Abstract. The onset of muscle cell differentiation is associated with increased transcription of muscle-specific mRNA. Studies from this laboratory, using 19-d embryonic rat skeletal muscle, suggest that additional, posttranscriptional controls regulate maturation of muscle tissue via a quantitative effect upon translation, and that the regulatory component may reside within the poly A- RNA pool (Nathanson, M. A., E. W. Bush, and C. Vanderburg. 1986. J. Biol. Chem. 261:1477-1486). To further characterize muscle cell translational control, embryonic and adult total RNA were separated into oligo(dT)cellulose-bound (poly A+) and -unbound (poly A-) pools. Unbound material was subjected to agarose gel electrophoresis to resolve constituents of varying molecular size and mechanically cut into five fractions. Material of each fraction was electroeluted and recovered by precipitation. Equivalent loads of total RNA from 19-20-d embryonic rat skeletal muscle exhibited a 40% translational inhibition in comparison to its adult counterpart. Inhibition was not due to decreased message abundance because embryonic, as well as adult muscle, contained equivalent proportions of poly A+ mRNA. An inhibition assay, based upon the translatability of adult RNA and its inhibition by embryonic poly A- RNA, confirmed that inhibition was associated with a 160-2,000-nt poly A- fraction. Studies on the chemical composition of this fraction confirmed its RNA composition, the absence of ribonucleoprotein, and that its activity was absent from similarly fractionated adult RNA. Rescue of inhibition could be accomplished by addition of extra lysate or mRNA; however, smaller proportions of lysate were required, suggesting a strong interaction of inhibitor and components of the translational apparatus. Additional studies demonstrated that the inhibitor acted at the level of initiation, in a dose-dependent fashion. The present studies confirm the existence of translational control in skeletal muscle and suggest that it operates at the embryonic to adult transition. A model of muscle cell differentiation, based upon transcriptional control at the myoblast level, followed by translational regulation at the level of the postmitotic myoblast and/or myotube, is proposed.

Differentiation of skeletal muscle begins with the appearance of myogenic regions within a previously homogeneous mass of mesenchymal cells (Searls et al., 1972). Each myogenic region consists of a group of proliferating, mononucleate myoblasts amid other postmitotic myoblasts that are fusing into syncyntial myotubes. It is now clear from in vitro studies that fusion is sensitive to factors such as nutrient supply (Konigsberg, 1971, 1977; O'Neil and Stockdale, 1972; Doering and Fischman, 1974; White et al., 1975) and fibroblast growth factor (Linkhart et al., 1980, 1981). The presence and/or addition of serum or fibroblast growth factor to cultured myoblasts is sufficient for myoblasts to remain in the proliferative pool rather than undergo fusion. A variety of substances other than serum and fibroblast growth factor influence fusion. Some of them, such as DMSO (Blau and Epstein, 1979) and transforming growth factor beta (Massague et al., 1986), inhibit both fusion and muscle-specific protein synthesis. Others, such as lowered Ca** concentrations or EGTA, affect only fusion (see below); however, not all species respond similarly to EGTA (Shainberg et al., 1971). In the well-characterized avian system, at least, and in nonfusing variants of an avian myogenic line (Ls; Kaufman et al., 1977), it is clear that withdrawal from the cell cycle is a requirement for fusion, but that fusion is not an obligate requirement for the initiation of muscle-specific protein synthesis.

Messenger RNA for muscle-specific proteins is known to occur in limited abundance in myoblasts before fusion (Young et al., 1975; Buckingham et al., 1976; Yablonka and Yaffe, 1977; Devlin and Emerson, 1979; Jain and Sarkar, 1979; Benoff and Nadal-Ginard, 1979; Doetschman et al., 1980; Zevin-Sonkin and Yaffe, 1980; Shani et al., 1981; Nadal-Ginard et al., 1982; Saidapet et al., 1982). Fusion is followed by a burst of transcription that enriches muscle-specific
mRNA concentrations up to several-hundredfold (Devlin and Emerson, 1979; Hastings and Emerson, 1982; Medford et al., 1983; see Paterson and Bishop, 1977). However, fusion is not the trigger for increased transcription, as fusion-blocked myoblasts show similarly increased rates of transcription (Yaffe and Dym, 1972; Dym et al., 1979) and protein synthesis (Emerson and Becker, 1975; Merlie and Gros, 1976; Moss and Strohman, 1976; see Trotter and Nameroff, 1976). Control at the transcriptional level has therefore received considerable attention.

While it is clear that a battery of muscle-specific genes are activated after fusion, young myotubes do not contain abundant myofibrils. It is also known that some muscle-specific proteins are not translated with equal efficiency or at the same time as others, in spite of the presence of their respective mRNAs (Nathanson et al., 1986).

A classic example of translational control has been studied in Escherichia coli, where synthesis of ribosomal proteins is controlled by feedback regulation of the proteins upon their mRNA (Yates and Nomura, 1981). In mammalian cells, there are also numerous examples where the control of gene expression occurs at the level of translation, including changes in protein synthetic rates during mitosis, nutrient deprivation, heat shock, and virus infection (Hershey et al., 1984). U-rich RNAs which act as inhibitors of translation have been described in Artemia embryos (Lee-Huang et al., 1977), rat calvaria (Zeichner and Breitkreutz, 1978), rat liver (Northemann et al., 1980), and rabbit reticulocytes (Dionne et al., 1982). The frequency with which posttranscriptional (i.e., translational) control mechanisms occur in eukaryotes suggests that this process may also play a regulatory role in protein synthesis of skeletal muscle.

Posttranscriptional regulation has been suggested to occur in skeletal muscle via release of mRNA from mRNP particles (Brawerman, 1974; Bester et al., 1975; Buckingham et al., 1976; Jain and Sarkar, 1979; Doetschman et al., 1980), or via increased stability of newly transcribed mRNA (Buckingham et al., 1974, 1976; Medford et al., 1983; Carniero and Schiebler, 1984). There is agreement that at least some muscle-specific mRNA occurs as mRNP, although it is not known with certainty how mRNPs control the availability of mRNA at the time of fusion, or whether newly transcribed mRNA is preferentially translated (Dym et al., 1979; Doetschman et al., 1980). The occurrence of message-specific initiation factors in skeletal muscle (Thompson et al., 1973) and other eukaryotic cells (Wigle, 1973; Nudel et al., 1973), as well as RNAs that inhibit translation (Mukherjee and Sarkar, 1981; Sarkar et al., 1981; Mroczkowski et al., 1984), have also been described. One of these translational control RNAs, tcRNA, has been isolated from elf-3 preparations of 19-d embryonic chick skeletal muscle (Heywood et al., 1974) and by polysomes and mRNP of 13–14-d embryonic skeletal muscle (Bester et al., 1975; Heywood and Kennedy, 1976). The tcRNA from 19-d embryonic skeletal muscle appears to be the component responsible for message-specific initiation of myoglobin synthesis (see Thompson et al., 1973), whereas the mRNP-associated tcRNA was capable of blocking translation of homologous (skeletal muscle) mRNA (Bester et al., 1975; Heywood et al., 1974, 1975) by binding the 5' and 3' poly A regions of mRNA (Kennedy et al., 1974, 1978; Bester et al., 1975; Heywood et al., 1975).

