Figure 18. Time-course of $^{14}$C-phenylalanine incorporation into $^{14}$C-polyphenylalanine by rabbit reticulocyte ribosomes in a cell-free assay containing saturating levels of poly U (Figure 17). Sixty micrograms of poly U were used as a saturating level. $^{14}$C-polyphenylalanine synthesis was measured by counting the radioactivity insoluble in 10% TCA, and rabbit reticulocyte ribosomes were prepared as described in Materials and Methods.
Figure 19. Time-course of myosin heavy-chain synthesis by 14-day embryonic chicken muscle polysomes in either the presence (O—O) or absence (e—e) of 0.5 mg of rabbit reticulocyte ribosomes and 0.015 M MgCl₂. Embryonic muscle polysomes and rabbit reticulocyte ribosomes were prepared, and myosin heavy-chain synthesis was measured as described in Materials and Methods.
Without Reticulocyte Ribosomes

With Reticulocyte Ribosomes

$^{3}$H-LEU dpm's in myosin heavy chains

REACTION TIME (MIN)
evidence that developing embryonic muscle contains specific initiation factors for myosin (Heywood, 1969; Heywood and Thompson, 1971; Rourke and Heywood, 1972; Heywood et al., 1974; Kennedy et al., 1974), the results obtained here show that addition of four times the normal levels of crude initiation factors obtained from muscle ribosomes caused no detectable initiation of muscle ribosomes onto myosin poly­somes (Figure 16). A critical examination of Heywood's data suggests that, although some initiation is unquestionably occurring in their systems, only a small proportion of the myosin mRNA's in their assays are actually involved in initiation; this proportion may possibly be as low as 1%. In addition, in an in vitro assay containing myosin mRNA and an excess of ribosomes and initiation factors (Heywood and Nwagwu, 1969), the largest polysomes detected contained only six ribosomes, and most of the polysomes contained only two or three ribosomes. Because the goal of this research project was to study the relationship between myosin polysome accumulation and myoblast fusion rather than to develop new methods and techniques for stimulating initiation of protein synthesis with purified initiation factors, no further attempts were made to increase sensitivity of the assay system for myo­sin polysomes by achieving initiation of ribosomes onto the myosin mRNA. The remainder of the assays for myosin
polysomes discussed in this dissertation were performed using an in vitro protein synthesizing assay that measured completion of nascent myosin heavy chains.

These preliminary experiments established that polysomes prepared and assayed according to the procedures used in this study were able to complete synthesis of all the peptide chains that they had initiated before their isolation (Figure 8). Consequently, only 0 and 20 min time-points were taken in all subsequent assays described in this dissertation, and the difference between incorporation at 0 and 20 min was used as a measure of synthesis of myosin heavy chains.

The assay described in the preceding paragraphs was applied to total polysomes prepared from 24-hr (mononucleated, unfused cells) and 72-hr (multinucleated, fused cells) muscle cell cultures to measure synthesis of myosin heavy chains by these polysomes. As indicated above, the assay used in this study measures run-off of peptide chains that had been initiated before isolation of the polysomes; therefore, measurement of the amount of myosin heavy chain synthesis in this system was a measure of amount of myosin polysomes in 24-hr and 72-hr cultures. Polysomes isolated from both 24-hr and 72-hr cultures directed synthesis of polypeptides that migrated with a molecular weight of 200,000 daltons on SDS-polyacrylamide gel electrophoresis
(Figure 20). Furthermore, expressing the results on the basis of radioactivity incorporated into myosin heavy chains per $10^7$ nuclei showed that total polysomes from nonfused muscle cell cultures support synthesis of approximately half as many myosin heavy chains as those from myotube cultures do (Figure 20). The accuracy of this quantitative comparison of myosin synthesis (and hence of total myosin polysomes) by polysomes from nonfused and fused muscle cell cultures obviously depends on complete recovery of all myosin polysomes from the initial cell homogenates. It is possible that some of the myosin-synthesizing polysomes originally present in the cultures remained in the pellet from the $12,000 \times g_{\text{max}}$ centrifugation of the cell lysate in 0.5% Triton X-100 either because of incomplete cell lysis or because of physical entrapment. Any myosin-synthesizing polysomes remaining in these $12,000 \times g_{\text{max}}$ pellets would not have been assayed and would therefore make the results shown in Figure 20 inaccurate. To eliminate this possibility, the $12,000 \times g_{\text{max}}$ pellets were reextracted in isolation buffer containing 0.5% Triton X-100 by a mild homogenization with a Dounce homogenizer (A pestle). This homogenate was centrifuged at $12,000 \times g_{\text{max}}$ for 10 min, and this second $12,000 \times g_{\text{max}}$ supernatant was also assayed for polysomes capable of synthesizing myosin heavy chains as described
Figure 20. Synthesis of myosin heavy chains by total polysomes isolated from 24-hr and 72-hr muscle cell cultures. Total polysomes were isolated, assayed, and the products analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The peak of $^3$H-leucine incorporation supported by polysomes from either 24-hr or 72-hr cultures migrated exactly the same distance in SDS-polyacrylamide gels as myosin heavy chains.
earlier. The results of these experiments suggest that although a slightly larger percentage of myosin-synthesizing polysomes were pelleted at $12,000 \times g_{\text{max}}$ in myotube lysates (Table 1) than in lysates from mononucleated myoblasts, this difference is small, and the data shown in Figure 20 accurately indicate the relative quantities of myosin-synthesizing polysomes in 24-hr and 72-hr myogenic cell cultures. The summary of a number of experiments like the one shown in Figure 20 indicates clearly that polysomes from nonfused muscle cultures have about half as much capability for incorporating $^{3}H$-leucine into a 200,000-dalton polypeptide as polysomes from myotubes do (Table 2, left column).

