Encapsulation of “Core” eIF3, Regulatory Components of eIF3 and mRNA into Liposomes, and Their Subsequent Uptake into Myogenic Cells in Culture

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ABSTRACT  Eukaryotic initiation factor 3 (eIF3), encapsulated in liposomes, is taken up by chick muscle cells in culture. The exogenously supplied factor (isolated from 14-d embryonic muscle) rapidly associates with 40S ribosomal subunits and particles sedimenting at 80-120S (the known sedimentation value of myosin heavy chain [MHC] mRNPs). In addition, exogenously supplied eIF3 has a specific stimulatory effect on myofibrillar protein synthesis. This stimulation is most apparent at the onset of cell fusion and after the accumulation of MHC-mRNPs. As previously reported (8), total eIF3 can be fractionated on an MHC-mRNA affinity column into a “core” eIF3 and a high affinity component (HAF) which dictates the discriminatory activity of core eIF3. Liposome-encapsulated core eIF3 delivered to cells is found predominantly in 40S ribosomal subunits and gives only a slight stimulation of total protein synthesis. When 3H-MHC-mRNA, preincubated with HAF, is introduced into myoblasts via liposomes, the mRNA is found in heavy polysomes. On the other hand, when the messenger alone or with core eIF3 is taken up by the cells, it is found only on small polysomes. Similar experiments, using viral RNA with the HAF, show no increase in the size class of polysomes. These results mimic the differences observed between myoblast and myotube utilization of MHC-mRNA previously observed (17). These results demonstrate the mRNA discriminatory activity of specific proteins associated with muscle eIF3 and suggest that these proteins play a role in mRNA activation and translation during muscle differentiation.

In eukaryotic cells the process of initiation has been found to be the rate-limiting step occurring in protein synthesis. Therefore, this process is considered to regulate the utilization of polysomal mRNAs (20). According to the model proposed by Lodish (14), the competition of cytoplasmic mRNAs for initiation factors is a mechanism used by eukaryotic cells to regulate the relative rates of synthesis of proteins. This model predicts that any reduction in the rate of initiation results in a preferential inhibition of the translation of mRNAs with low initiation rate constants (14). The initiation rate constant is a measure of the affinity of the mRNAs for initiation factors, presumably resulting from the primary structure of the initiation region of the mRNAs.

We have suggested that, in addition to the purely competitive model for translational control, eukaryotic initiation factor 3 (eIF3) can be modulated by associated polypeptides and thereby discriminate between mRNAs (8, 9). In this manner an eIF3 containing discriminatory polypeptides would have a higher affinity for particular mRNA(s) and thereby increase the rate of initiation. Such discriminatory proteins (high affinity fraction [HAF]), having a high affinity for myosin heavy chain (MHC)-mRNA have been separated from “core” eIF3 using an MHC-mRNA affinity column (8, 9). The isolated HAF endows either reticulocyte or muscle “core” eIF3 with a discriminatory property such that MHC-mRNA is preferentially translated in a cell-free system containing optimal amounts of eIF3 and equimolar concentrations of MHC and globin mRNAs (8).

In vitro translational assays have generally been used to study translational control mechanisms. Although, certainly of value because of the ability to alter the concentrations of various reactants, cell-free systems can introduce numerous artifacts. The question remains as to the validity of interpretation of the results in these systems as compared to the in vivo situation. By inserting macromolecules into living cells and observing their effects and utilization a comparison of the
results obtained from cell-free systems could be made. We have recently observed that deproteinized mRNA can be taken up by myogenic cells in culture and that MHC-mRNA is differentially translated in myoblasts as compared to myotubes (17). Recent advances in liposome technology, specifically with regard to the use of large unilamellar vesicles (LUV), allow the introduction of macromolecules into cells (22). A wide spectrum of molecules and molecular complexes have been encapsulated into LUV and delivered to cells while still retaining their biological activity (4, 19). Little effect on cell viability is observed upon the incubation of cells with LUV and in certain cases an increase in the activity of LUV-encapsulated substances has been reported (4, 19, 22).

In this report we have used LUV to deliver mRNAs, unfractionated eIF3, “core” eIF3, and HAF to myogenic cells in culture. It has been observed that a substantial proportion of the molecules and macromolecular complexes rapidly associate with the cellular protein synthetic machinery in a specific manner and subsequently alter the utilization of both exogenously added and endogenous mRNA. These results demonstrate the mRNA discriminatory activity of HAF associated with eIF3 and suggest that it plays a role in mRNA utilization during the terminal differentiation of muscle.