Another RNA capable of inhibiting translation, has been isolated from 10 S, postsosomal RNP particles of embryonic chick muscle (Sarkar et al., 1981; Pluskal and Sarkar, 1981). This RNP (termed iRNP) contained a 4 S RNA (iRNA) that was nonspecific in its inhibitory activity. The proteins of iRNP could not reassociate with globin mRNA, suggesting that there was specificity to the association of iRNA and iRNP proteins (Mukherjee et al., 1981). The proteins themselves were devoid of inhibitory activity (Mukherjee and Sarkar, 1981). Mechanistic studies suggested that iRNA acted at the level of initiation to inhibit binding of mRNA to 43 S initiation complexes (Winkler et al., 1983).

Recent studies in this laboratory have also implicated translational control as a posttranscriptional regulatory step in the differentiation of skeletal muscle (Nathanson et al., 1986). Our data suggested that skeletal muscle gene regulation occurred at three levels. Alpha actin was regulated transcriptionally, whereas alkali light chains were controlled at the translational level, and regulation of myosin heavy chain and light chain 2 was of the mixed transcriptional–translational type. One feature of these studies was that the inhibitory component could not be identified in adult skeletal muscle, suggesting that translational regulation may be a feature of embryonic tissue. Data from another laboratory was consistent with these findings and suggested that muscle "housekeeping" proteins were subject to transcriptional control, whereas muscle-specific proteins such as alpha and beta tropomyosin were subject to transcriptional–translational regulation, and others were subject to purely translational regulation (Endo and Nadal-Ginard, 1987).

In skeletal muscle there appears to be two major methods of exerting posttranscriptional control over translationally competent mRNA. One is by sequestering mRNA as mRNP, while the second acts upon translation itself, presumably via direct interaction of an RNA with the translational apparatus. The present studies were undertaken to further characterize translational regulation of embryonic vs. adult skeletal muscle and to identify the component(s) responsible for inhibited translation in embryonic tissue.

Materials and Methods

Glassware was baked (250°C for 5 h) and solutions were autoclaved (121°C for 15 min) to destroy nucleases. Water was deionized (Mill-Q, 18 megohm/cm) and treated with 0.1% diethylpyrocarbonate to inactivate nucleases; plasticware was soaked in 0.1% diethylpyrocarbonate to inactivate nucleases. Glassware was baked (250°C for 5 h) and solutions were autoclaved (121°C for 15 min) to destroy nucleases. Water was deionized (Mill-Q, 18 megohm/cm) and treated with 0.1% diethylpyrocarbonate to inactivate nucleases. Glassware was baked (250°C for 5 h) and solutions were autoclaved (121°C for 15 min) to destroy nucleases. Water was deionized (Mill-Q, 18 megohm/cm) and treated with 0.1% diethylpyrocarbonate to inactivate nucleases. Glassware was baked (250°C for 5 h) and solutions were autoclaved (121°C for 15 min) to destroy nucleases.
purchased from Eastman Kodak Co. (Rochester, NY). RNAsin was from Promega Biotech (Madison, WI). Ethidium bromide was from International Biotechnologies Inc. (New Haven, CT). Gelcode silver stain was from the Pierce Chemical Co. (Rockford, IL). Quick Blot mRNA isolation kits and Elutrap membranes were from Schleicher and Schuell, Inc. (Keene, NH). Acrylamide stock solutions (Acrygel) and scintillation cocktail (Ultrafluor or Ecoscint) were purchased from National Diagnostics, Inc. (Somerville, NJ). Treated pregnant Sprague-Dawley albino rats were purchased from Taubman's (Germantown, NY). Reagents were certified as RNAse-free or nuclease-free whenever possible; all other components were of reagent grade.

**Isolation and Fractionation of RNA**

RNA was isolated from 20-d embryonic rat thigh muscle as described previously (Nathanson et al., 1986). Tissue was extracted by the guanidinium isothiocyanate method (Chirgwin et al., 1979) and RNA was isolated in a pellet by isopycnic density gradient centrifugation through cesium trifluoroacetate (d-1.30 g/ml). Gradients were established by centrifugation at 46,000 g at 20°C in a model SW 50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA). Pelleted material was washed twice with ice-cold 70% ethanol, dissolved in 10 mM Tris-HCl, pH 8, containing 1 mM EDTA (TE), and SDS was added to a final concentration of 0.1%.

**In Vitro Translation**

In vitro translation of total RNA or oligo(dT)-selected poly A+ mRNA was performed with a message-depleted reticulocyte lysate system. Each 12.5-μl translation contained 0.25 μCi [35S]methionine, and RNA at concentrations described below. The final translation mixture contained 2.2 mM magnesium and 100 mM potassium (as acetate). Before translation, RNA samples were heated in a 65°C water bath for 10 min and quenched on ice. Translation was performed at 30°C for 60 min. After translation, RNA was degraded by the addition of 40 μg of RNase A and reincubation at 30°C for 30 min. This step was repeated during nuclear induction experiments. Translation products were analyzed by SDS-PAGE using 15% resolving gels. Products were visualized by autoradiography. Translation products were analyzed by SDS-PAGE using 10% separating gels (10% T, 3% C; not of Hjerden, 1962) with 4% stacks (Laemmli, 1970), at 40 mA per gel, and at 20°C. Samples were prepared for electrophoresis by dilution with 4 vol of sample buffer (0.1 M Tris-HCl, 10 mM magnesium and 1 mM EDTA, and SDS was added to a final concentration of 0.1%.

**TCA Precipitation**

Aliquots of 1.5 μl were removed from in vitro translations, placed into 1 ml of prewarmed 1 N NaOH containing 1.5% hydrogen peroxide, and incubated at 37°C for 10 min. 4 ml of ice-cold 25% TCA containing 2% casamino acids were added and the mixture held on ice for 30 min. Each sample was then vacuum filtered through GF/C filters (Whatman Inc., Clifton, NJ) and washed once with 10 ml of ice-cold 8% TCA and twice with 1 ml each of acetone. 25 μg cpm were determined by scintillation counting of the dried filters in 10 ml of cocktail in a scintillation counter (model LS-8100; Beckman Instruments, Inc.) in the automatic quench correction mode.