Because a small but consistent level of fusion exists in 24-hr cultures (Figure 2), it is critical to determine whether myosin-synthesizing polysomes in the 24-hr cultures could originate entirely from the small number of fused myotubes present in those cultures. The number of nuclei was thus determined as described in Materials and Methods and scoring these nuclei as from either fused or nonfused cells. By using this measure of total nuclei present in multinucleated myotubes, and by using the total dpm's incorporated into myosin heavy chains in 24-hr and 72-hr cultures (left column, Table 2), it is possible to calculate the amount of myosin polysomes that would be present
Table 1. Myosin synthesis by polysomes from 24-hr and 72-hr muscle cultures\(^a\).

<table>
<thead>
<tr>
<th>Source of polysomes</th>
<th>% of myosin-synthesizing polysomes in each supernatant(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24-hr cultures 72-hr cultures</td>
</tr>
<tr>
<td>First 12,000 x (g) supernatant</td>
<td>94.9 84.9</td>
</tr>
<tr>
<td>Second 12,000 x (g) supernatant</td>
<td>5.1 15.1</td>
</tr>
</tbody>
</table>

Polysomes in the first 12,000 x \(g\) supernatant from both 24-hr and 72-hr muscle cultures were prepared and assayed as described in Materials and Methods. The pellet from the first 12,000 x \(g\) centrifugation was reextracted with isolation buffer plus 0.5% Triton X-100 to test for complete extraction of myosin-synthesizing polysomes. This second extract was also centrifuged at 12,000 x \(g\) for 10 min, and the supernatant was assayed for polysomes capable of incorporating \(^3\text{H}\)-leucine into myosin heavy chains as described in Materials and Methods.

\(^{b}\) % of myosin-synthesizing polysomes = (myosin dpm's by polysomes in each supernatant/sum of myosin dpm's by polysomes in both supernatants) x 100.

Table 2. Myosin heavy-chain synthesis per \(10^7\) nuclei and per \(10^7\) fused nuclei by polysomes from 24-hr and 72-hr muscle cell cultures\(^a\).

<table>
<thead>
<tr>
<th>Culture age</th>
<th>(^3\text{H})-leucine dpm's in myosin heavy chains/(10^7) nuclei</th>
<th>(^3\text{H})-leucine dpm's in myosin heavy chains/(10^7) fused nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hr</td>
<td>498</td>
<td>12,475</td>
</tr>
<tr>
<td>72 hr</td>
<td>914</td>
<td>1,406</td>
</tr>
</tbody>
</table>

Incorporation of \(^3\text{H}\)-leucine into myosin heavy chains and number of nuclei in single cells and in multinucleated myotubes were determined by the procedures described in Materials and Methods.
per $10^7$ nuclei in multinucleated myotubes if it were assumed that mononucleated myoblasts in the 24-hr and 72-hr cultures contained no myosin polysomes. The results of this calculation (right column, Table 2) show that the quantity of myosin polysomes per $10^7$ nuclei would be nearly ninefold greater in the myotubes in 24-hr cultures than in the myotubes in 72-hr cultures if the small myotubes in 24-hr cultures were the only source of myosin-synthesizing polysomes. In addition, when the myosin synthesis curve in Figure 2 is expressed on the basis of myosin synthesis per $10^{10}$ fused nuclei, the rate of myosin heavy chain synthesis per unit of DNA is fourfold greater in 16-hr myotubes than it is in 72-hr myotubes (Figure 21). Because it seems incongruous to suggest that myotubes in 24-hr myogenic cell cultures should contain ninefold greater amounts of myosin polysomes than myotubes in 72-hr myogenic cultures, or that myotubes in 16-hr cultures are synthesizing myosin fourfold faster than myotubes in 72-hr cultures, these results indicate that mononucleated muscle cells after 24 hrs in cultures contain significant levels of myosin-synthesizing polysomes and these mononucleated muscle cells are therefore also probably synthesizing myosin at a significant rate.

The conclusion that mononucleated myoblasts contain approximately half as many myosin-synthesizing polysomes
Figure 21. Kinetics of fusion and myosin heavy-chain synthesis per $10^{10}$ fused nuclei in myogenic cell cultures pulse labeled at different culture ages with $^3$H-leucine. Number of nuclei within multinucleated myotubes and rate of myosin synthesis were determined as described in Materials and Methods. Points shown for myosin heavy-chain synthesis are averages of two determinations.
per $10^7$ nuclei as multinucleated myotubes (Figure 20, Table 2) is partly based on the assumption that average number of ribosomes per myosin-synthesizing polysome is identical in 24-hr and 72-hr muscle cell cultures, because total run-off of initiated peptide chains was used to measure amount of myosin-synthesizing polysomes in the cells in these two cultures. To test this assumption, total polysomes from 24-hr and 72-hr cultures were fractionated on sucrose density gradients. The gradients were collected in eight different fractions, and the polysomes in each fraction were pelleted and then assayed for ability to incorporate $^3$H-leucine into myosin heavy chains as described in Materials and Methods. As expected (Heywood et al., 1967), polysomes capable of synthesizing myosin were located near the bottom in density gradients of total polysomes prepared from either 24-hr or 72-hr cells (Figures 22 and 23). Average size of the myosin-synthesizing polysomes from 24-hr and 72-hr myogenic cell cultures was similar, although the peak of myosin-synthesizing activity was in density-gradient fraction 3 for polysomes from 24-hr cultures but in density-gradient fraction 2 for polysomes from 72-hr cultures (Figures 22 and 23). These results indicate that mononucleated cells contain at least half as many myosin-synthesizing polysomes per $10^7$ nuclei as multinucleated cells do. Indeed, if, as the data in Figures 22 and 23
Fractionation of total polysomes from 24-hr myogenic cell cultures on a 15-40% sucrose gradient. Density-gradients were fractionated into eight different fractions, and polysomes in each fraction were isolated and assayed for ability to synthesize myosin heavy chains as described in Materials and Methods. Direction of sedimentation is from right to left. Points shown for amount of myosin heavy-chain synthesis are averages of two determinations.
24 HOUR CULTURES

![Graph showing absorbance at 260nm and 3H-LEU dpm's in myosin vs fraction number.](image-url)
Figure 23. Fractionation of total polysomes from 72-hr myogenic cell cultures on a 15-40% sucrose gradient. Density-gradients were fractionated into eight different fractions, and polysomes in each fraction were isolated and assayed for ability to synthesize myosin heavy chains as described in Materials and Methods. Direction of sedimentation is from right to left. Points shown for myosin heavy-chain synthesis are averages of two determinations.
72 HOUR CULTURES

ABSORBANCE AT 260nm

3H-LEU dpm's IN MYOSIN

FRACTION NUMBER

1 2 3 4 5 6 7 8
suggest, the average number of ribosomes per myosin-synthesizing polysome is actually slightly less in mononucleated cells than it is in multinucleated cells, then the procedure for measuring myosin-synthesizing polysomes quantitatively by measuring run-off of initiated peptide chains would underestimate the number of myosin-synthesizing polysomes in mononucleated cells.

