THE SYNTHESIS OF MYOSIN mRNA AND MYOSIN
IN THE
EARLY DEVELOPMENT OF XENOPUS LAEVIS EMBRYOS

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Department of ZOOLOGY)

We accept this thesis as conforming
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THE UNIVERSITY OF BRITISH COLUMBIA
July, 1978

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A biochemical approach was used to detect the appearance of the heavy chain of skeletal myosin (HCSM) and myosin mRNA during the early development of *Xenopus laevis* embryos.

An antibody against the HCSM of adult *X. laevis* muscles was biochemically characterized and shown to be monospecific. This anti-myosin antibody reacted with embryonic polysomes synthesizing myosin and with tadpole tail myosin. This indicates that the myosins of adult muscles, early embryonic muscles and tadpole tails are sufficiently homologous to share some antigenic determinants.

Polysomes from various stages of *X. laevis* embryogenesis were reacted with the anti-myosin antibody. Analysis of these reactions showed that myosin synthesis begins in stage 20 embryos, in which about 7 somites have segregated.

The RNA from stage 12, stage 16/17 and stage 20 embryos was then analyzed for the presence of the heavy chain myosin mRNA in order to determine whether the synthesis of myosin is under translational or transcriptional control. Total RNA preparations from staged embryos were fractionated on oligo(dT)-cellulose columns and fractions that did and did not bind were translated in a wheat germ cell-free protein synthesizing system. The translational products were precipitated with the anti-myosin antibody and characterized biochemically. Myosin mRNA was detected by this method in stage 16/17 embryos.

We conclude that somite segregation results in the appearance of
new myosin mRNA molecules in *X. laevis* embryos. It seems likely, by all the evidence considered, that a large pool of untranslated myosin mRNA molecules is not responsible for muscle myosin synthesis. Therefore, the synthesis of certain proteins in early development is under transcriptional control.
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INTRODUCTION

A major goal of developmental biology is to understand the regulatory mechanisms that ensure the synthesis of specific proteins. For the most part, the study of differentiation has become the study of differential gene expression. Such studies have dealt, because of methodological limitations, with the terminal steps of pathways giving rise to highly specialized tissues, such as skeletal muscle.

Efforts to understand the mechanisms that are involved in cell differentiation favor two dominant points for control: (1) Transcriptional control and (2) translational control. Transcription includes the synthesis of the initial precursor mRNA, the processing of this RNA into mature mRNA and its transport to the cytoplasm (Darnell, 1976). Transcriptional control implies that qualitative and/or quantitative changes in the pattern of RNA synthesis lead to alterations in the pool of mRNA sequences available to the protein translating machinery of a cell. Thus, the concentration of a particular mRNA in the cell determines the rate of synthesis of a particular protein.

The term translational control includes situations in which gene expression is modulated by mechanisms which select or activate particular mRNAs from a pre-existing pool of untranslated mRNA. Thus, all cells having the same genes will transcribe all those genes into RNA. Differences among cells would result from the ability of each cell to select which message to translate.

It has been shown in unfertilized eggs of sea urchins that
various species of mRNAs are synthesized and some of them are translated in the cytoplasm. Galau et al. (1976) have demonstrated the existence of so-called "complex class" RNAs (present in 1-5 copies per cell), in the unfertilized sea urchin egg, which probably code for metabolic enzymes (housekeeping enzymes) and so-called "morphogenetic determinants" (Davidson, 1976). It has also been shown that histone and tubulin proteins are synthesized prior to, as well as after, fertilization in the cytoplasm of the sea urchin egg (Davidson, 1976; Gurdon, 1974).

When sea urchin eggs are treated at fertilization with actinomycin D, the embryos are able to undergo cleavage and develop up to the gastrula stage (Terman and Gross, 1965). These results imply that protein synthesis in early development occurs on preformed templates. Hence, it appears that proteins synthesized on complex RNA templates and housekeeping RNA templates (such as histone and tubulin) are under the influence of a post-transcriptional developmental program.