**Enzyme Digestions**

Experiments to determine protease K sensitivity were performed for up to 20 min at 30°C in a 20 μl reaction mixture containing 10 μg RNA, 50 U of protease K, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Digestion with RNase A was performed for up to 1 h at room temperature in a 20-μl reaction mixture containing 10 μg RNA, 5 μg of RNase A; 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Aliquots of protease K and RNase A digestions were taken at the times indicated under Results. Digestion with DNase I was performed for 30 min at room temperature in a 50-μl reaction volume containing 10 μg RNA, 10 U of RNase-free DNase I, 40 mM Tris-HCl, pH 7.9, 10 mM NaCl and 6 mM MgCl2. RNase A digestion of a protein molecular weight standard was used to confirm the absence of protease activity in this preparation and was performed for 40 min in the presence of 500 ng of the protein standard. Enzymatic digestions of RNA were stopped by extraction with phenol. Digests were analyzed by electrophoresis as described above. For these experiments, the distal portion of each 1.5% gel was replaced with 30% agarose to trap potential digestion products.

**Quantitation of DNA and Poly A+ RNA from Embryonic Muscle of Various Developmental Stages**

DNA and RNA were isolated as nuclear and cytoplasmic fractions of skeletal muscle obtained from adult, 6-wk-old, 5-d neonate, 20-d embryonic, and 16-d embryonic rats. Leg and thigh muscle from animals of each of these ages was collected by dissection as described previously (Nathanson et al., 1986) and enzymatically disaggregated into single cell suspensions (Nathanson et al., 1978). Cell suspensions were washed 3 times on the centrifuge in Hanks' balanced salt solution (HBSS), containing 50 μg/ml cycloheximide, 2 U/ml RNasin, and 5 mM DTT, at 300 g for 5 min, and resuspended in a fresh aliquot of the same solution. Fractionation into nuclear and cytoplasmic pools was performed using a modification of the protocol supplied with the Quick-Blot kit (Schleicher and Schuell, Inc.). 1 ml of each cell suspension was mixed with 50 μl of Brij 35 and gently vortexed. 50 μl of sodium deoxycholate was then added and, after gentle vortexing, the mixture was incubated on ice for 5 min. The mixture was then centrifuged at 10,000 g for 30 min. The supernatant was removed from the pellet and treated with RNase-free DNase I and proteinase K, phenol extracted, and precipitated with ethanol as described above. Nuclear pellets were homogenized by sonication in sterile water, on ice, for 30-40 s in a volume of 500 μl. Quantitation of total DNA content in each of the nuclear pellets
was carried out using a fluorometric method described previously (Nathanson and Hay, 1980). RNA contents of cytoplasmic fractions were normalized with respect to the DNA content of the respective nuclear pellet. An aliquot of each cytoplasmic fraction equivalent to a volume representing 200 ng of DNA was assayed for total RNA content by UV spectrophotometry at 260 nm and corrected for nonnucleic acid absorption at 300 nm. This RNA was then mixed with 50 μl of oligo(dT)cellulose in binding buffer, and incubated with shaking for 1 h at room temperature. The slurry was centrifuged at 9000 g for 5 min to pellet the oligo(dT)cellulose matrix. Supernatants were carefully removed and assayed for their RNA content as above. Oligo(dT)cellulose pellets were washed once with 50 μl of binding buffer, resuspended in 50 μl of elution buffer, and incubated for 1 h at room temperature with shaking. Oligo(dT)cellulose was pelleted as before, and the eluted supernatants were assayed for their RNA content.

**Nuclease Induction**

Nuclease induction as a means of limiting translation in vitro was studied using an end-labeled 7.5-kb marker RNA as a substrate in translations lacking isolate and primed with RNAs isolated above. Potential degradation products were separated by methylmercury-agarose gel electrophoresis. The marker RNA (150 pmol) was dephosphorylated with 9.0 U of calf alkaline phosphatase. Dephosphorylation was carried out in a buffer consisting of 0.05 M Tris-HCl, pH 9.0, 1 mM MgCl₂, 0.1 mM ZnCl₂, and 1 mM spermidine for 30 min at 75°C in a volume of 200 μl. 40 μl of 10× STE buffer (100 mM Tris-HCl, pH 8.0, 1 M NaCl, 10 mM EDTA), 20 μl of 10% SDS, and 140 μl of water was then added and the mixture incubated at 60°C for 15 min in order to inactivate the enzyme. The mixture was extracted twice with phenol/chloroform (1:1) followed by two extractions with chloroform, and finally precipitated with 2.5 vol of absolute ethanol, 0.1 vol of 3 M sodium acetate, and 1 μg of glycogen. 50 pmol of 5'-dephosphorylated RNA ends were end-labeled with 30 U of T4 polynucleotide kinase, in a reaction mixture containing 100 μCi gamma(32p)ATP (New England Nuclear; 3,000 Ci/mmol) and 10 μl of 10× kinase buffer (0.5 M Tris-HCl, pH 7.6, 0.1 M MgCl₂, 50 mM DTT, 1 mM spermidine, 1 mM EDTA) in a total volume of 50 μl at 37°C for 30 min. The reaction was stopped by addition of 2 μl of 0.5 M EDTA, extracted with phenol/chloroform, and precipitated with ethanol. By virtue of the poly A tails on the 5-kb RNA marker, labeled RNA was separated completely from unincorporated isotope by oligo(dT)cellulose chromatography as described previously (Nathanson et al., 1986). Two peaks of 32P activity were identified by counting of the fractions eluted from the oligo(dT) column. The second peak contained the end-labeled RNA; these fractions were pooled, precipitated with ethanol, and recounted. After in vitro translation in the presence of 20,000 cpm per sample of the end-labeled RNA, translations were digested with proteinase K, phenol extracted, and ethanol precipitated before loading 5000 cpm per sample onto composite 1.5% 30% agarose-methylmercury gels (see above) along with DNA molecular weight standards. Standards consisted of phage lambda DNA, digested to completion with Hind III endonuclease, and labeled with alpha (32p)dATP. Restriction digests, containing 2 pg of input DNA, were loaded in a 10× reaction volume, received 3 μl of nucleotide (3000 CI/mM), 5 μl of 0.2-mM unlabeled dNTPs (lacking dATP), and 1 μl of DNA polymerase (Klenow). Labeling was carried out at room temperature for 30 min and stopped by the addition of 70 μl of TNE buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1× EDTA), 5 μl of 0.2 M EDTA, and 5 μl of 10% SDS. Unincorporated isotope was removed by Sephadex G-50 chromatography, and labeled lambda DNA was precipitated with ethanol in the presence of 1 μg of glycogen. Electrophoresis was carried out for 24 h at 30 mA constant current, in an electrophoresis buffer consisting of 35 mM Tris base, 35 mM NaH₂PO₄, 1 mM EDTA, pH 7.7. Agarose gels were fixed in 8% TCA for 1 h at room temperature and blotted to dryness on filter paper (type 3MM; Whatman Inc.). Autoradiography was carried out for 1 h at room temperature using a single intensifying screen (type Cronex Quanta-III; DuPont Co., Wilmington, DE).