Because myosin polysomes in myogenic cells after 24-hr and 72-hr in culture had slightly different numbers of ribosomes per myosin mRNA, it seemed possible that blocking termination with the antibiotic, trichodermin (Stafford and McLaughlin, 1973) might result in "stacking" of ribosomes to maximum capacity on myosin mRNA. This stacking would eliminate any inherent differences in the number of ribosomes per myosin mRNA. Moreover, it seemed possible that stacking ribosomes on the myosin mRNA might result in a sharper distribution of myosin polysomes on sucrose density gradients and thereby permit a substantial purification of myosin polysomes from polysomes of other sizes also present in the total polysome extracts.

To test the effect of trichodermin on protein synthesis in myogenic cells, 76-hr cultures were pulse-labeled with $^3$H-leucine for 2 hr in the presence of several different levels of trichodermin, total protein was precipitated with 5% TCA, and radioactivity in the TCA-insoluble
protein was measured. The resulting incorporation of $^3$H-leucine into all proteins precipitated by 5% TCA at each concentration of trichodermin was expressed as a percentage of incorporation in the control cultures (Figure 2). Less than 1 µg trichodermin/ml caused 50% inhibition of protein synthesis, and 12 µg trichodermin/ml caused 95% inhibition (Figure 2). In all subsequent experiments, 12 µg/ml of trichodermin was used to inhibit protein synthesis.

The distribution of polysomes isolated from both control and trichodermin-treated myotubes (Figures 25 and 26) indicates that even though total protein synthesis is 95% inhibited (Figure 2), polysomes from the trichodermin-treated 72-hr myogenic cells are ostensibly intact (Figure 26). Indeed, a greater proportion of total ribosomes seems to be attached to mRNA's in the form of polysomes in the trichodermin-treated cells than in the control cells because absorbance was consistently higher on gradients of trichodermin-treated cells than on gradients of control cells. Trichodermin treatment, however, causes no obvious increase in proportion of large polysomes that sediment farther into the gradient (cf. Figures 25 and 26).

Seven fractions from both the control and trichodermin-treated gradients were collected and assayed in vitro for ability to incorporate $^3$H-leucine into myosin
Figure 24. Percentage inhibition of total protein synthesis in 76-hr myogenic cell cultures by different levels of the antibiotic, trichodermin. Cultures were pulse labeled with $^3$H-leucine, and the radioactivity insoluble in 5% TCA was determined as described in Materials and Methods.
Figure 25. Fractionation of total polysomes from 76-hr myogenic cell cultures on a 15-40% sucrose gradient. Density-gradients were fractionated into seven different fractions, and polysomes in each fraction were isolated and assayed for ability to synthesize myosin heavy chains as described in Materials and Methods. Direction of sedimentation is from right to left. Points shown for myosin heavy-chain synthesis are averages of two determinations.
A graph showing absorbance at 260 nm against fraction number with peaks at 40% sucrose and a rise in absorbance at 3% sucrose. The x-axis represents fraction number ranging from 1 to 7, and the y-axis represents absorbance.
Figure 26. Fractionation on a 15-40% sucrose gradient of total polysomes from 76-hr myogenic cell cultures treated with 12 μg/ml trichodermin for 0.5 hr prior to polysome isolation. Density-gradients were fractionated into seven different fractions, and polysomes in each fraction were isolated and assayed for ability to synthesize myosin heavy chains as described in Materials and Methods. Direction of sedimentation is from right to left. Points showing myosin synthesis are averages of two determinations.
wood chains. Trichodermin treatment produced, at best, only a slight sharpening of the distribution of polysomes having ability to incorporate $^3H$-leucine into myosin heavy chains compared to the distribution of these polysomes from control cells (cf. Figures 25 and 26). Trichodermin also caused no detectable shift of the myosin polysomes toward the bottom of the sucrose density gradient (cf. Figures 25 and 26). Moreover, total myosin-synthesizing capacity (i.e., the sum of the myosin dpm's from all 7 fractions) of the trichodermin-treated cultures was only 60% of the total myosin-synthesizing capacity of the control cultures. This finding suggests that binding of trichodermin to muscle polysomes is not as reversible as it is for yeast polysomes (Stafford and McLaughlin, 1973). Because it did not cause ribosomes to accumulate on myosin mRNA to the capacity of myosin mRNA to bind to ribosomes, and because it inhibited completion of nascent chains, trichodermin was not useful in standardizing the number of ribosomes per myosin mRNA in 24-hr and 72-hr muscle cultures.

As indicated earlier, the 72-hr myogenic cell cultures contained approximately 1.5 times more total polysomes than the 24-hr cell cultures (Figure 3 and left column, Table 3), and no more than two times more myosin-synthesizing polysomes than 24-hr myogenic cell cultures (Figures 20-23 and
Table 3. Total polysome content and myosin heavy chain synthesis per unit of polysomes by polysomes from 24-hr and 72-hr muscle cell cultures.

<table>
<thead>
<tr>
<th>Culture age</th>
<th>mg total polysomes/10^7 nuclei</th>
<th>^H-leucine dpm's in myosin heavy chains/mg of total polysomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hr</td>
<td>0.242</td>
<td>2,058</td>
</tr>
<tr>
<td>72 hr</td>
<td>0.370</td>
<td>2,470</td>
</tr>
</tbody>
</table>

Polysome content was measured as described in Materials and Methods.