MATERIALS AND METHODS

eIF3 was prepared from 14-d embryonic chick leg muscle as previously described (8) except that only a single sucrose density gradient centrifugation was employed to isolate the 15S factor complex. The final gradient was omitted to obtain a higher yield of both eIF3 and HAF. Approx. 40% of the material is lost during the final dialysis, concentration, and recentrifugation steps. Under the procedures used, 400-500 μg of eIF3 is obtained from 600 embryos. The preparation of the MHC-mRNA affinity column and subsequent fractionation of eIF3 into “core” eIF3 and HAF were as previously described (8). Normally 75–100 μg of eIF3 was applied to the MHC-mRNA affinity column. Of this 20–30% did not bind, ~1% eluted as HAF, and the remainder was recovered as core eIF3. Reductive methylation of eIF3 and the affinity column fractions of eIF3 was performed using the Na(CN)BH₃ procedure of Jentoft and Dearborn (11) and MacKean et al. (15). Briefly, the proteins to be labeled were dialyzed against 0.1 M HEPES (pH 7.5), 0.28 M KCl, 5% glycerol, and concentrated to 1 mg/ml using Sephadex G-200. The solution was adjusted to 2 M HCl/maldehyde (New England Nuclear, Boston, Mass.) and 20 mM Na(CN)BH₃, and incubated 16 h at 4°C. MHC-mRNA was prepared as previously described (5) and a sample tested in a reticulocyte lysate before its use in the experiments described in this report. The translation of this mRNA results in the synthesis of a 200,000-dalton protein that is precipitable with anti-MHC antibodies. MHC-mRNA was radioactively labeled by the addition of 20 μCl [3H]thymidine to 24-h muscle cell cultures (see below) and harvested at 48 h.

LUV were prepared according to the procedures of Wilson et al. (25) with the following modifications: 0.2 ml of phosphatidylserine (Sigma Chemical Co., St. Louis, Mo.) was evaporated to dryness under nitrogen. The resultant film was immediately resuspended by brief vortexing in 5 ml of buffer (0.1 M NaCl, 0.002 M histidine, 0.02 M Tris-HCl [pH 7.4]) and 1 h of sonication under nitrogen. The temperature did not exceed 35°C. 0.5 ml of 0.1 M CaCl₂ was added and the solution incubated for 1 h at 37°C. The flocculent precipitate was pelleted by centrifugation at 10,000 rpm for 20 min at 15°C using a Sorvall SS34 rotor. The resulting pellets may be stored at −20°C for up to 4 wk. Material to be encapsulated was dialyzed against the LUV buffer (above) containing 0.001 M MgCl₂. The frozen LUV pellet was slowly thawed and resuspended in 0.5 ml of the material to be encapsulated by utilizing a vortex mixer. After the addition of 25 μl of neutralized 0.1 M EGTA the samples were rapidly mixed and incubated at 37°C for 30 min. The resulting vesicles containing the encapsulated material were collected by centrifugation at 14,000 rpm for 30 min at 15°C. These vesicles were washed two times in the 0.1 M NaCl buffer containing 0.001 M MgCl₂ followed by centrifugation. Finally, the loaded vesicles were resuspended in 0.5 ml serum-free medium for addition to the cell cultures. After encapsulation and washing, the radioactivity remaining with the pelleted vesicles is considered encapsulated and expressed as the percent total radioactivity in the original 0.5 ml of buffer.

Breast muscle cell cultures, obtained from 12-d chick embryos, were prepared as previously described (23). Briefly, pectoralis muscle was mechanically dissociated and the cells were plated at an initial density of 6 × 10⁴/100-mm culture dish. The cells were grown on F-10 medium (Gibco Diagnostics, Gibco Invgenex Div., Chagrin Falls, Ohio) supplemented with 3% chick embryo extract and 10% horse serum. The cultures were refed after 24 h. Before addition of liposomes to the cells, the cultures were washed four times in serum-free medium. The liposomes were added in enough serum-free medium (2 ml) to cover the cells and incubated for 3 h (except where noted). In experiments in which protein synthesis is measured, 5 μCi [³H]lysine was added to each culture immediately after the addition of LUV. In this case, each data point consists of 15 cell cultures. After incubation the cells were washed extensively with PBS and harvested using a rubber policeman at 4°C in 0.15 M KCl, 0.005 M MgCl₂, 0.02 M Tris-HCl (pH 7.4), 0.008 M β-mercaptoethanol. The cells were lysed as previously described (16).