Similar results have been obtained when Xenopus (amphibian) embryos have been treated with actinomycin D (Brachet et al., 1964). Again, cleavage in these embryos does not seem to be affected by the drug, although gastrulation and neurulation are blocked. Furthermore, histones and tubulin are synthesized prior to and after fertilization in Xenopus eggs (Pestell, 1975; Woodland and Adamson, 1977). In addition, Darnbrough and Ford (1976) have shown the existence of very long-lived messages in the cytoplasm of unfertilized Xenopus eggs. Another case of documented translational control in Xenopus embryos concerns the appearance of enzymes involved in the conversion of tyrosine to melanin, via the enzyme tyrosine-DOPA oxidase (Benson and Triplett, 1974; Davidson, 1976). The synthesis of this protein is first detectable at
the neurula stage, well in advance of the appearance of enzyme activity in melanophores at the hatching stage. Thus, it appears that some genes in amphibians are active in the egg and are necessary for early development.

It follows from this discussion that some genes expressed in early development are controlled by some form of translational control.

However, it is possible that genes coding for molecules such as skeletal muscle myosin may not be included in this category since the products of these genes do not make their appearance until after gastrulation (Gurdon, 1974; Davidson, 1976). From all available data, the genes coding for such "cell type-specific" products are single-copy sequences whose mRNA products are found in high concentrations in cells (Galau et al., 1976; Paterson and Bishop, 1977). Therefore, their mRNAs are not part of complex class mRNAs or of mRNAs synthesized from highly repeated genes (histone genes) (Galau et al., 1976; Davidson, 1976; Adamson and Woodland, 1977). Experimental evidence also seems to indicate that messenger RNA synthesis in specialized cells such as the chick oviduct and muscle cells in tissue culture is under transcriptional control (Palmiter, 1975; Paterson and Bishop, 1977); this suggests that this may also be true of embryos (Gurdon, 1974).

We have examined the regulation of myosin gene expression during somite formation in Xenopus embryos. Differentiating muscle in Xenopus laevis embryos undergoes a series of distinct morphological changes. The molecular events which determine and accompany these changes in this embryonic system, as well as in others, have not been fully characterized. Thus, production of myosin and other muscle proteins in X. laevis embryos is a favourable system for studying the molecular
aspects of myogenesis and sequential gene expression during vertebrate development. The first somites in *Xenopus* embryos appear in the head region at the end of neurulation and myosin-like thick filaments are first visible with the light and electron microscope at the early tail-bud stage (Hamilton, 1969; Muntz, 1975; Kullberg et al., 1977). Myoblasts in the somites do not appear to fuse until the onset of metamorphosis (Muntz, 1975). Therefore, the different muscle populations are easily distinguished during the development of *Xenopus* embryos. Furthermore, pre-fusion myoblasts are well-separated in time from fused myoblasts.

**Transcriptional versus translational controls in development.**

Direct evidence for transcriptional and translational controls has come from developmental systems. Intensive studies on erythropoietic cells and the chick oviduct have now provided some of the most convincing evidence for transcriptional control. Translational control appears to be responsible for the quantitative changes in protein synthesis which occur at fertilization in some species. The first evidence for message-specific translational components has come from work on translation of chick myosin mRNA *in vitro*. However, there is also strong evidence that the synthesis of muscle-specific proteins in chick muscle cultures is mainly controlled at the transcriptional level. The purpose of the present discussion is to examine the evidence for the existence of transcriptional and translational controls during development.

Studies in a few well-defined systems indicate that cells in the terminal compartment of certain lineages synthesize proteins and
mRNAs that are qualitatively different from those found in the progenitor lineage cells (Holtzer et al., 1975). Experiments by Axel et al. (1973) and Groudine et al. (1974), for example, provide evidence that chicken hematocytoblast cells do not appear to synthesize globin mRNAs at the same rate as erythroblasts. Similarly, hybridization experiments by these workers, using a labelled cDNA probe complementary to adult globin mRNA, show that if a hematocytoblast transcribes globin mRNA, it is at a rate at least $10^5$ times lower than that transcribed by erythroblasts. It has also been shown that the cDNA hybridized with RNA made by reticulocyte chromatin but not with hRNA made by liver chromatin (Axel et al., 1973). Groudine and Weintraub (1975) also demonstrated that hemoglobin mRNA sequences are not detectable in the total RNA of normal chick fibroblasts. It is clear, therefore, that with respect to the presence or absence of hemoglobin message, the difference among reticulocyte, liver and fibroblast cells is transcriptional in nature. However, long-term growth in tissue culture of chick fibroblasts results in the production of small amounts of globin message as detected by the cDNA probe (Humphries et al., 1976).