Values in this column were obtained by dividing the numbers in the left column of Table 2 by the numbers in the left column of Table 3.

These data can be used to estimate what proportion of total polysomes in 24-hr and 72-hr cultures is myosin-synthesizing polysomes. The results of this calculation show that the proportion of myosin-synthesizing polysomes relative to total polysomes is only 1.2 times greater in 72-hr, multinucleated myogenic cell cultures than it is in 24-hr, mononucleated myogenic cell cultures (right column, Table 3). Yet, rate of myosin heavy-chain synthesis is approximately 7 times higher in 72-hr myogenic cell cultures than in 24-hr cell cultures (Figure 2). Consequently, cell fusion and the onset of bulk synthesis of myosin heavy chains is not accompanied
by a dramatic increase in either proportion of myosin-synthesizing polysomes relative to total polysomes or total amount of myosin-synthesizing polysomes. Whether this ostensible disparity between rate of myosin heavy-chain synthesis and amount of myosin-synthesizing polysomes indicates that myosin polysomes in multinucleated cells have a greater translation rate is presently unknown.

As discussed in the Literature Review, and in contrast to the data presented in this dissertation, Strohman and coworkers have reported that prefusion cultures of embryonic chicken myoblasts contain no myosin mRNA (Pryzbyla et al., 1973; Pryzbyla and Strohman, 1974; Strohman et al., 1974). Careful evaluation revealed that the procedure used by Strohman and coworkers to prepare polysomes for mRNA extraction differed from the procedure used in the present study in a way that could possibly explain the disparity in results. The polysome extraction procedure outlined in Materials and Methods of this dissertation involves cell lysis by dissolving the outer cell membrane with 0.5% Triton X-100, sedimentation of nuclei and mitochondria at 12,000 x g_max for 10 min, and assay of the polysomes remaining in the supernatant for ability to incorporate radioactivity into myosin heavy chains. The polysome extraction procedure used by Pryzbyla and Strohman
(1974), however, involves disrupting cell membranes by homogenization with a loosely fitting Dounce homogenizer followed by centrifugation at 10,000 x $g_{max}$ for 10 min. The supernatant from this centrifugation was then used as a source of polysomes for isolation of mRNA. Membrane-bound polysomes can be sedimented at 10,000 x $g_{max}$ (Diegelmann et al., 1973; Venkatesan and Steele, 1972), and if myosin-synthesizing polysomes in prefusion myoblasts were associated with membranes, Strohman and co-workers would have discarded most of the myosin-synthesizing polysomes in prefusion myogenic cells in the pellet sedimented at 10,000 x $g_{max}$. Membrane-bound ribosomes are known to be active in synthesis of proteins that are to be secreted from the cell (Diegelmann et al., 1973; 0'Toole and Pollack, 1974; Shafritz, 1974). The possibility that myosin heavy chains are either synthesized on membrane-bound ribosomes for intracellular use or that prefusion myoblasts secrete myosin was intriguing enough to warrant a careful investigation into whether myosin-synthesizing polysomes are membrane-bound in these cells. Furthermore, conclusively demonstrating that myosin-synthesizing polysomes in mononucleated myoblasts are membrane-bound would clarify a major discrepancy in published accounts of myosin mRNA synthesis in prefusion muscle cultures.

Muscle cell cultures were fractionated by two procedures
designed to determine whether myosin-synthesizing polysomes are membrane-bound intracellularly. In the first procedure, 24-hr and 72-hr muscle cell cultures were homogenized with a tightly fitting Dounce homogenizer (B pestle) so that membrane-bound ribosomes would not be dissociated from the membranes. The cell lysate was centrifuged at $700 \times g_{\text{max}}$ for 10 min to sediment nuclei as well as any intact cells (Figure 27). The supernatant from this centrifugation was centrifuged at $10,000 \times g_{\text{max}}$ for 10 min to sediment membrane-bound polysomes. Those polysomes sedimenting at $10,000 \times g_{\text{max}}$ as well as free polysomes not sedimenting at $10,000 \times g_{\text{max}}$ were then pelleted as outlined in Figure 27 and assayed for ability to incorporate $^3\text{H}$-leucine into myosin heavy chains by using the cell-free protein-synthesizing assay described in Materials and Methods. In the second treatment, 24-hr and 72-hr muscle cell cultures were homogenized very gently with a loosely fitting Dounce homogenizer (A pestle) in 0.5% Triton X-100 to dissolve endoplasmic reticular membranes and therefore release membrane-bound ribosomes (Figure 27). The cell lysate was then treated as above, and the polysomes sedimenting at either $10,000 \times g_{\text{max}}$ or at $150,000 \times g_{\text{max}}$ were assayed for ability to incorporate $^3\text{H}$-leucine into myosin heavy chains. Measurement of myosin-synthesizing polysomes from 24-hr and 72-hr muscle cells
Figure 27. Flow sheet showing the fractionation scheme developed for localization of myosin-synthesizing polysomes as either free or membrane-bound in 24-hr and 72-hr muscle cultures. See text for explanation of rationale used in developing this fractionating procedure. Polysomes sedimenting at 105,000 x $g_{max}$ in each fraction were assayed for ability to synthesize myosin heavy chains as described in Materials and Methods.
I. Muscle cell cultures
   (a) Cells scraped from culture dishes were lysed either by homogenization 5 times in Dounce homogenizer (B pestle) in 0.25 M KCl, 0.01 M MgCl₂, 0.01 M Tris, pH 7.4, or by gentle homogenization 5 times in Dounce homogenizer (A pestle) in the same buffer containing 0.5% Triton X-100.
   (b) Centrifuge at 700 x g max for 10 min.