**Initiation of Protein Synthesis**

The assay for initiation of translation was performed using an in vitro system based on the formation of an NH₂-terminal dipeptide, fMet-Val, in response to globin mRNA, as described (Cenatiempo et al., 1983).

**Results**

Embryonic and Adult Rat Skeletal Muscle Contain Equivalent Proportions of Translatable mRNA

We have previously reported that RNA isolated from 19-20 embryonic rat skeletal muscle (total RNA) directs less incorporation of labeled methionine into TCA-precipitable material than equivalent amounts of total RNA from its adult counterpart (Nathanson et al., 1986). SDS-PAGE confirmed the inhibition of protein synthesis. However, selection of poly A+ mRNA via oligo(dT)cellulose chromatography resulted in restoration of translation to levels comparable to that of adult total RNA. Based upon the additional finding that isolated embryonic and adult RNA both contained equivalent proportions of poly A+ RNA (1-2% of total RNA), and that only poly A+ RNA (mRNA) was translatable in vitro, we sought to rule out variations in transcriptional activity that may give rise to an apparent enhancement of translation in the adult.

Total RNA was isolated from cytoplasmic extracts of skeletal muscle from 16-d embryonic through adult stages and selected for translatable species by oligo(dT)cellulose chromatography. Data were normalized to "cell number" by DNA assay of the nuclear fractions using the DNA content of a single cardiac muscle cell nucleus (0.0065 pg) as a standard. Cell number in these experiments refers to number of nuclei rather than actual number of cells. This convention is necessitated by the fact that skeletal muscle cells are multinucleate; production of mRNA is thus related to mRNA producing units. It is impossible to express this data on a tissue mass or protein basis as these multinucleate cells contain greater proportions of stable protein in myofibrils in the adult, in comparison to the embryo. The increased volume of adult cells may also not clearly relate to nuclear content as message turnover rates are not known for all messages likely to be encountered. The results of this experiment indicated that cytoplasmic extracts at each stage contained approximately equivalent amounts of mRNA, and that its recovery was consistent throughout the experiment (Table 1). The results suggest that transcription of mRNA was constant from the onset of myoblast fusion (>16 d of gestation) until adulthood, or that the stability of translatable species was not clearly related to nuclear content.

**Partial Purification of Translational Inhibitor Activity**

The protocol for preparation of RNA used in the remainder of these experiments included extraction by guanidine isothiocyanate and recovery of RNA as a pellet from cesium...
Fractionation of embryonic skeletal muscle poly A⁺ RNA. (a) Preparative agarose gel. Lane 1, RNA ladder (2 µg); lane 2, embryonic rat skeletal muscle poly A⁺ RNA (5 µg). Sizes of components of the RNA ladder are indicated on the left margin. The arrow indicates the position of a band migrating near 10 kb. (b) Size estimation of RNA fractions. Tic marks on the right margin correspond to the RNA ladder in lane 7 and are shown in a of this figure. Preparative gels were cut into five fractions as indicated on the right margin. Lane 1, bovine ribosomal RNA (0.5 µg); lane 2, rabbit tRNA (0.25 µg); lane 3, adult total RNA (3 µg); lane 4, embryonic total RNA (3 µg); lane 5, adult poly A⁺ RNA (3 µg); lane 6, embryonic poly A⁺ RNA (3 µg); lane 7, RNA ladder (1 µg). (c) Re-electrophoresis of fractions 1-5 excised from preparative gels and recovered by electroelution. Each lane contains 5 µg of individual fractions as indicated. Overlap in the leading and trailing edges of fractions 2-5 indicated the possibility of overlap in RNA content, however, each fraction contained a single major RNA band.

Trifluoroacetate gradients. Residual protein was degraded by protease K, followed by phenol and chloroform extraction (see Materials and Methods). OD_{260/280} ratios of 1.8 or higher demonstrated that the preparations were largely devoid of protein. Insofar as the inhibitory activity associated with embryonic total RNA could be removed by oligo(dT)-cellulose chromatography (Nathanson et al., 1986), the starting material for these experiments consisted of oligo(dT)-cellulose-unbound material. Oligo(dT)-unbound material was fractionated by methylmercury-agarose gel electrophoresis and recovered by electroelution. In preparative gels, samples contained well-defined bands near 10 kb (Fig. 1 a, lane 2, arrow). Below 10 kb, the RNA migrated as a continuous distribution with only weak evidence of banding. Conspicuous features included bands of ribosomal RNAs, and these bands were used as arbitrary guides for slicing of each lane into five fractions (see below).

Initially, preparative gels were run in the presence of molecular size markers, however, the consistency with which each of the five fractions were resolved made the inclusion of standards unnecessary as a routine procedure. Accurate sizing of each fraction was performed on analytical gels and an example is shown in Fig. 1 b. Standards included bovine ribosomal RNA (lane 1), rabbit tRNA (lane 2), and an RNA ladder (lane 7; tic marks on right margin refer to these same standards as shown in Fig. 1 a, lane 1). For comparative purposes, both adult and embryonic total RNA (lanes 3 and 4, respectively) and adult and embryonic oligo(dT)-unbound material (lanes 5 and 6, respectively) were included on this gel. We note that embryonic material differed from its adult counterpart by decreased ethidium bromide staining <28 S. Adult material also contained well-defined bands <18 S; these same bands appear faintly in embryonic samples, but are obscured by other more abundant species. The right margin of Fig. 1 b indicates the slicing scheme applied to the preparative gels. Fraction 1 contained the band near 10 kb, fraction 2 contained material from fraction 1 up to, but not including, the second major band (18 S rRNA), fraction 3 contained 18 S rRNA, fraction 4 contained material migrating faster than 18 S to above a band of 5.8 S rRNA (160 nt), and fraction 5 included all smaller species. In anticipation of data to be presented below, we note that fraction 4 contained a heterogeneous population that fell within the size range of 160-2,000 bases and was well-separated from tRNA. When each individual fraction was resubjected to electrophoresis, they contained large leading and trailing shoulders and some degree of overlap was apparent (Fig. 1 c). However, each fraction contained sequences unique to that fraction.

An assay for translational inhibitory activity was devised, based upon our observation that adult skeletal muscle total RNA translated in an uninhibited fashion. Thus, addition of fractionated, embryonic oligo(dT)-unbound material to adult total RNA should yield inhibition of in vitro translation if that fraction contained such activity. Further, 6 µg of each embryonic, oligo(dT)-unbound fraction failed to direct protein synthesis in vitro (data not shown) and should not contribute to the fluorographic patterns. Fluorographs prepared...
Figure 2. Cotranslation of fractionated embryonic poly A RNA with adult total RNA. Each of the five fractions obtained from embryonic poly A RNA (cf. Fig. 1c) was added to in vitro translations containing 250 ng of adult muscle total RNA. The amount of each fraction added is indicated below each lane. Two microgram amounts of any fraction was found to be inhibitory, indicating nonspecific overload of the translation system. Only fraction 4 was able to inhibit translation in a specific fashion. Translation in a completely uninhibited system is indicated in the lane on the far left.