The chick oviduct has proved to be an especially useful system for the study of gene expression. Administration of estrogen to a newborn chick results in the differentiation of three distinct epithelial cell types from the homogeneous population of mucosal cells (Palmiter, 1975). One of these cell types, the tubular gland cells, synthesize the protein ovalbumin after about 18 hours. After hormonal treatment is stopped, ovalbumin synthesis declines and becomes undetectable. When estrogen or progesterone (secondary stimulation) is administered a second time, ovalbumin is induced again in the existing tubular gland
cells after a lag of about 3 hours. This induction precedes a second stage of cellular proliferation. Experiments performed on the chick oviduct, which has been secondarily stimulated by steroid hormones to synthesize ovalbumin, show that the increase in the rate of ovalbumin synthesis is quantitatively correlated with an increase in the ovalbumin mRNA content of the oviduct (Palmiter, 1973). Titration of the ovalbumin mRNA sequence, with a cDNA probe complementary to this message, shows that the message is undetectable in the absence of steroids. After secondary hormone stimulation, there are 70,000 molecules of ovalbumin mRNA present per tubular gland cell (Cox et al., 1974; Harris et al., 1975) and most of the ovalbumin mRNA (93%) is present on polysomes.

Recently, Thomas and Palmiter (1977) found that ribonucleoprotein (RNP) particles, isolated from nuclei of estrogen-stimulated chick oviducts, contain ovalbumin mRNA. Upon hormone withdrawal, the concentration of ovalbumin mRNA in the RNP particles rapidly decreases within 24 hours with kinetics equal to the decrease in numbers of estrogen receptors in the nucleus (The numbers of nuclear receptors correlates with the rate of ovalbumin mRNA production). These results imply that ovalbumin mRNA may be stored in RNP particles for a short time prior to translation. However, the essential control for ovalbumin synthesis appears to be transcriptional although secondary controls probably exist at the translational and post-translational levels. This hypothesis is strengthened by the fact that the induction of other proteins specific to the oviduct is mediated by different levels of hormone and has different rate constants (Palmiter et al., 1972). If regulation of ovalbumin and other oviduct-specific proteins was strictly at the translational level, then all the respective mRNAs should be synthesized
at the same rate upon hormone stimulation.

There are other cell types which show similar developmental kinetics to the chick oviduct (Gurdon, 1974; Baker and Shapiro, 1977; Reeves, 1977). For example, vitellogenin mRNA is undetectable in livers of male *Xenopus laevis* (Baker and Shapiro, 1977). For 3 hours after primary estrogen administration, vitellogenin mRNA remains undetectable. However, 4 1/2 hours after hormone stimulation, these mRNA sequences are detectable. Sixty to 65 days after hormone stimulation, the concentration of vitellogenin mRNA molecules drops down to very low levels. The results of these studies imply that the production of vitellogenin is transcriptionally controlled.

The main points to emerge from this review are the following: Gene transcription is controlled in systems whose developmental program is initiated by an external stimulus such as a hormone. However, the hormones can only affect those cells which have, in the course of their differentiation, acquired the appropriate receptors. Thus, these agents enhance a regulatory mechanism which is already present in the cells that respond.

**Translational control in muscle differentiation.**

Evidence for translational control mechanisms during muscle differentiation has come from the work of Gros, Heywood, Sarkar and their colleagues.

A study of RNA synthesis during muscle development has been carried out by Buckingham *et al.* (1974) on primary cultures from fetal calf muscle. Ribonucleic acid extracted from the muscle cells
after pulse-labelling with $[^3]H$-uridine was analyzed on sucrose gradients, and mRNA was identified on the basis of its binding to poly(U) filters. It was observed that a labelled peak of 26S RNA was present in dividing precursor myoblasts. This peak was presumed to be myosin mRNA on the basis of its sedimentation rate. Examination of the cytoplasmic localization of the 26S RNA indicated that it was predominantly non-polysomal. The half-life of the 26S RNA was 10 hours before fusion of cells and increased by a factor of 5-6 just before fusion. After fusion, the 26S RNA was found mostly on polysomes. These data suggest that post-transcriptional controls, acting on messenger stability and utilization, may be important in the terminal differentiation of muscle. The weakness in this argument lies in the failure of these workers to convincingly identify the 26S RNA. The pre-fusion 26S RNA may code for non-skeletal myosin or for a protein other than myosin.