A. Sediment
   (Discard)

II. Supernatant
   (a) Centrifuge at 10,000 x g max for 10 min.

B. Sediment
   (1) Resuspend in 0.5% Triton X-100, 0.25 M KCl, 0.01 M MgCl₂, 0.01 M Tris, pH 7.4, by gentle homogenization 5 times in Dounce homogenizer (A pestle).
   (2) Centrifuge at 150,000 x g max for 2 hr through 1.5 M sucrose, 0.25 M KCl, 0.01 M MgCl₂, 0.01 M Tris, pH 7.4.

C. Sediment
   (membrane-bound ribosomes)
   (1) Assayed for myosin-synthesizing polysomes.

D. Sediment
   (Discard)

III. Supernatant
   (a) Centrifuge at 150,000 x g max for 2 hr through 1.5 M sucrose, 0.25 M KCl, 0.01 M MgCl₂, 0.01 M Tris, pH 7.4.

IV. Supernatant
   (Discard)

B. Sediment
   (free ribosomes)
   (1) Assayed for myosin-synthesizing polysomes.
fractionated by this procedure indicated that only a very small percentage of myosin-synthesizing polysomes are membrane-bound in either 24-hr or 72-hr cells (Table 4).

Although the above experiments suggest that most myosin-synthesizing polysomes in nonfused muscle cells are not membrane-bound, fractionation by the very simple scheme in Figure 27 is based on two assumptions that, if invalid, could seriously affect the results. First, it is assumed that centrifugation of the cell lysate at 700 x $g_{\text{max}}$ sediments none of the membrane-bound polysomes. This assumption is not completely justified (Venkatesan and Steele, 1972), because fragments of endoplasmic reticulum are continuous with the nuclear membrane and these fragments are pelleted along with nuclei. Second, it is assumed that centrifugation at 10,000 x $g_{\text{max}}$ quantitatively sediments membrane-bound polysomes. Although it is true that large fragments of rough endoplasmic reticulum can be sedimented at such centrifugal forces (Diegelmann et al., 1973), higher forces are generally employed (O'Toole and Pollak, 1974; Shafritz, 1974).

Because of the two questionable assumptions discussed in the preceding paragraph, and because of the significance of a precise answer as to whether myosin-synthesizing polysomes are associated with membranes in mononucleated myoblasts, a more rigorous cellular fractionation scheme
Table 4. Percentage of myosin-synthesizing polysomes sedimenting at 10,000 \( x g_{\text{max}} \) or at 150,000 \( x g_{\text{max}} \) from 24-hr and 72-hr muscle cultures after cell lysis by homogenization or by treatment with 0.5% Triton X-100a.

<table>
<thead>
<tr>
<th>Pellet</th>
<th>% myos-synthesizing polysomes in each pelletb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24-hr cultures</td>
</tr>
<tr>
<td></td>
<td>Homogenization</td>
</tr>
<tr>
<td>10,000 ( x g_{\text{max}} )</td>
<td>3.2</td>
</tr>
<tr>
<td>150,000 ( x g_{\text{max}} )</td>
<td>97.8</td>
</tr>
</tbody>
</table>

Cells from 24-hr and 72-hr muscle cultures were lysed either by homogenization with five strokes of a Dounce homogenizer (B pestle) or by using 0.5% Triton X-100 and gentle homogenization, and polysomes were prepared as shown in Figure 27. Polysomes sedimenting at 10,000 \( x g_{\text{max}} \) for 10 min or at 15,000 \( x g_{\text{max}} \) for 2 hr were assayed for ability to incorporate 3H-leucine into myosin heavy chains as described in Materials and Methods.

\[ \% \text{myos-synthesizing polysomes} = \frac{\text{dpm's incorporated into myosin heavy chains by polysomes in either pellet}}{\text{sum of dpm's incorporated into myosin heavy chains by polysomes in both pellets}} \times 100. \]

(Figure 28) was adapted from the very complex fractionation procedures described by O'Toole and Pollack (1974) and Venkatesan and Steele (1972). Muscle cells were homogenized in the absence of Triton X-100 with a Dounce homogenizer (B pestle), and the homogenate was centrifuged at 12,000 \( x g_{\text{max}} \) for 10 min. The 12,000 \( x g_{\text{max}} \)-pellet was
Figure 28. Flow sheet showing the fractionation scheme developed for quantitative localization of myosin-synthesizing polysomes as either free or membrane-bound in 24-hr and 72-hr muscle cell cultures. Rationale behind this fractionation scheme is discussed in text. Polysomes sedimenting in each fraction were assayed for ability to synthesize myosin heavy chains as described in Materials and Methods.
I. Muscle cell cultures
(a) Cells scraped from culture dishes were lysed by homogenization 5 times with Dounce homogenizer (B pestle) in 0.25 M KCl, 0.01 M MgCl₂, 0.01 M Tris, pH 7.4.
(b) Centrifuge at 12,000 x $g_{\text{max}}$ for 10 min.

A. Sediment
(1) Resuspend in 0.25 M KCl, 0.01 M MgCl₂, 0.01 M Tris, pH 7.4, by aspirating with Pasteur pipet.
(2) Homogenize 5 times with Dounce homogenizer (B pestle).
(3) Centrifuge at 12,000 x $g_{\text{max}}$ for 10 min.

B. Sediment
(1) Resuspend in 0.5% Triton X-100, 0.25 M KCl, 0.01 M MgCl₂, 0.01 M Tris, pH 7.4, by aspirating with Pasteur pipet.
(2) Homogenize gently 5 times with Dounce homogenizer (B pestle).
(3) Centrifuge at 12,000 x $g_{\text{max}}$ for 10 min.

V. Supernatant
(a) Centrifuge at 105,000 x $g_{\text{max}}$ for 16 hr through 2.0 M sucrose, 0.25 M KCl, 0.01 M MgCl₂, 0.01 M Tris, pH 7.4.

D. Sediment
(membrane-bound ribosomes)
(1) Assayed for myosin-synthesizing polysomes.

VI. Supernatant
(Discard)
III. Supernatant
(a) Mix thoroughly.
(b) Divide into two equal parts.

IIIa. Supernatant
(a) Add Triton X-100 to final concentration of 0.5%.
(b) Homogenize gently 5 times with Dounce homogenizer (A pestle).
(c) Centrifuge at 105,000 × g_{max} for 16 hr through 2.0 M sucrose,
0.25 M KCl, 0.01 M MgCl₂, 0.01 M Tris, pH 7.4.