The cellular localization of eIF3 and the MHC-mRNA affinity column fractions of eIF3 was performed by sucrose density gradient analysis of the 10,000 g supernatant from the cultured cells. These experiments were repeated a minimum of five times and found to be highly reproducible in both the localization of LUV-inserted materials and the amount of recoverable radioactivity. The 10,000 g cellular supernatates were analyzed on 10–30 wt/wt sucrose gradients containing 0.15 M KCl, 0.005 M MgCl₂, 0.02 M Tris-HCl (pH 7.4), 0.008 M β-mercaptoethanol by centrifugation at 40,000 rpm for 2 h in an IEC SB283 rotor (Damon/IEC Div., Damon Corp., Needham Heights, Mass.). The sucrose density gradients were analyzed by continual monitoring at 260 nm using a spectrophotometer (Gillford Instrument Laboratories Inc., Oberlin, Ohio). Fractions were collected and the radioactivity was determined directly for dpm using a Searle Mark III Liquid Scintillation System (Searle Diagnostics Inc., subisd. of G. D. Searle & Co., Des Plaines, Ill.) in which background was subtracted.

Radioactivity associated with myofibrillar proteins was determined after isolation by multiple ionic precipitation as previously described (21). SDS electrophoresis, and fluorography. Radioactivity of total cellular protein and nonmyofibrillar proteins was determined after TCA precipitation by the method of Bensadoun and Weinstein (2).

Gel electrophoresis was performed according to Laemmli (12) on 7.5–12% polyacrylamide gels. Fluorography was done as described by Laskey and Mills (13). The pre-exposure of the film using a Kodak deep orange filter allows quantitative analysis of fluorograms. The fluorographic images were subsequently analyzed with a microdensitometer (Joyce, Loebi & Co., Ltd., Gateshead-on-Tyne, England).

RESULTS

If a dilute aqueous solution of phosphatidylserine is sonicated in the presence of Ca²⁺, coelate cylinders are formed. Upon the removal of Ca²⁺ by a chelating agent the cylinders are transformed into LUV. Macromolecules as well as macromolecular complexes present in solution during this transformation are passively encapsulated and can subsequently be introduced into cells in culture (22). By use of this technique, the efficiency of both entrainment and subsequent uptake by myogenic cells of ¹⁴C-labeled proteins has been determined. As shown in Table I, there is no apparent correlation between either the molecular size or complexity and the entrainment or cellular uptake of the proteins tested. The lower entrainment of myofibrillar proteins is likely a result of the solubility properties

| Material entrapped | % Entrapped | Myoblast | Myotube |
|--------------------|-------------|----------|---------|
| Myoglobin           | 48          | 2.5      | 6.5     |
| Thyroglobulin       | 39          | 4.2      | 7       |
| Myofibrillar proteins| 14.5       | 1.2      | 2.8     |
| eIF3                | 28          | 2.2      | 4.1     |

All proteins were radioactively labeled by reductive methylation with [¹⁴C]formaldehyde as described in Materials and Methods. The percent entrainment and cellular uptake are expressed relative to total radioactivity present in the 0.5 ml encapsulation volume (1 × 10⁶–1.5 × 10⁷ dpm). The cultures designated as myoblasts are 30-h cell cultures while those designated as myotubes are 60-h cultures.
of these proteins in the buffer used in LUV preparation. The results indicate that 60-h muscle cell cultures, consisting primarily of differentiating myotubes, can more readily incorporate proteins from LUV than can myoblasts (30 h of culture). This is likely a result of the increased membrane fluidity of myotubes as compared to myoblasts (7). It has been reported that cells with greater membrane fluidity are more responsive to the uptake of materials from liposomes (18). To determine whether the exogenously supplied proteins were significantly altered after uptake into the myogenic cells, the cells were lysed after 3 h of incubation with LUV-encapsulated 14C-labeled proteins and the proteins were analyzed by denaturing acrylamide gel electrophoresis (Fig. 1). These results indicate that little if any proteolytic degradation of encapsulated proteins occurs. This substantiates previous reports that LUV uptake by cells does not involve significant lysosomal activity (22).