Bag and Sarkar (1976) have isolated a myosin heavy chain mRNP particle (MHC mRNP) from the non-polysomal fraction of homogenates of 14 day-old chick embryonic muscles. They showed that the particle and the protein-free RNA derived from the particle were effective in programming the synthesis of myosin in a reticulocyte cell-free system as judged by gel electrophoresis. Furthermore, it was shown that the mRNP particles exist as 2 distinct types of macromolecular complexes in different fractions of the cytoplasm: a relatively protein-deficient particle in the polysomes where the mRNAs are translated and a protein-rich particle in the non-polysomal compartment where they are not translated. The tissue studied by Bag and Sarkar is differentiated and it is not clear as to whether the non-polysomal mRNA particles are merely present in excess of the translational capacity of the cell.
Another proposal to account for control of protein synthesis, derived from experiments on chicken embryo leg muscle, is that of the existence of message-specific factors for the translation of skeletal myosin. Rourke and Heywood (1972) found that only muscle initiation factors (proteins removed from muscle ribosomes by washing with salt) were capable of directing myosin heavy chain synthesis and that reticulocyte factors could not substitute for muscle factors when salt-washed muscle ribosomes were used. Since the initiation factors isolated by Heywood and colleagues were not shown to be completely pure, it is possible that other factors present in the ribosomal salt wash might act selectively to allow completion of the intact myosin molecule, which otherwise might be produced only as an undetected fragment. It will be shown later in this thesis that the problem of premature termination of polypeptide chains in cell-free systems has to be considered (Atkins et al., 1975). Heywood and Kennedy (1976) have also suggested that a substance with some of the properties of RNA (tcRNA) was isolated from dialysates of muscle initiation factors and this tcRNA can inhibit translation of myosin mRNA in cell-free systems. However, questions concerning the degree of specificity of this reaction and the function of this tcRNA in vivo remain to be answered.

Is myosin synthesis regulated at the translational level? The experiments presented in this section do not provide an answer to this question. Translational control as a primary mechanism for the regulation of myosin synthesis presupposes that myosin mRNA exists in dividing muscle precursor cells in an unstable form or in an untranslatable form. When the muscle precursor cells stop dividing, the myosin mRNA
would be stabilized or activated. Such a mechanism cannot be unambiguously ruled out. However, a translational mechanism of this sort cannot account for the rapid increase in myosin mRNA observed during differentiation in vitro (Strohman et al., 1977). It has been clearly established that *Xenopus* oocytes and eggs have no mechanism by which they can exclude the translation of messages characteristic of other cell types (Gurdon, 1974; Laskey et al., 1977). It seems likely that translational control is used to make quantitative adjustments to a pattern of protein synthesis determined primarily by the synthesis of new messages (Gurdon, 1974).

**Transcriptional control in muscle differentiation.**

The experiments described in this section appear to demonstrate that the essential control for myosin synthesis is transcriptional, although secondary controls may exist at the translational and post-translational levels.

Holtzer et al. (1957) showed, by the use of fluorescein-labelled antibodies, that skeletal myosin synthesis is temporally correlated with the segregation of somites in chick embryos in vivo. This implies that skeletal myosin is not synthesized in muscle precursor cells. However, this result has to be interpreted with caution since the myosin antibody may have not been able to detect small quantities of myosin. Subsequently, Bischoff and Holtzer (1970) showed that muscle precursor cells cultured in vitro synthesize a myosin heavy chain that is electrophoretically similar to myosin isolated from myotubes. Furthermore, myosins from muscle precursor cells or such non-myogenic cells as fibroblasts, nerve cells, smooth muscle cells or gut epithelial
cells do not react with skeletal muscle antibody in Ouchterlony double-diffusion tests (Chi et al., 1975; Holtzer et al., 1976). However, myosins from non-dividing myoblasts, myotubes and mature muscle do react with skeletal myosin antibody. These results suggest that muscle precursor cells synthesize myosins that are the products of structural genes different from those that transcribe myosin mRNAs in post-mitotic myoblasts.