IIIb. Supernatant
(a) Homogenize gently 5 times with Dounce homogenizer (A pestle).
(b) Centrifuge at 105,000 × g_{max} for 16 hr through 2.0 M sucrose,
0.25 M KCl, 0.01 M MgCl₂, 0.01 M Tris, pH 7.4.

E. Sediment
IVa. Supernatant F.
(membrane-bound and free ribosomes)
(1) Assayed for myosin-synthesizing polysomes.

(Discard)

IVb. Supernatant (free ribosomes)
(1) Assayed for myosin-synthesizing polysomes.

(Discard)

Total free ribosomes = 2(F)
Total membrane-bound ribosomes = 2(E - F) + D

Figure 28 (Continued)
resuspended and again homogenized to insure complete cell rupture in the absence of detergent. This second homogenate was also centrifuged at 12,000 x $g_{\text{max}}$ for 10 min. The material sedimenting under these conditions should contain nuclei, mitochondria, and a portion of the membrane-bound ribosomes (largely those attached to nuclei). Treatment of this pellet with 0.5% Triton X-100 should dissolve endoplasmic reticular membranes and, therefore, free all ribosomes into solution. Centrifugation again at 12,000 x $g_{\text{max}}$ sediments only nuclei and mitochondria, whose membranes are not solubilized by Triton X-100, and the supernatant contains only those ribosomes which were formerly membrane-bound. These ribosomes were then sedimented at 105,000 x $g_{\text{max}}$ for 16 hr through 2.0 M sucrose and assayed for ability to incorporate $^3$H-leucine into myosin heavy chains (Figure 28).

The two supernatants from the 12,000 x $g_{\text{max}}$ centrifugation of the initial cell homogenate and the reextraction of the pellet sedimented from this initial homogenate (Figure 28) were combined and thoroughly mixed. These combined supernatants contain all free polysomes as well as all membrane-bound polysomes that do not sediment at 12,000 x $g_{\text{max}}$ for 10 min. The free and membrane-bound polysomes in these combined supernatants can be distinguished by taking advantage of the fact that membrane-
bound polysomes will not sediment through 2.0 M sucrose at 105,000 \times g_{\text{max}} for 16 hr, whereas free polysomes and ribosomes will sediment under these conditions. Therefore, the thoroughly mixed 12,000 \times g_{\text{max}} -supernatants were carefully divided into two equal aliquots, and Triton X-100 was added at a final concentration of 0.5% to one of these aliquots to solubilize all membranes. Free polysomes were then sedimented from both these aliquots at 105,000 \times g_{\text{max}} for 16 hr through 2.0 M sucrose (Figure 28). Thus, the pellet obtained from the aliquot of the 12,000 \times g_{\text{max}} -supernatant that had been treated with Triton X-100 contained both polysomes that had originally been free in the initial cell homogenate as well as those polysomes solubilized from small fragments of endoplasmic reticulum. On the other hand, the polysome pellet obtained from the aliquot of the 12,000 \times g_{\text{max}} -supernatant that had not been treated with Triton X-100 contained only those polysomes which had originally been free in the cytoplasm of the cells because membrane-bound polysomes would not sediment through 2.0 M sucrose. Any difference between the free plus membrane-bound polysomes and the free polysomes in amount of myosin heavy-chain synthesis supported should be due to myosin synthesized by membrane-bound polysomes. The expressions given at the bottom of Figure 28 can be employed to calculate the proportion of
myosin-synthesizing polysomes that are free and membrane-bound.

The results of several experiments done according to the procedure described in Figure 28 clearly indicate that only a very small proportion of myosin-synthesizing polysomes are membrane-bound in either 24-hr or 72-hr muscle cell cultures (Table 5). The existence of a significant amount of membrane-bound myosin-synthesizing polysomes in 24-hr muscle cell cultures, therefore, cannot be invoked to explain the inability of Strohman and coworkers to isolate from extracts of mononucleated myoblasts a mRNA that will code for myosin heavy chains in a reticulocyte cell-free assay (Pryzbyla et al., 1973; Pryzbyla and Strohman, 1974; Strohman et al., 1974). Consequently, the discrepancy between the results obtained in the present study and by other workers (Buckingham et al., 1974; Morris et al., 1972; Stockdale and O'Neill, 1972; Rubenstein et al., 1974) and the findings reported by Strohman and coworkers remains unexplained.

Based on existing knowledge of the mechanism of protein synthesis, the dramatic activation of myosin heavy-chain synthesis immediately after myoblast fusion must occur at either the transcriptional or the translational level, or at both of these levels. If myosin heavy-chain synthesis is regulated exclusively at the transcriptional
level, concentration of myosin mRNA should be very low in mitotically dividing myogenic cells, but should increase rapidly soon after initiation of myoblast fusion. On the other hand, if myosin heavy-chain synthesis is regulated exclusively at the translational level, concentration of myosin mRNA should be the same in mitotically dividing myoblasts and in nondividing, multinucleated myotubes, and the large increase in rate of myosin heavy-chain synthesis observed immediately after fusion would originate entirely from an increase in rate of translation of existing myosin mRNA molecules.
The results of the studies described in this dissertation suggest that rate of myosin heavy-chain synthesis during muscle cell differentiation is controlled at both the transcriptional and translational levels. The finding that myosin polysomes exist in prefusion, mitotically dividing myogenic cells and that amount of myosin polysomes in myogenic cells increases less than twofold during fusion of mononucleated myoblasts into multinucleated myotubes (Table 2) whereas rate of myosin heavy-chain synthesis increases approximately sevenfold during this same period (Figure 2) clearly demonstrates that myosin heavy-chain synthesis is not controlled solely at the transcriptional level during muscle cell differentiation. Because free myosin mRNA containing no bound ribosomes was not assayed in the present study, it is impossible to determine whether regulation of myosin heavy-chain synthesis during muscle cell differentiation occurs largely or exclusively at the translational level. It is conceivable, for example, that mononucleated myoblasts contain large amounts of free myosin mRNA that has no bound ribosomes, and that fusion results in binding of ribosomes to this free myosin mRNA to produce myosin polysomes. Hence, the amount of total myosin mRNA (free and containing bound ribosomes) might be identical in mononucleated myoblasts and multinucleated myotubes, but the binding of ribosomes to a pool of free
myosin mRNA in myoblasts could cause the twofold increase in amount of myosin polysomes observed in the present study. On the other hand, the data in Table 3 of the present study show that proportion of myosin polysomes to total polysomes changes very little during fusion of myoblasts that are synthesizing little myosin to multinucleated myotubes that are synthesizing large amounts of myosin, and it is not clear why proportion of myosin polysomes to total polysomes would not increase substantially if fusion resulted only in aggregation of ribosomes to preexisting myosin mRNA without an increase in the amount of myosin mRNA. The ability to reach definite conclusions concerning the quantitative amounts of myosin heavy chains and myosin mRNA in prefused and fused myogenic cells is further complicated by the lack of information on turnover rates of myosin heavy chains and myosin mRNA in prefused and fused cells.