Figure 3. Cotranslation of fractionated adult poly A RNA with adult total RNA. Five fractions were also obtained from adult muscle poly A preparations and cotranslated with 250 ng of adult muscle total RNA. Nonspecific inhibition was again observed at the 2-μg level; however, no inhibition by adult fraction 4 was detected. A blank (no RNA) and an uninhibited translation are shown in the first two lanes.
from SDS-PAGE-separated translation products indicated complete inhibition when fraction 4 was present at 1–2 times the concentration of translatable (adult) RNA (Fig. 2). No other fraction exhibited comparable activity. It is important to note that all fractions gave rise to partial inhibition when present at an eightfold excess; partial inhibition appeared to be the result of nonspecific inhibition, related to overload of the in vitro translation system by nontranslatable sequences. We also sought to confirm that inhibition was not present after addition of adult-derived fraction 4; the data has confirmed this point (Fig. 3). Data from translations containing adult fractions also yielded inhibition when an eightfold excess was added, consistent with nonspecific inhibition as described above. Evidence of inhibitory activity associated with embryonic fraction 4 indicates that this fraction represents a partially purified, embryo stage–related, inhibitor of translation.

We note again that the activity of fraction 4 appears to mimic the condition encountered in the embryo, that translation of a given amount of total RNA is inhibited in comparison to adult skeletal muscle. One concern is that the assay does not artificially add back RNA (i.e., fraction 4) in greater proportions than actually occur in embryonic and adult muscle. Embryonic RNA does not resolve into bands, as does DNA, but appears as a continuous distribution of sizes. Our fractionation procedure, while reproducible, is limited by random losses encountered during precipitation and electrophoresis, and consequently cannot result in recovery of equivalent percentages of fraction 4 from each preparation. We cannot reliably estimate the amount of fraction 4 RNA in a given total RNA pool. The answer to this important question must await purification and/or cloning of the active species. However, with regard to the preparations shown in Figs. 2 and 3, embryonic poly A+ RNA contained 8.8% fraction 4, while its adult counterpart contained 13.5%. These values are similar enough to suggest that our procedure of adding back a constant amount of each fraction 4 does not result in artificial inhibition. We believe that data presented in Figs. 2 and 3 is an accurate demonstration of in vivo conditions insofar as adult fraction 4 does not show inhibitory activity.

Some lanes shown in Fig. 2, associated with fractions devoid of inhibitory activity, do indicate less translation products than would be expected (lanes containing 0.25 μg of each fraction). The entire series of experiments shown in Figs. 2 and 3 have been repeated twice, and on the basis of the combined data it appears that this is due to random variation in quantitation of RNA. This data is not consistent with the presence of inhibitory activity in any fraction except fraction 4.

Preliminary experiments, using unfractionated oligo(dT)-unbound material, demonstrated that translational inhibition could be achieved when this and adult total RNA were mixed together and held overnight at 4°C. Secondary structure was reduced by heat denaturation before translation. These mixtures demonstrated a dose–dependent inhibition, requiring at least 10 μg of embryonic oligo(dT)-unbound material to inhibit translation of 0.25 μg of adult total RNA. After fractionation as described above, it no longer was necessary to preassociate the RNAs. We now detect inhibition when 250–500 ng of fraction 4 is added to a reaction containing 250 ng of adult total RNA (Fig. 2), demonstrating purification of the inhibitory activity 20–40-fold.

**The Inhibitory Material of Fraction 4 is Ribonucleic Acid**

Oligo(dT)cellulose–unbound material was subjected to enzymatic digestion with either DNase I, protease K, or RNase A for varying periods of time to determine its composition. Analytical scale 1.5% methylmercury gels were used for separation and were cast with distal plugs of 3% agarose to retain digestion products. Nucleic acid was visualized after staining with ethidium bromide. Preliminary analysis demonstrated that the 10-kb band (cf. Fig. 1 a, lane 2) was the only component sensitive to DNase I. Digestion data confirmed that this band was composed of DNA, which isolated with oligo(dT)-unbound material (Fig. 4 a; compare undigested fraction 1 in lane 4 with digested material in lane 5). Examination of the electrophoretic pattern obtained after protease K digestion of fraction 1 demonstrated no alterations in the ethidium bromide profile, even after digestion for up to 20 min. Similarly, protease K digestion failed to alter the ethidium bromide staining pattern of total RNA preparations treated with DNase I. Undigested RNA (Fig. 4 b, lane 2) migrated in an identical fashion to material treated with the enzyme for up to 60 min (Fig. 4 b, lanes 3–6). Control digestions indicated that RNase A, while devoid of protease activity, did contain DNase activity. To avoid complications, digestion with RNase A was performed on a pool of fractions 2–5 (Fig. 4 c, lane 2). The data demonstrated degradation of the entire pool within 5–10 min (Fig. 4 c, lanes 3–8).

The use of ribonuclease was avoided when digested material was to be translated in vitro. Poor or reduced translation control (adult) mRNA indicated that phenol extraction, or even protease K digestion, failed to remove all added nuclease. In an attempt to circumvent this problem, material of fraction 4 was digested with micrococcal nuclease, taking advantage of the fact that the fraction was free of DNA and protein. Digestion conditions were designed to maintain EGTA in a concentration similar to that of lysate. The data indicated that 5 min of micrococcal nuclease digestion was sufficient to degrade 500 ng of fraction 4 to the point that it no longer inhibited translation of adult skeletal muscle mRNA (Fig. 5, lane 6). Patterns of (35S)methionine incorporation were equivalent among samples of 5–30-min digests (Fig. 5, lanes 3–5) and adult total RNA in the absence of any addition (Fig. 5, lane 1). The presence of translational inhibitory activity associated with fraction 4, its sensitivity to ribonuclease, and the absence of inhibition after degradation of RNA, confirms the presence of translational inhibitory activity associated with fraction 4, and that the inhibitor is indeed an RNA and is not dependent upon the presence of protein or ribonucleoprotein for its in vitro activity.

**Quantitation of the Inhibitory Effect of Fraction 4**

Translational inhibition has been operationally defined as the point at which translation products can no longer be visualized by fluorography in comparison to control lanes without inhibitor. This procedure does not, however, lend itself to a quantitative analysis of translation. Quantitation by measurement of TCA-precipitable, (35S)methionine incor-
Sensitivity of embryonic poly A- RNA to enzyme digestions. (a) Digestion with DNase I. Lane 1, DNA standard containing 10 μg of Hind III digested lambda DNA; lane 2, DNase I digest of the DNA standard; lane 3, 10 μg of embryonic muscle poly A- RNA; lane 4, 10 μg of fraction 1 as defined in Fig. 1; lane 5, DNase I digest of 10 μg of fraction 1. (b) Protease K digestion of DNase I-treated poly A- RNA. Lane 1, 2 μg of an RNA ladder; lane 2, 5 μg of undigested RNA; lanes 3-6, digestion for 1, 5, 10, and 20 min, respectively. The sizes of the components of the RNA ladder are indicated on the left margin. (c) Digestion with ribonuclease A. Lane 1, RNA ladder; lane 2, 10 μg of a pool of fractions 2-5; lanes 3-8, digestion for 1, 5, 10, 20, 40, and 60 min, respectively. The data indicated sensitivity to RNase, confirming the RNA composition of fraction 4.