A study of RNA synthesis during myogenesis has been carried out by Strohman et al. (1977) on primary cultures of 12-day chick embryo breast muscle. Ribonucleic acid extracted from pre-fusion and post-fusion cells was translated in a reticulocyte assay system and the translational products were precipitated with an antibody against skeletal muscle myosin. It was found that unfused cultures of cells contained a small amount of myosin mRNA, which could be synthesized by the small amount of precocious myotubes and myoblasts present in unfused cultures. In fused cultures, however, they found a 30-fold increase in myosin mRNA activity. Thus, this result suggests that myoblast fusion, which is an early and easily recognized phenotypic manifestation of muscle differentiation, is associated with synthesis of myosin mRNA and myosin. Paterson and Bishop (1977) analyzed the sequence complexity, frequency distribution and coding capacity of the mRNA populations of primary chick embryo muscle cultures at different stages of myogenesis. Pre-fusion cultures and fused myofibrillar cultures all contained 17,000 different mRNA sequences. The myofibril cultures also contained about 2500 sequences in higher concentration and six sequences in exceptionally high concentration, each present in about 15,000 copies per nucleus. These sequences were shown to be 10 times less common in pre-myogenic
cultures. The concentration of these sequences in myofibrillar cultures correlated well with the capacity of the mRNA to stimulate the cell-free synthesis of muscle-specific proteins such as myosin, as judged by comparison of tryptic peptides of cell-free synthesized myosin and authentic myosin.

The evidence presented in this section strongly supports the conclusion that under both in vivo and in vitro conditions, myogenesis is probably regulated by transcriptional rather than translational control mechanisms. The skeletal myosin synthesized by pre- and post-fusion chick embryo myoblasts has been characterized by precipitation with skeletal myosin heavy chain antibody and by tryptic peptide analysis. The 26S RNA coding for this embryonic skeletal myosin has been translated in cell-free systems and the products characterized by antibody precipitation and by tryptic peptide fingerprinting. Therefore, chick myosin mRNA does not appear to be stored in precursor muscle cells in an inactive form. However, these results do not rule out changes in the stability of selected mRNA sequences as the mechanism regulating the concentration of mRNA in the cytoplasm.

Myogenesis in Xenopus laevis embryos.

In Xenopus embryos, the first indication of somite formation is found at stage 17, the late neural fold stage (Nieuwkoop and Faber, 1967). The somites are masses of mesodermal cells with a small central cavity. The dorsal part of the inner wall of the somite is the source of somatic muscle in a vertebrate's body and is called the myotome. The cells of the myotome are originally orientated with their long axes vertical to the body axis and are arranged around a central cavity
called the myocoelic cavity. The process of somite segregation progresses in a cranio-caudal direction. For example, by stage 20, when the neural folds are fused, six to seven anterior somites have been individualized. As the somites become segregated from the somitic mesoderm, their cellular differentiation also proceeds in a cranio-caudal direction. Hamilton (1969) observed that the myocoel is obliterated before segmentation starts and that the final longitudinal orientation of myoblasts is achieved by a rotation of the myotome cells through \( 90^\circ \).

The gradual rotation of the myotome cells can be seen in sequence along the axis, since it starts in the most cranial somites and follows on in a cranio-caudal sequence through the series. Between stage 20, when the neural tube has closed and stage 22, when the eyes are beginning to protrude, there is no sign of striated myofibrils in the somites when viewed with the light microscope. Also, the embryo does not respond to any form of stimulation, implying that the myotome cells are incapable of contraction (Muntz, 1975). By stages 22 to 24, the embryo contains 12-15 somites and a few thin myofibrils are visible by light microscopy in the myotomes. At these stages, the embryo responds to direct mechanical and electrical stimulation in the neck region. By the time the embryo is free-swimming (stages 32-46), the myotome cells contain myofibrils but remain uninucleate. It is only at the onset of metamorphosis (stages 48-50) that myotome cells become multinucleate, possibly by fusion with satellite cells at the ends of the fibres (Muntz, 1975). This occurs in myotome muscles long after contractility and nervous control have appeared. Thus, the myotome cells of *Xenopus* develop to a fully functional state and large size.
while they remain uninucleate (Muntz, 1975). In the early stages of metamorphosis, when the myotome muscle cells become multinucleate, the hind limb musculature also develops, but its structure is quite different from the myotome muscle because it appears to be multinucleate from the very onset, when the striated myofibrils first appear (Muntz, 1975). Therefore, in the myotome muscle cells the establishment of contractility and multinucleation (that is, fusion) are not causally related as may be the case with hind limb muscle.