The conclusions of this dissertation are supported by several previous studies that have also reported that rate of myosin synthesis during the initial stages of differentiation of muscle cells in culture is not dictated solely by the concentration of myosin mRNA, but that some degree of translational control must also be involved, although a few details in some of the earlier studies seemingly disagree with the findings reported in this dissertation.
Yaffe and Dym (1972) have found that myoblast fusion is accompanied by a moderate increase in the rate of myosin heavy chain synthesis even when the antibiotic, actinomycin D, is administered several hours before initiation of fusion. Actinomycin D is a potent inhibitor of RNA polymerase, and the presence of actinomycin D prevents synthesis of mRNA. Hence, any protein synthesis that occurs after administration of actinomycin D must be directed by preexisting mRNA. Yaffe and Dym's results, therefore, indicate that some myosin mRNA exists in prefusion myoblasts and that either the rate of translation or the stability of this mRNA increases immediately after fusion. Recent RNA-DNA hybridization studies (Buckingham et al., 1974) also substantiate the suggestion that both transcriptional and translational controls are involved in regulating the burst of myosin synthesis that follows myoblast fusion. DNA complementary to fetal calf myosin mRNA was synthesized using reverse transcriptase, and this complementary DNA was then used to measure the rate of 26S, presumptive-myosin mRNA synthesis in cultures of fetal calf muscle cells at different stages of differentiation by hybridizing RNA isolated from these cultures in the presence of excess complementary myosin DNA (Buckingham et al., 1974). These experiments showed that presumptive myosin mRNA is synthesized at approximately the same rate in
mononucleated myoblasts and multinucleated myotubes and that the half-life of this mRNA increased about fivefold during differentiation. Consequently, both Yaffe and Dym (1972) and Buckingham et al. (1974) agree with the results presented in this dissertation (Table 2) demonstrating that mononucleated, prefusion muscle cells contain myosin mRNA.

In contrast to the results shown in this dissertation, however, Buckingham et al. (1974) found that presumptive myosin mRNA isolated from mononucleated fetal calf muscle cells was not associated with polysomes on sucrose density gradients but instead was free in the cytoplasm of the cells. Approximately the same time that fusion started, myosin mRNA could be isolated both from fractions very near the top of gradients and from fractions associated with polysomes in the lower third of the gradients. The reason for the discrepancy between this finding by Buckingham et al. (1974) and the results presented in Figure 22 of this dissertation that indicate that at least a portion of myosin mRNA in mononucleated myoblasts is associated with large polysomes is not known. Yaffe and Dym's results (Yaffe and Dym, 1972) with actinomycin D do not differentiate between free myosin mRNA and myosin mRNA containing bound polysomes, but simply require that some myosin mRNA be present in prefusion myoblasts. Hence, Yaffe and Dym's
results are compatible with both those presented in this dissertation and those found by Buckingham et al. (1974). Heywood and coworkers (Heywood, 1970b; Heywood et al., 1974; Kennedy et al., 1974; Thompson et al., 1973) have proposed that myosin heavy-chain synthesis is regulated at initiation of ribosomes onto the myosin mRNA. The results in Figure 22 of this dissertation, however, show that at least some of the myosin mRNA's in mononucleated myoblasts are associated with ribosomes to form polysomes which will complete nascent chain synthesis in a cell-free protein-synthesizing assay. Consequently, the presence of myosin polysomes in prefusion myoblasts is also inconsistent with the hypothesis that the final controlling step in myogenesis is synthesis of messenger-specific initiation factors. Indeed, the results described in this dissertation imply that rate of translation of myosin mRNA is slower in mononucleated myoblasts than it is in multinucleated myotubes. As indicated previously, the techniques used in this study would not measure myosin mRNA not associated with ribosomes, and it is impossible, therefore, to determine from the present results whether a fraction of myosin mRNA in embryonic chicken mononucleated muscle cells is not associated with ribosomes, and hence, whether some regulation might actually exist at the initiation level of protein synthesis. It should be pointed out
in this discussion that a large number of recent studies indicate strongly that myosin is used for a number of cell movement functions in addition to muscle contraction, and that all animal cells therefore must contain some myosin, although the amounts might be much less than that contained by muscle cells. If so, then mononucleated myoblasts cannot be devoid of myosin polysomes but must contain sufficient levels of such polysomes to permit synthesis of the low levels of "nonmuscle myosin" required by these cells. In this view then, the function of any regulation by myosin messenger-specific initiation factors would be in control of the bulk synthesis of myosin associated with muscle cells. Hence, the suggestion of Heywood and coworkers (Heywood, 1970b; Heywood et al., 1974; Kennedy et al., 1974; Thompson et al., 1973) that synthesis of myosin is regulated by messenger-specific initiation factors is not incompatible with the results of the present study showing that mononucleated myoblasts contain myosin polysomes. Comparison of results from the three studies discussed in this paragraph are further complicated because muscle cells from three different species were used: fetal calf by Buckingham et al. (1974), fetal rat by Yaffe and Dym (1972), and embryonic chick in the present study. With exception of the results of Strohman and coworkers (Pryzbyla et al., 1973; Pryzbyla and Strohman, 1974;
Strohman et al., 1974), however, there is general consensus that mononucleated myoblasts contain myosin mRNA.