Because of the size similarity between thyroglobulin and eIF3, the kinetics of cellular uptake of LUV encapsulated proteins were determined utilizing thyroglobulin (Fig. 2). In this case, either freshly prepared LUV or those stored at −20°C as cochrleate cylinders were incubated with both myoblasts (30-h cultures) and myotubes (60-h cultures). The LUV preparations that had been stored at −20°C generally show a more efficient delivery of 14C-thyroglobulin into cells. Again, it is noted that myotubes are more responsive in taking up LUV-encapsulated materials than myoblast cell cultures. It was decided that −20°C stored LUV and an incubation time of 3 h would be utilized in subsequent experiments.

We have previously reported that muscle eIF3 could be fractionated by MHC-mRNA affinity column chromatography (8). When the eluted fractions are reductively labeled with [14C]formaldehyde and subsequently analyzed by electrophoresis under denaturing conditions, the pattern of polypeptides shown in Fig. 3 is observed. The 0.1-M KCl wash through fraction is identical in composition to the 0.5-M KCl-eluted core eIF3. If the 0.1-M KCl fraction is reapplied to the affinity column it does not bind (unpublished results), indicating that [14C]formaldehyde and subsequently analyzed by electrophoresis under denaturing conditions. The cytoplasmic extracts of the 5-h cultures were analyzed on denaturing acrylamide gels. The autoradiographs of these gels demonstrate that the thyroglobulin is still intact at this time period (results not shown).

![Figure 1](image1.png) **Figure 1** Analysis of liposome-delivered molecules retrieved from cells in culture. All proteins were reductively labeled with [14C]formaldehyde, encapsulated in liposomes, and incubated with cell cultures as detailed in Materials and Methods. After cell lysis, total cellular proteins were analyzed by gel electrophoresis as described by Laemmli (12). After electrophoresis the gels were examined by fluorography. Lane 1, myofibrillar protein encapsulated in liposomes; lane 2, total myoblast protein retrieved from cells of material shown in lane 1; lane 3, myoglobin encapsulated in liposomes; lane 4, total myoblast protein retrieved from cells after delivery of material shown in lane 3; lane 5, impure eIF3 encapsulated in liposomes; lane 6, total myoblast protein retrieved from cells after delivery of eIF3 shown in lane 5. Bars between lanes 1 and 2 and lanes 3 and 4 indicate the position of major protein bands.

![Figure 2](image2.png) **Figure 2** Cellular uptake kinetics of LUV-encapsulated thyroglobulin. Thyroglobulin labeled with [14C]formaldehyde as described in Materials and Methods was encapsulated in freshly prepared LUV (●, ●) as well as preparations stored at −20°C at the cochleate cylinder stage (■, ■). This material was delivered to myoblast cell cultures, 30 h (■, ■) and myotube cell cultures, 60 h (●, ●). Percent incorporation as cellular uptake is determined relative to radioactivity in the 0.5-mL encapsulation volume as detailed in Materials and Methods and Table I. The uptake of thyroglobulin was measured by hot TCA-precipitable radioactivity. The cytoplasmic extracts of the 5-h cultures were analyzed on denaturing acrylamide gels. The autoradiographs of these gels demonstrate that the thyroglobulin is still intact at this time period (results not shown).
FIGURE 3  Electrophoretic analysis of MHC-mRNA affinity column fractions of eIF3. Acrylamide gel electrophoresis of fractions was done as previously described (9). Reductive methylation with ["C"]formaldehyde was as described in Materials and Methods. Lane 1, 0.1-M KCl wash through of affinity column; lane 2, core eIF3 obtained by elution with 0.5-M KCl buffer; lane 3, HAF eluted with 2 M LiCl as previously described (9). The original sample of eIF3 applied to the column was 75 μg and the autoradiograph (lanes 1–3) represents the total amount of each fraction eluting from this column.