The preceding description of *Xenopus* embryo myogenesis raises several questions:

1. Do presumptive myoblasts, that is, those cells present in the somites prior to segregation, synthesize all the molecules present in a functional myoblast but at lower levels?

2. When in embryonic development is it possible to detect skeletal myosin mRNA and myosin protein?

3. Since many non-myogenic cells synthesize actin and myosin, are these proteins the product of the same gene(s) as skeletal muscle myosin?

4. Is the myosin synthesized in the myotome cells of *Xenopus* embryos prior to fusion, the product of the same gene(s) as the myosin of adult skeletal muscle?

5. Is the transcription and translation of skeletal myosin mRNA coupled with fusion of myoblasts?

The present study was designed to be an exploratory base for answering some of the questions asked in this review. A monospecific antibody against the heavy chain of adult *Xenopus* skeletal muscle myosin
was prepared and characterized. The antibody was then used to
demonstrate the developmental appearance of myosin mRNA and myosin
protein in *Xenopus* embryos. It appears that myogenesis in *Xenopus*
somites is regulated at the level of transcription.
MATERIALS AND METHODS

Chemicals.

Unless otherwise stated, all chemicals used were of highest purity and were obtained either from Sigma, Fisher or Mallinkrodt. Phenylmethylsulfonylfluoride (PMSF) was obtained from Sigma. Enzyme grade ammonium sulphate was obtained from Schwarz/Mann. DEAE-cellulose and Sepharose 4B were obtained from Pharmacia. Ribonuclease-free sucrose was obtained from Schwarz/Mann. Diethylpyrocarbonate was obtained from Sigma. Acrylamide and N,N-methylene bisacrylamide came from Eastman Organic Chemicals. Sodium dodecyl sulphate (SDS) was obtained from BDH. Na$^{125}$I was purchased from New England Nuclear.

Preparation of skeletal myosin.

Myosin was prepared from the back and superficial thigh muscles of *X. laevis* adult frogs by the procedure of Wikman-Coffelt (1973) with some modification. The tissue was minced in 2.5 volumes of wash buffer (5 mM sodium phosphate (pH 7.0)), 1 mM EDTA, 1 mM sodium pyrophosphate, 1 mM PMSF and 5 mM dithiothreitol (DTT) and homogenized in a Sorvall Omni-mixer at full speed. The homogenate was centrifuged at 9,000 x g for 5 minutes and the resulting pellet washed twice more in wash buffer followed by centrifugation at 9,000 x g for 5 minutes. Most of the soluble proteins were removed by this procedure. The pellets from the above centrifugations were then extracted with 1 volume of 50 mM sodium phosphate buffer (pH 7.5), 0.5 M KCl, 10 mM sodium pyrophosphate,
5 mM ATP, 5 mM DTT, 0.2 mM PMSF and 1 mM EDTA. After 10 minutes on
ice, the suspension was centrifuged at 20,000 x g for 20 minutes. The
pellet was re-extracted and the supernatants were combined. Those
supernatants are called the "actomyosin fraction". The supernatants
were made 38% in ammonium sulfate at 4°C and the fractions precipitating
between 38% and 45% saturation with (NH₄)₂SO₄ were collected. The
precipitation with ammonium sulfate was repeated once more and the
precipitates were dissolved in the necessary buffers made 50% v/v in
glycerol and stored at -20°C.

ATPase activity.

For myosin ATPase determinations, myosin in 50% (v/v) glycerol was
precipitated at low ionic strength (9 volumes of 1 mM Tris-Hcl (pH 7.0),
1 mM EDTA and 0.2 mM PMSF) and resuspended in 0.05 M sodium pyrophosphate
(pH 7.5), 0.5 M NaCl, and 1 mM DTT. The solution was applied to a 1.4
cm x 50 cm column of DEAE-cellulose (Whatman DE-52), equilibrated with
the same buffer. Myosin was eluted with a linear gradient of 0 to
0.5 M NaCl in 20 mM Na pyrophosphate, pH 7.5. Fractions collected were
scanned at A₂₅₀ nm and the myosin peak was identified by sodium dodecyl
sulphate/gel electrophoresis (SDS/GE). For some determinations, myosin
was applied to a 1.4 cm x 50 cm column of Sepharose 4B (Pharmacia)
equilibrated with 0.6 M NaCl, 10 mM sodium phosphate (pH 7.5), 1 mM
sodium pyrophosphate, 1 mM sodium azide, 2 mM DTT. Eluted fractions
were collected and monitored for myosin as previously.