Although little conclusive information is yet available on factors regulating rate of myosin synthesis in differentiating muscle cell cultures, even less information is available on factors regulating synthesis of muscle proteins other than myosin. Polysomes responsible for synthesis of tropomyosin (Heywood and Rich, 1968), actin (Heywood and Rich, 1968; Paterson et al., 1974), and myosin light chains (Low et al., 1967) have been tentatively identified, but have not been extensively studied. Paterson et al. (1974) have compared the relative rates of actin synthesis in cultures of embryonic chicken muscle cells with the relative amounts of actin mRNA in these cells and have tentatively concluded, in contrast to the results discussed above for myosin, that rate of actin synthesis depends directly on concentration of actin mRNA present in the cells at all stages of differentiation.

In conclusion, although it is clear that a great deal of biochemical differentiation must occur in mononucleated myogenic cells before the onset of morphological differentiation in the form of fusion of mononucleated cells into multinucleated myotubes, it is also manifestly clear that basic knowledge about how developmental pathways of
myogenic cells are integrated so that functional muscle fibers are produced is still very rudimentary.
Mononucleated myoblasts and multinucleated myotubes were obtained by culturing embryonic chicken skeletal muscle cells. Comparison of total polysomes isolated from these mononucleated and multinucleated cell cultures by density-gradient centrifugation and electron microscopy revealed that mononucleated myoblasts contain polysomes similar to those contained by multinucleated myotubes and large enough to synthesize the 200,000-dalton subunit of myosin. When placed in an in vitro protein-synthesizing assay containing $^3$H-leucine, total polysomes from both mononucleated and multinucleated myogenic cultures were active in synthesizing myosin heavy chains as detected by measurement of radioactivity in slices through the myosin band on SDS-polyacrylamide gels. Fractionation of total polysomes on sucrose density-gradients showed that myosin-synthesizing polysomes from mononucleated myoblasts may be slightly smaller than myosin-synthesizing polysomes from myotubes. Multinucleated myotubes contain approximately two times more myosin-synthesizing polysomes per unit of DNA than mononucleated myoblasts, and the proportion of total polysomes constituted by myosin polysomes is only 1.2 times higher in multinucleated myotubes than it is in mononucleated myoblasts. Because rate of myosin synthesis is seven times higher in multinucleated myotubes than it
is in myoblasts, these results suggest that rate of translation of myosin mRNA is more rapid in multinucleated myotubes than in mononucleated myoblasts. Separation of polysomes into membrane-bound and free fractions indicate that myosin-synthesizing polysomes are free in both mononucleated and multinucleated myogenic cells. The results of this study show that mononucleated myoblasts contain significant amounts of myosin messenger RNA before the burst of myosin synthesis that accompanies muscle differentiation and that a portion of this messenger RNA is associated with ribosomes to form polysomes that will actively translate myosin heavy chains in an in vitro protein-synthesizing assay.
VI. CONCLUSIONS

As a result of this study, the following conclusions are justified.

1. Cultures of embryonic skeletal muscle cells used in this study were indistinguishable from those reported by other workers when evaluated on the basis of growth rates, fusion kinetics, myosin synthesis rate, ribosome accumulation, and ability to form contractile myofibers.

2. Mononucleated myoblasts contain polysomes similar to those contained by multinucleated myotubes when compared both by distribution of polysomes after sucrose density-gradient centrifugation and by electron microscope determination of the number of ribosomes present in the large polysomes isolated from the lower portion of sucrose density gradients. These large polysomes isolated from both these cell types contain enough ribosomes to be capable of synthesizing a polypeptide as large as the 200,000 dalton subunit of myosin.

3. Total polysomes isolated from both mononucleated and multinucleated myogenic cultures are active in incorporating $^3$H-leucine into 0.025 M KCl-insoluble polypeptides that migrate with a molecular weight indistinguishable from that of the 200,000-dalton subunit of myosin when the
products of cell-free protein-synthesis are subjected to SDS-polyacrylamide gel electrophoresis. The protein-synthesizing assay used for these studies was able to complete synthesis of all the nascent myosin chains that had been initiated on the polysomes before their isolation.

4. Fractionation of total polysomes from nonfused and fused muscle cell cultures on sucrose density gradients into eight fractions followed by assaying the ability of the polysomes in each fraction to incorporate $^3$H-leucine into myosin heavy chains revealed that the average size of myosin-synthesizing polysomes from mononucleated myoblasts is slightly less than that from multinucleated myotubes. This difference in myosin polysome size, however, is not sufficient to prevent reasonable estimates of the relative amounts of myosin-synthesizing polysomes in pre-fused and fused cultures by assuming that the number of ribosomes per myosin mRNA is identical in mononucleated myoblasts and multinucleated myotubes.

5. Multinucleated myotubes contain approximately two times more myosin-synthesizing polysomes per unit of DNA than mononucleated myoblasts, and the proportion of total polysomes constituted by myosin-synthesizing polysomes is only 1.2 times greater in multinucleated myotubes than it is in mononucleated myoblasts.

6. Because rate of myosin heavy chain synthesis in
cultures of embryonic chicken muscle cells increases approximately sevenfold between 24 and 72 hr in culture, but myosin-synthesizing polysome content increases only two-fold during this same interval, it is concluded that rate of translation of myosin mRNA is greater in multinucleated myotubes than it is in mononucleated myoblasts.

7. Essentially all myosin-synthesizing polysomes in mononucleated myoblasts and in multinucleated myotubes are free in the cytoplasm of the cells rather than associated with membranes.
VII. BIBLIOGRAPHY


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