The in vitro use of radioactively labeled initiation factors has led to a model of the assembly of the initiation complex (1, 8, 24). It is reasonable to assume that the path taken by LUV-encapsulated eIF3 introduced into cultured cells can be followed in an analogous manner. Total muscle eIF3, labeled with ["C"]formaldehyde, was delivered to the muscle cells at 24, 42, and 72 h of culture. Under the culture conditions used, these time periods represent dividing myoblasts, pre- to early-fusion myotubes, and myotubes, respectively. The intracellular location of the exogenously supplied eIF3 was determined by sucrose density gradient analysis. The overall distribution of radioactivity changes slightly as differentiation proceeds. There is an apparent shift in the eIF3-associated radioactivity from the top to the bottom portions of the sucrose density gradient. The meaning of this observation is unclear at this time. Nevertheless, these results suggest that exogenously supplied eIF3 will associate with the translational machinery of the cells.

It has been previously shown that total eIF3 from 14-d embryonic muscle demonstrates a discriminatory property on the competitive translation of mRNAs (8). Therefore, it was of interest to determine whether exogenously supplied eIF3 exerts a similar effect on the protein synthetic pattern of differentiating muscle cells. Cell cultures were incubated with LUV containing eIF3 in the presence of [3H]leucine. The myofibrillar proteins were subsequently isolated and examined after electrophoresis. Fig. 5 A, B, C are fluorograms and densitometric scans of those myofibrillar protein preparations from 24-, 42-, and 72-h cultures. Although there is an increase in myofibrillar protein synthesis at all times with the addition of eIF3 to the cells, it is most apparent in the 42-h muscle cell cultures, a time when the majority of myogenic cells have stopped dividing and are beginning to fuse. The areas under the MHC chain and actin-tropomyosin peaks, as determined from the densitometric scans, for the three culture periods have been compared (Fig. 6). At 42 h of culture there is a 200% increase in the incorporation of radioactivity in MHC and a 400% increase in actin-tropomyosin in cell cultures in which eIF3 has been introduced to the cells via LUV. At 72 h of culture (predominantly myotubes) the effect of exogenously added eIF3 is diminished with the increase in MHC reduced to 100% and that of the actin-tropomyosin to 60%. It is apparent that at 42 h the cells are most influenced by eIF3 additions. The small effect at 24 h is likely a result of the absence of the muscle-specific mRNAs in dividing myoblasts (3, 6), while the diminished effect at 72 h may be due to the decrease in the number of dividing myoblasts at this time.
h may result from increased concentrations of endogenous cellular factor(s) involved in translational control, specifically HAF.

Because muscle eIF3 can be separated into three fractions (an inactive 0.1-M KCl fraction; core eIF3, that functions indiscriminately with any mRNA; and HAF, which imposes a discriminatory function on core eIF3) by MHC-mRNA affinity column chromatography (8), it was of interest to determine the behavior of these fractions after their entry into muscle cells. The 3H-labeled affinity column fractions were introduced to the 42-h cell cultures using LUV and the cellular localization was determined by sucrose density gradient centrifugation. Again, because of the low amounts of recovered radioactivity, each experiment was repeated five times and an absolute reproducibility was obtained with regard to the position of the

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**FIGURE 5** Exogenously supplied total eIF3 alters myofibrillar protein synthesis. The myofibrillar proteins were isolated as described in Materials and Methods after incubating cultured muscle cells with [3H]leucine and LUV containing total eIF3 as described in

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**FIGURE 6** Effect of exogenous total eIF3 on the synthesis of individual myofibrillar proteins. The areas under the fluorometric scans in Fig. 5 A–C were calculated for the MHC peak and the pooled actin and tropomyosin peaks. All data are expressed relative to control values obtained with the 24-h cultures. The four curves represent: MHC less eIF3 (○); MHC with exogenously added eIF3 (■); actin, tropomyosin less eIF3 (□); actin, tropomyosin with exogenously added eIF3 (▲).