ATPase activity of the column-purified myosin was measured by
incubating samples with 2 mM ATP, 10 mM Hepes (pH 7.0), 0.6 M KCl and
either 2 mM EDTA, 10 mM CaCl₂ or 5 mM MgCl₂. The rate of orthophosphate
production was measured by the method of Taussky and Schorr (1972) and was linear with time for up to 1 hour. Column-purified myosin had a specific activity ($\mu$moles Pi/mg or protein/min) of 2.0 in the presence of EDTA, 0.45 in the presence of Ca$^{2+}$ and no activity was detectable in the presence of Mg$^{2+}$. Protein determinations were carried out by the method of Lowry et al. (1951), using appropriate protein reference standards.

**Biological methods.**

Embryos were obtained and handled using procedures described by Gurdon (1967) and staged according to Nieuwkoop and Faber (1967). Embryos were dejellied with 2% cysteine-HCl (pH 7.8) containing 0.001% each of sodium benzyl penicillin and streptomycin sulfate, washed in sterile pond water and frozen at -70°C.

**Preparation and fractionation of polysomes.**

Polysomes were prepared essentially as described by Woodland (1974). The homogenization buffer contained 0.3 M KCl, 10 mM MgCl$_2$, 200 mM Tris (pH 7.4, at room temperature), 4 $\mu$g/ml of polyvinyl sulphate, 0.13% of diethylpyrocarbonate (DEP) or 50 $\mu$g/ml of heparin. All solutions were sterilized with 10 $\mu$l/100 ml of DEP. Excess DEP was destroyed by warming at 70°C for 1 hour.

Homogenates were prepared with embryos which had been stored at -70°C. 500-2000 embryos were homogenized in 5-15 ml of homogenizing buffer using a loose-fitting Dounce homogenizer. After five strokes with the pestle, the homogenate was brought to 0.5% in sodium deoxycholate and NP-40. Three to four more strokes with the pestle were
followed by centrifugation at 12,000 x g for 5 minutes at 4°C. The supernatant fluid remaining after centrifugation was layered onto 2.0 ml of homogenization buffer containing 2.5 M sucrose and was centrifuged in a Beckman SW 27 rotor at 25,000 RPM for 4.0 hours. Pelleted polysomes were resuspended in homogenization buffer and layered over a 20-50% sucrose gradient (12.0 ml). The gradients were centrifuged at 20,000 RPM for 2 hours in the Beckman SW 27 rotor at 4°C. Gradients were monitored for absorption at 254 nm in a LKB flow cell or fractions were collected and manually scanned in the model 2000 Gilford spectrophotometer.

When Xenopus liver or chick embryo thigh muscle homogenates were prepared, fresh tissue was used and the polysomes were isolated as described above.

**Immunoprecipitation of polysomes.**

Immunoprecipitation of polysomes was performed as described by Palmiter et al. (1972) and Rhoads et al. (1973). Sucrose gradient fractions were suspended in immunoprecipitation buffer (0.6 M NaCl, 5 mM MgCl₂, 10 mM Tris, pH 7.5) and reacted with 250 µg of [¹²⁵I]-anti-myosin antibody dissolved in the same buffer. After 12 hours of incubation at 4°C, pure antigen and unlabelled antibody were added to the reaction mixture and incubated for 12 hours. The precipitated complex was centrifuged three times through a discontinuous gradient of 0.5 M and 1.0 M sucrose (1% Triton X-100, 1% sodium deoxycholate, 0.6 M NaCl). The pellets were washed in immunoprecipitation buffer containing 1% Triton X-100 and 1% sodium deoxycholate, digested with 0.7 ml of Protosol (New England Nuclear) overnight and counted in a scintillation
counter.

Iodination of proteins.