Sensitivity of fraction 4 to micrococcal nuclease digestion. RNase sensitivity of fraction 4 was assessed by treating DNA and protein-free preparations with micrococcal nuclease; the enzyme should only degrade RNA under these conditions. Enzyme activity was inhibited by EGTA (see Materials and Methods) and aliquots containing the original equivalent of 0.5 μg of fraction 4 were added to in vitro translations. Lane 1, 0.25 μg adult muscle total RNA in the absence of fraction 4; lane 2, 0.25 μg adult muscle total RNA plus 0.5 μg undigested fraction 4; lanes 3-7, 0.25 μg adult muscle total RNA in the presence of fraction 4 digested for 30, 20, 10, 5, and 1 min, respectively; lane 8, (32P)protein molecular weight markers; lane 9, blank containing no added RNA. The data indicate that 5 min of nuclease treatment destroyed inhibitory activity, thus confirming the RNA composition of the inhibitor.

Figure 5. Sensitivity of fraction 4 to micrococcal nuclease digestion. RNase sensitivity of fraction 4 was assessed by treating DNA and protein-free preparations with micrococcal nuclease; the enzyme should only degrade RNA under these conditions. Enzyme activity was inhibited by EGTA (see Materials and Methods) and aliquots containing the original equivalent of 0.5 μg of fraction 4 were added to in vitro translations. Lane 1, 0.25 μg adult muscle total RNA in the absence of fraction 4; lane 2, 0.25 μg adult muscle total RNA plus 0.5 μg undigested fraction 4; lanes 3-7, 0.25 μg adult muscle total RNA in the presence of fraction 4 digested for 30, 20, 10, 5, and 1 min, respectively; lane 8, (32P)protein molecular weight markers; lane 9, blank containing no added RNA. The data indicate that 5 min of nuclease treatment destroyed inhibitory activity, thus confirming the RNA composition of the inhibitor.

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One unsuspected finding was that embryonic poly A⁺ RNA directed a more rapid incorporation of label into protein during the initial 15 min of translation than adult poly A⁺ RNA (compare solid lines of Fig. 6, a and b). Thus, not only is the demonstrated inhibition not due to transcriptional control, but it is also unaffected by the efficiency of the embryonic mRNA; embryonic poly A⁺ RNA appears to be a more efficient message than adult. Differences were also noted among absolute levels of incorporation, with adult poly A⁺ RNA demonstrating continued translation through

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**Figure 6.** Quantitative analysis of translational inhibition. (a) 500 ng of fraction 4 RNA was added to in vitro translations primed with 250 ng of either adult total or adult poly A⁺ RNA. Fraction 4 inhibited translation of total RNA by 75% and poly A⁺ RNA by 45% at 60 min. (b) 500 ng of fraction 4 RNA was added to in vitro translations primed with 250 ng of either embryonic total or embryonic poly A⁺ RNA. Addition of fraction 4 inhibited translation of total RNA by 40% and poly A⁺ RNA by 69% at 60 min. (-----) adult and embryonic total RNA; (----------) adult and embryonic total RNA plus fraction 4; (——) adult and embryonic poly A⁺ RNA; (———) adult and embryonic poly A⁺ RNA plus fraction 4.

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**Figure 7.** Absence of nuclease activity in the presence of fraction 4. (a) A ³²P end-labeled, 7.5-kb RNA marker was added to in vitro translations primed with adult total RNA and embryonic fraction 4 (see text), recovered, and analyzed via methylmercury-agarose gel electrophoresis to detect nuclease activity. The marker was assumed to be intact since no changes in its mobility were detected in comparison to controls lacking input RNAs. Lanes 1 and 12, 5,000 cpm of 7.5-kb RNA marker (no translation); lane 2, 5,000 cpm of HinF I pBR322 ladder; lane 3, 1,000 cpm gamma(³²P)ATP did not resolve at the exposure used for the remainder of the lanes and its position is indicated by the arrow; lanes 4 and 5, 5,000 cpm of 7.5-kb RNA added to in vitro translations lacking input RNAs for 60 and 90 min, respectively; lanes 6–8, 5,000 cpm of 7.5-kb RNA plus 250 ng of adult muscle total RNA translated in vitro for 30, 60, and 90 min, respectively; lanes 9–11, 5,000 cpm of 7.5-kb RNA plus 250 ng of adult muscle total RNA and 500 ng of fraction 4 translated in vitro for 30, 60, and 90 min, respectively. The 3% agarose altered the migration of standards in lane 2 and only those bands of verifiable length are indicated on the left margin. (b) Verification of size of the 7.5-kb marker RNA after translation in vitro. Hind III fragments of lambda DNA were used as standards. The data indicated that the marker RNA ran at a position of 7.5-kb before and after in vitro translation in inhibited and uninhibited systems. Lanes 1 and 8, 5,000 cpm of ³²P-labeled Hind III lambda DNA ladder (fragment length indicated on left margin); lane 2, 5,000 cpm of 7.5-kb RNA marker; lane 3, 5,000 cpm of 7.5-kb RNA added to an in vitro translation lacking input RNA for 90 min; lanes 4 and 5, 5,000 cpm of 7.5-kb RNA plus 250 ng of adult muscle total RNA translated in vitro for 60 and 90 min, respectively; lanes 6 and 7, 5,000 cpm of 7.5-kb RNA plus 250 ng of adult muscle total RNA and 500 ng of fraction 4 RNA translated in vitro for 60 and 90 min, respectively.
Inhibition Is Not Due to Nuclease Activity or Ribonucleoprotein

The factor(s) responsible for the latter differences remain unclear at the present time.

Figure 8. Absence of protein from embryonic muscle poly A^-RNA. RNase A digested preparations of embryonic poly A^-RNA were analyzed by SDS-PAGE followed by silver staining. A plug of 15% acrylamide was used to trap digestion products. Lane 1, 500 ng protein molecular weight standard digested with RNase A for 40 min. Molecular weights are indicated on the left border. Lanes 2-3, 10 µg embryonic poly A^-RNA digested with RNase A for 20 and 40 min, respectively; lane 4, blank; lane 5, 20 ng RNase A; lane 6, 10 µg undigested bovine total RNA; lane 7, 5 µg undigested RNA molecular weight standard; lane 8, 10 µg undigested embryonic poly A^-RNA; lane 9, 10 µg undigested rabbit tRNA; lane 10, 5 µg Hind III lambda DNA ladder.