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Materials and Methods. The proteins were analyzed by electrophoresis as described by Laemmli (12) and subsequent fluorography was performed. The figures represent the fluorograms and densitometric tracings of control PBS containing LUV (—) and eIF3-treated (—) cultures. (A) 24-h cultures, (B) 42-h cultures, (C) 72-h cultures. MHC indicates myosin heavy chain at 200,000 daltons; A indicates position of actin; T indicates position of tropomyosin. The fluorograms are labeled C and 3 for control and eIF3-treated cells.
radioactive peaks in the gradients. As shown in Fig. 7A, the 0.1-M KCl fraction of eIF3, which fails to bind MHC-mRNA, sediments as one predominant peak at 15S and does not appear to become associated with 40S ribosomes or the 80–120S region previously noted in Fig. 4. This fraction was previously found to be inactive in cell-free systems, and these results suggest that it may be similarly inactive in intact cells. By contrast, the 0.5-M KCl-eluted, core eIF3 is judged to contain active eIF3 molecules by its ability to bind the 40S ribosomal subunits (Fig. 7B). This agrees with our previous data showing this fraction to be active in cell-free systems (8). It is noted that this core eIF3 also sediments on the 80–120S region of the sucrose gradient but to a lesser extent than unfractionated eIF3 (compare Fig. 7B and Fig. 4B). The HAF fraction is found to sediment in the sucrose density gradients in a highly characteristic manner (Fig. 7C). It is found free on the top of the gradient and possibly complexed with cellular eIF3 sedimenting at 15S. In addition, some HAF is found sedimenting at 40S, presumably resulting from eIF3 associated with 40S ribosomal subunits. Strikingly, the major peak observed sediments at 80–120S. This is the known sedimentation of MHC-mRNPs and, therefore, may represent the binding of HAF to MHC-mRNA. Comparing the 80–120S sedimentation behavior of unfractionated eIF3 (Fig. 4B) and core eIF3 (Fig. 7B) to the HAF (Fig. 7C), it is apparent that essentially all of the binding of unfractionated eIF3 to 80–120S material results from the HAF present in unfractionated eIF3. It is unclear, at the present time, why radioactivity is found in the polysomal regions of the sucrose density gradients. Possibly, it results from the one initiation complex found on each polyribosome.

Both the core and HAF fractions of eIF3 were tested for their effects on myofibrillar as well as total cellular protein synthesis after insertion into cells at 42 h of culture. The histograms in Fig. 8A and B show [3H]leucine incorporation into total and nonmyofibrillar proteins, respectively, after incubation with core eIF3. A slight stimulation of incorporation is evident in the 42-h cultures. The fluorograms of myofibrillar proteins from these same muscle cell cultures revealed no detectable stimulation of [3H]leucine into these proteins (Fig. 9). Similarly, the HAF revealed no detectable stimulation of radioactive incorporation into total, nonmyofibrillar or myofibrillar protein synthesis at any time point (results not shown). This inability of HAF to effect protein synthesis may result from the relatively small amount found in the 40S ribosomal subunit area of the sucrose density gradient as compared to the 80–120S material (Fig. 7C). These results are seemingly in conflict with our previous studies utilizing cell-free systems (8, 9). However, in these cases, the HAF was preincubated with the mRNAs before their addition to these in vitro systems, allowing specific binding to occur in the absence of interfering substances present in the cell lysate (8). A similar nonspecific binding may occur upon entry into the cells or, as suggested in Fig. 7 C, HAF may bind specifically to inactive MHC-mRNAs stored in the cytoplasm and thus be removed from the active protein synthetic machinery. Therefore, [3H]uridine mRNA was complexed with HAF before its encapsulation into LUV and subsequent uptake into the cells. In this case, 24-h dividing myoblast cell cultures were used, as it is known that they contain little if any MHC-mRNA. In addition, we have demonstrated previously that MHC-mRNA can be translated in these cells, but much less efficiently than in differentiating myotubes (17). The position of [3H]MHC-mRNA when delivered alone and after preincubation with the HAF is displayed...
FIGURE 9. Effect of core eIF3 on muscle-specific protein synthesis. Muscle cells were incubated with LUV containing core eIF3 in the presence of [3H]leucine, and myofibrillar proteins were subsequently isolated, electrophoresed, and prepared for fluorography as described in Fig. 5 and Materials and Methods. C, control vesicles containing only PBS; 3, vesicles containing core eIF3. A, actin; T, troponin.

in Fig. 10A and B, respectively. It is noted that the association of the mRNA with the HAF has resulted in a shift of the MHC-mRNA from polysomes of four to five ribosomes each to those of 20 or more. Fig. 11A and B show the results of a similar experiment involving preincubation with core eIF3 and HAF, respectively. The nondiscriminatory core eIF3 has no effect on the number of ribosomes associated with MHC-mRNA (Fig. 10A) when compared to the mRNA alone (Fig. 9A). To determine whether the discriminatory nature of HAF could be detected in this type of experiment, [3H]uridine viral mRNA (vesicular stomatitis virus [VSV]) was similarly preincubated with HAF and inserted in 24-h cell cultures using LUV. As can be seen, VSV-mRNA readily associates with ribosomes and appears in polysomes (Fig. 12A) and no appreciable change in the sedimentation of VSV polysomes is noted if VSV mRNA is preincubated with HAF. These results give further evidence suggesting the mRNA discriminatory nature of HAF. It is realized that the control experiment utilizing viral mRNA is not completely adequate, for this mRNA may already be maximally associated with ribosomes in this cell system. An adequate control must await the finding of an mRNA which can be shown to be translated with <100% efficiency in myoblast cells. The inability of HAF to effect the sedimentation pattern of VSV-RNA makes it unlikely that the shift in MHC-mRNA polysome size is caused by a nonspecific aggregation phenomenon. However, to rule out this possibility, puromycin was added to the cells 5 min before the addition of the liposome-encapsulated HAF-[3H]myosin mRNA complex and maintained at a concentration of 1 mM throughout the duration of the experiment. As was previously shown (Fig. 10) the MHC-mRNA was found predominantly in the large polysome size class when added in the presence of HAF (Fig. 13A); however, when puromycin is present, the mRNA is not associated with polysomes (Fig. 13B). These results indicate that protein synthesis is required for the HAF-mediated shift of MHC-mRNA from small to large polysomes and suggests that mRNA aggregation and nonspecific binding are not occurring.

DISCUSSION

Using whole cell lysates (9) and a partially fractionated cell-free protein synthesis system (8), we have shown that eIF3 from chick muscle can preferentially stimulate the translation of MHC-mRNA in the presence of equimolar concentrations of rabbit globin mRNA. This discriminatory behavior of muscle eIF3 is mediated by a protein(s) having a very high affinity for MHC-mRNA. Furthermore, this component can modulate the activity of core eIF3 from either muscle or reticulocytes so as to render either factor discriminatory for MHC-mRNA (8, 9). These results, utilizing cell-free extracts, have led us to postulate a regulatory role for these HAF proteins in altering the cytoplasmic utilization of mRNAs in eukaryotic cells.

The developmental significance and the actual in vivo occurrence of these events has remained a matter of speculation.

![Figure 10](http://www.jcb.org/data/...)

**Figure 10.** HAF of eIF3 changes the size of polysomes synthesizing MHC in vivo. The high affinity fraction of eIF3 was preincubated with [3H]-labeled myosin mRNA. The complex was then encapsulated in LUV and delivered to 24-h cell cultures as detailed in Materials and Methods. In this case the cells were only in the presence of LUV for 20 min before addition of cycloheximide and subsequent cell lysis. 50 μg/ml of cycloheximide was present during lysis and polysome preparation. The cell lysates were centrifuged through 15-40% wt/wt sucrose density gradients for 90 min in an IEC SB283 rotor at 4°C and 38,000 rpm in the presence of 20 OD (260 nm) of polysome and ribosome carrier obtained from 13-d embryonic chick leg muscle. (A) MHC mRNA alone; (B) MHC mRNA preincubated with the HAF of eIF3. B and T indicate bottom and top of gradient, respectively.
FIGURE 11 Effects of core eIF3 and HAF of eIF3 on the size of polysomes synthesizing MHC in vivo. Core eIF3 and HAF were preincubated with 3H-MHC-mRNA. The mixture was entrapped in LUV and incubated with 24-h cell cultures. The cell lysates were analyzed on sucrose density gradients as described in Materials and Methods and Fig. 10. (A) MHC-mRNA plus core eIF3, (B) MHC-mRNA plus HAF, B and T represent bottom and top of gradients, respectively.

In a recent study, Mroczkowski et al. (17) have demonstrated that muscle cells in culture can actually take up and utilize added mRNAs. Furthermore, these studies suggested that MHC-mRNA was less efficiently translated in myoblasts than in myotubes. The introduction of cytoplasmic control elements and mRNAs into differentiating muscle cells utilizing liposomes will provide a basis for comparison of results obtained utilizing cell-free extracts.