120 min, whereas embryonic preparations plateaued at 30 min. The factor(s) responsible for the latter differences remain unclear at the present time.

Inhibition Is Not Due to Nuclease Activity or Ribonucleoprotein

Inhibition associated with fraction 4 appears to be due to the action of an RNA, although it is conceivable that a ribonuclease could have been co-isolated, or induced within reticulocyte lysate by fraction 4. This activity, if present, would degrade input RNA in a time-dependent fashion, leading to reduced protein synthesis. Experiments to assay for ribonuclease activity were performed by adding a 5' end-labeled, 7.5-kb RNA to in vitro translations primed with 250 ng translatable RNA and 500 ng of fraction 4. Degradation of the labeled marker RNA was measured by methylmercury gel electrophoresis after translation for up to 90 min and autoradiography of the dried gel. Control translations were performed in the absence of fraction 4. The data demonstrated no degradation of end-labeled RNA in either the uninhibited or inhibited samples. The labeled marker RNA (Fig. 7 a, lanes 1 and 12) did not show altered mobility or degradation after translation in the absence of input RNA (lanes 4 and 5), or in the presence of adult total RNA (lanes 6-8), or adult total RNA and embryonic fraction 4 (lanes 9-11). Lanes 6-8 and 9-11 represent translation for up to 90 min. Degradation products were also absent at the position of free ([35]P)ATP (Fig. 7 a, arrow). Lastly, the marker RNA was recovered from translations in the presence of adult total RNA or adult total plus fraction 4 and subjected to electrophoresis in the presence of a DNA ladder. These data confirmed that the marker remained undegraded at 7.5 kb (Fig. 7 b).

Each RNA preparation had been treated with protease K during its initial preparation. OD260/280 ratios of 1.8-1.9 indicated the absence of protein. Absence of changes in the ethidium bromide staining pattern after a second protease K treatment (cf. Fig. 4) lends assurance that ribonucleoprotein contamination of fraction 4 did not occur. As a further check on the presence of ribonucleoprotein, RNA samples were degraded by protease-free RNase A and analyzed for the presence of residual protein by silver staining after SDS-PAGE (Fig. 8). 4-10% gradient gels were cast above 15% acrylamide to retain digestion products. After degradation of all RNA, no stainable material was detected, suggesting that ribonucleoprotein was removed during preparative procedures.

The Inhibitor Acts at the Level of Initiation

Experiments designed to clarify the mechanism of action of fraction 4 were performed by attempting to rescue translation through addition of either increased proportions of reticulocyte lysate or increased amounts of translatable RNA. Lysate contributes eukaryotic initiation and elongation factors, and ribosomes. Incorporation of [35]Smethionine into protein was followed by SDS-PAGE and fluorography and quantitated by TCA precipitation. Addition of fraction 4 was performed either simultaneously with components of the translation reaction, or to lysate 15 min before adding message (Fig. 9). The results indicated that after simultaneous addition of all components, inhibition could be rescued by a fourfold increase in translatable message over that used in our standard assay or an 8% increase in lysate. However, when fraction 4 was mixed with lysate 15 min before adding RNA and beginning the reaction, it required a sixfold increase in translatable message to overcome inhibition. Addition of fraction 4 to lysate 15 min before adding a constant amount of translatable RNA required a >20% increase in lysate to overcome inhibition. The results indicate an interaction with both mRNA and lysate, and a particularly strong interaction with components of lysate. On a mechanistic level, the data do not suggest a catalytic activity, but direct interference with protein synthesis. Absence of incomplete peptides in the fluorograms suggested further that the activity of fraction 4 was directed towards initiation.

To study initiation in vitro, we have used a translation system lacking factors for elongation (with the exception of eEF-2; necessary for translation from the A site to the P site of the ribosome), and termination (Cenatiempo et al., 1983). In the presence of purified rabbit globin mRNA, and the first two amino acids of the beta globin polypeptide, the system should generate a dipeptide and indicate initiation. The dipeptide (f-Met-[3H]val) was isolated chromatographically and quantitated by liquid scintillation counting. The results demonstrated that in the presence of fraction 4, initiation was blocked in a dose-dependent manner (Fig. 10).
Discussion

Differentiation of skeletal muscle, both in vivo and in vitro, is primarily regulated by transcriptional control mechanisms. However, the role of posttranscriptional regulation is gaining increased recognition in skeletal muscle and other eukaryotic systems. Posttranscriptional events, including transport of mRNA from the nucleus to cytoplasm in the form of a ribonucleoprotein particle (Schwartz and Darnell, 1976), storage of mRNA in repressed RNP form (Darnell et al., 1973), processing of heterogeneous nuclear RNA (hnRNA; Lewin, 1975), degradation of mRNA (Kafatos and Gelinas, 1974), and formation of RNA: RNA hybrids by antisense RNA (Green et al., 1986) have all been shown to control the "availability" of message for translation in eukaryotes. Further, binding of initiation factors to RNA (Grifo et al., 1983), unwinding of RNA secondary structure (Ray et al., 1985), mRNA cap binding protein complex effects (Edery et al., 1984), selection of translational start sites (Kozak, 1984), and regulation of initiation, elongation, and termination factors associated with ribosomes (Moldave, 1985; Weissbach and Ochoa, 1976) have all been shown to regulate translation of mRNAs which are "available."

The role of RNA molecules other than mRNA or rRNA in posttranscriptional control has also been studied. These "control" RNAs are usually of small size, may or may not be associated with protein, and vary in their function and site of action upon posttranscriptional processes. One of these, small nuclear RNAs (snRNA; see Busch and Reddy, 1985 for review), are known to be involved in the processing and transport ofhnRNA and in the binding of regulatory proteins. Low molecular weight cytoplasmic RNAs, which affect translation of mRNA, have been identified as well. These RNAs differ from snRNA in that they are active in vitro and influence translation of mRNAs that are fully processed and available for translation (Sarkar, 1984).

We have isolated an embryo-related poly A+ RNA fraction from embryonic rat skeletal muscle. This 160–2,000-nt fraction has been shown to inhibit in vitro translation of skeletal muscle mRNA in an apparently nonspecific fashion.

Figure 9. Rescue of translational inhibition. Rescue was attempted by addition of additional translatable (adult) RNA or lysate to standard translation assays containing 250 ng adult RNA and 500 ng fraction 4. Controls consisted of translation mixes in the absence of input RNA and translation of adult RNA alone. Adult RNA loads and the percent increase in lysate (vol) are indicated beneath each lane. Two types of experiments were performed for each series, including simultaneous addition of all components and addition of fraction 4 to lysate 15 min before adding adult RNA. See text for details.