When unfractionated muscle eIF3 is taken up by the cells, it is found to associate with 40S ribosomal subunits, suggesting that it is biologically active. Similar association of 14C-labeled eIF3 with ribosomes has been observed in cell-free systems (1, 8). While some of the radioactivity associated with unfractionated eIF3 is found to sediment with the 80–120S fraction, a significant proportion of the HAF is found in the 80–120S region of the sucrose density gradient. On the other hand, core eIF3, stripped of HAF, sediments primarily with the 40S ribosomes (24). The small fraction of 14C-HAF found sedimenting with 40S ribosomal subunits is likely a result of the HAF binding eIF3 found on these ribosomal subunits. Taken together, these results suggest that both the core eIF3 and the HAF are biologically active after entry into the myogenic cells. Significantly, HAF is found associated with an 80–120S particle, the known sedimentation value of MHC-mRNP (10). Upon isolation of the MHC-mRNP by separation from the ribosomes on metrizamide density gradients, the 14C-HAF is found only in the mRNP fraction (unpublished results). The fact that HAF is found to associate with MHC-mRNPs as well as eIF3 suggests that HAF may originally be found on MHC-mRNPs. If this is the case, upon the activation of the mRNP, a nondiscriminatory core eIF3, participating in the initiation of the MHC-mRNA may then become associated with HAF. This HAF-eIF3 is now discriminatory and would favor the initiation of MHC-mRNA. Through this process of sequestering cellular core eIF3, the translational efficiency of MHC-mRNA would be increased.

The fact that the eIF3 introduced into the cells can exert a specific influence on protein synthesis further demonstrates that the procedures used do not eliminate biological activity and that mRNA discrimination is observed much as it was utilizing cell-free extracts (8, 9). Indeed, it is observed that the muscle eIF3 prepared from 14-d embryonic muscle exerts rather specific stimulatory effects in myofibrillar protein synthesis with little effect on total protein synthesis. This suggests that the discrimination is not only for MHC-mRNA. However, it is not known at this time whether HAF isolated from MHC-mRNA affinity column chromatography is responsible for the effects observed on the other myofibrillar proteins, or whether indeed different HAF proteins will be isolated utilizing different mRNA affinity columns. The fact that the myogenic cells which are just beginning to fuse (42 h) show the greatest effect of added eIF3 on myofibrillar protein synthesis is likely a result

FIGURE 12 HAF of eIF3 and the polysome size of VSV-mRNA polysomes. The HAF obtained from muscle eIF3 was preincubated with 3H-labeled VSV-mRNA and subsequently entrapped in LUV. Incubation with myoblast cell cultures is as described in Materials and Methods. The cell lysates were analyzed on sucrose density gradients as described in Materials and Methods and Fig. 10. (A) VSV-mRNA plus HAF, (B) VSV-mRNA alone.

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that HAF is a developmentally programmed regulatory process in myoblasts (24 h) and (b) the presence of discriminatory eIF3 + HAF in the presence of puromycin. Cultures were incubated with 1 mM puromycin for 5 min before the addition of liposomes and maintained at this concentration throughout the duration of the experiment.

Figure 13. Puromycin inhibits the effect of HAF on MHC-mRNA association with polysomes. This experiment was performed as described in Fig. 10. (A) MHC-3H-mRNA + HAF, (B) MHC-3H-mRNA + HAF in the presence of puromycin. Cultures were incubated with 1 mM puromycin for 5 min before the addition of liposomes and maintained at this concentration throughout the duration of the experiment.

The fact that we could not detect an effect on protein synthesis when HAF was inserted into the cells may be a result of (a) its dilution or (b) its binding to stored mRNAs. Since pre-association of HAF with mRNA has been shown to be required for maximal discriminatory effects in cell extracts (8, 9), preincubated MHC-mRNA and HAF complexes were inserted into the cells. While MHC-mRNA appeared on larger polysomes after being preincubated with HAF, no effect could be seen on the association of viral mRNA with polysomes. These experiments suggest that HAF has a discriminatory role in the intact cell. The fact that MHC-mRNA, inserted in myoblasts in the presence of HAF, wastranslated with a similar efficiency as previously observed in myotube cell cultures (17) suggests that HAF is a developmentally programmed regulatory protein appearing during myogenesis. These experiments lend strong support to our previous in vitro studies, suggesting that mRNA discriminatory elements play a significant role in the cytoplasmic utilization of mRNAs during muscle differentiation.

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