Figure 10. Inhibition of initiation by fraction 4 RNA. Synthesis of the first dipeptide of beta globin (f-met-val) in vitro is dependent upon initiation in a reconstructed translation system lacking elongation factors. The dipeptide was quantitated by counting f-met-(H)valine after a 1 h translation at 37°C. Each point represents the average of three determinations. Blanks consisted of reactions containing fraction 4, but lacking beta-globin mRNA; incorporation in blanks has been subtracted from each point shown above. Dipeptide formation by beta-globin mRNA, in the absence of fraction 4, is indicated as zero inhibitor. A dose-dependent decrease in initiation was associated with addition of fraction 4 RNA to the system.

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The ribonucleic acid composition of this inhibitor has been confirmed by differential enzymatic digestion. The inhibitor does not inhibit translation by induction of ribonuclease activity or through the presence of ribonucleoprotein that may have been co-isolated. The inhibitory mechanism is not fully understood at this time, but apparently involves a blockage of the initiation step of protein synthesis. This may ultimately be a consequence of the interaction of inhibitor with message or the translational apparatus, or perhaps through competition for a ribosomal binding site. We propose that this inhibitor functions in translational control of muscle-specific protein synthesis, acting in conjunction with transcriptional regulation of skeletal muscle differentiation.

Low molecular weight inhibitory RNAs, and the RNPs that they associate with, have been isolated from a variety of sources. Eller et al. (1984) has isolated a >4-S RNA from cytoplasmic RNP of human term placenta which is an active inhibitor of translation in vitro. This inhibitor acts either in rabbit reticulocyte lysates or wheat germ extracts to inhibit, nonspecifically, the translation of added mRNA. Activity of the placental inhibitor is not dependent upon the presence of a cap structure, and appears to function by blocking initiation through an interaction with a component of the wheat germ extract. Bag et al. (1980) reported a very similar 4.4-S RNA from embryonic chicken muscle, which is found as a part of a 10-15-S RNP. The RNP molecule and a 4.4-S RNA component were both active as translational inhibitors, but the protein components were inactive in the absence of RNA. However, it should be noted that one protein, a polypeptide with a molecular weight of 36,000 identified in the RNP preparation, was absent from cellular mRNP and its role in posttranscriptional regulation is presently unclear. Another inhibitory RNA (iRNA) has been reported by Sarkar et al. (1981). Isolated from a 10-S iRNP of chick embryonic muscle, the 4-S iRNA was a potent inhibitor of translation in vitro. Winkler et al. (1983) has shown that iRNA inhibits translation at the initiation step by inhibiting mRNA binding to 43-S initiation complexes. He suggested that since iRNA blocks mRNA binding, it is possible that the relative affinity of message or inhibitor for the 43-S initiation complex acts to regulate translation based upon iRNA concentrations in the cell. Although we have not proven that the initiation block seen using our rat skeletal muscle inhibitory RNA is due to the mechanism proposed by Winkler et al., this mechanism would explain the ability of additional message or lysate to rescue translation in our experiments. If we extend this proposed mechanism to include an interaction of inhibitory RNA with the ribosome, causing increased numbers of 43-S initiation complexes, it may also explain the apparent increases in inhibition associated with adding inhibitor to the lysate 15 min before translation. Furthermore, the 40-fold increase in inhibitor purity that we report in the present experiments, would end the need for holding inhibitor and lysate together for extended periods to cause inhibition.

Although the mechanism proposed above may suffice for all inhibitory RNAs that act nonspecifically, it does not explain the mode of action of the myosin-specific tcRNA described by Heywood et al. (1975). tcRNA has most recently been described as a naturally occurring antisense RNA of eukaryotes (Heywood, 1986). Heywood has shown that the 3' end of tcRNA contains significant homology to 5' non-translated sequence of fast myosin heavy chain mRNA from chicken. Cross-linking studies show close association between these RNAs, and Heywood proposes translational inhibition of myosin heavy chain synthesis due to blockage of initiation by RNA: RNA hybridization. tcRNA is isolated from embryonic muscle of chicken, as is iRNA. It is not clear whether a true dichotomy of inhibitor types is present in this muscle or if some aspects of an identical process are misunderstood.

It is particularly interesting to note that many of the small cytoplasmic inhibitory RNAs have been isolated from embryonic sources, such as chicken muscle (Sarkar et al., 1981; Heywood et al., 1975), Artemia embryos (Pirot et al., 1984), rabbit reticulocytes (Dionne et al., 1982), or from glandular tissues such as rat liver (Kuhn, 1982), placenta (Eller et al., 1984), Chinese hamster ovary (CHO; Jelinek, 1978), and lactating guinea pig mammary gland (Bathurst et al., 1980). It is feasible that inhibitory RNAs are transcriptionally expressed based on developmental cues or during a state of secretory activity in a tissue. We have investigated the embryo-related nature of rat skeletal muscle inhibitory RNA by comparison of inhibitory activity of fractions from adult and embryonic sources. We found that inhibitor is present in the embryo, but not in the adult. This may be due to transcriptional control of inhibitor production or posttranscriptional control of inhibitor activity. Endo and Nadal-Ginard (1987) have recently uncovered evidence that protein synthesis in fusion-blocked rat myoblasts is subject to translational control. These authors found that translation of muscle-specific proteins was inhibited in fusion-blocked myoblasts and proposed that Ca++ dependent mechanisms, such as ribosomal protein phosphorylation, are responsible for translational arrest, although the factors involved in the translational control they described have not yet been identified.

What are the advantages of inhibitory translational control to a developing or secretory cell type already under the influence of well-defined transcriptional controls? Transcriptional control in eukaryotes must be far more complex than that of prokaryotes owing to the diversity of proteins produced. If a class or classes of transcript production are suddenly activated within a eukaryotic cell, it may be advantageous to create a "lag time" in which new transcripts can be fully processed and transported or stored in the cytoplasm before translation. Degradation of obsolete messages takes time to occur after cessation of their transcription. Translationally inhibitory RNAs could also serve to balance the amounts of each protein produced in a cell by giving the transcriptional apparatus time to produce the correct proportions of message before translation. These proportions are undoubtedly critical to the structural requirements of a differentiating cell.

We have shown that postfusion myoblasts contain translationally active muscle specific mRNAs at, or near, adult levels, but the production of proteins from these messages is delayed for a number of days, apparently due to translational control. We are currently investigating the quantities of inhibitory RNA in embryonic stages from the time of myoblast fusion to adulthood. We suggest that translational control RNAs, which by themselves are subject to transcriptional and posttranscriptional control, act in conjunction with classical transcriptional and posttranscriptional mechanisms to fine tune the timing of muscle differentiation.
The dipetide assay, used to determine initiation, was performed in the laboratory of Drs. Nat Brot and Bob Schuman at the Roche Institute of Molecular Biology, Nutley, NJ. Their assistance is gratefully acknowledged.

Received for publication 6 November 1987, and in revised form 10 May 1988.

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