Iodination of myosin and anti-myosin antibody was performed as described by Palacios et al. (1972). The specific activity of myosin ranged from $9 \times 10^6$ cpm/mg of protein to $9 \times 10^9$ cpm/mg of protein. For anti-myosin antibody the specific activity ranged from $4 \times 10^7$ cpm/mg of protein to $1.0 \times 10^9$ cpm/mg of protein (45% counting efficiency).

Preparation of anti-myosin antibody.

Ammonium sulfate-fractionated myosin or column-purified myosin was dissolved in Laemmli electrophoresis sample buffer (Laemmli, 1970) and 2.5 mg of the material was applied to a preparative 5% SDS-polyacrylamide slab gel (Laemmli, 1970). After electrophoresis, side strips were cut from the gels and stained with Coomassie Blue to determine the location of the myosin bands. Alternatively, the whole gel was stained with Coomassie Blue. The regions of the gel containing the myosin band were cut out, and the protein was eluted from the gel by electrophoresis of the gel slice, which was packed into a disposable Pasteur pipette covered at the anode end with a dialysis bag (Reeves, personal communication). The protein purified in this manner was homogeneous when analyzed on a second SDS-polyacrylamide gel and migrated with the same mobility (210,000 daltons) as standard myosin. No C proteins or M proteins appeared in the eluted myosin bands.

Antiserum to the electrophoretically-purified heavy chain skeletal myosin was prepared by inoculating rabbits by standard
procedures (Clausen, 1969). Primary injections into three rabbits were
done in the presence of Freund's complete adjuvant and were performed
intramuscularly. Secondary injections in the presence of incomplete
adjuvant were given subcutaneously. Serum was tested for anti-myosin
activity by immunodiffusion against crude muscle homogenates and
electrophoretically-purified myosin. Gammaglobulin was prepared from
the rabbit serum according to published methods (Clausen, 1969) and,
when necessary, freed of ribonuclease by passage over combined CMC-
DEAE-cellulose columns according to Shapiro et al. (1974). After repeated
ammonium sulphate precipitations the serum gammaglobulin fraction was
dissolved in water, lyophilized and stored at -20°C. For use, portions
of the dry powder were dissolved in appropriate buffers at concentrations
of 30 to 50 A_{280} units of protein per ml.

Gel electrophoresis.

Polyacrylamide slab gels were polymerized and run according to
the method of Laemmli and Favre (1973). Stacking and separating gels
were made from stock solutions containing one part of bisacrylamide to
37.5 parts of acrylamide (recrystallized). Separating gels were used
24 hours after manufacture. Samples were diluted with 63 mM Tris-HCl
(pH 6.8), 3% sodium dodecyl sulphate, 1% 2-mercaptoethanol, 10% glycerol
and 0.002% Bromophenol Blue. The amount of protein in the samples was
always determined to insure that the SDS to protein ratio was in excess
of 1.4 g of SDS to 1 g of protein. Electrophoresis was carried out at
room temperature for 30 minutes at 50 volts, then at 100 volts for about
5 hours and stopped when the Bromophenol Blue front had migrated 10 cm
into the separating gel.
When concentrations of proteins or myosin were less than 1 mg/ml in a volume of less than 30 μl, the SDS-acrylamide electrophoretic procedure of Paterson and Strohman (1972) was adopted. The use of a stacking gel was omitted. The pH of the sample buffer was raised from 6.8 to 8.6. The samples were heated in a boiling water bath for 4 minutes instead of 1 minute. Under these conditions (higher pH of the sample buffer and heating of the sample for longer than 1 minute) myosin preparations do not precipitate as much at the origin of the SDS-acrylamide gel. Electrophoresis was carried out at 45 volts for about 12 hours and stopped when the Bromophenol Blue front had migrated 10 cm into the gel. Gels were stained for 1-4 hours in 0.25% (w/v) Coomassie Blue, 35% (w/v) methanol, 10% (w/v) acetic acid and destained in 35% (v/v) methanol, 10% (w/v) acetic acid. For preparative gels, it was important to stain and destain the gels in the minimum amount of time to prevent damage to proteins. Gels were placed in 5% (v/v) methanol, 7.5% acetic acid in order to restore the original size. Gel slabs were dried for autoradiography.

**Protein determination.**

Protein concentrations were estimated by the method of Lowry et al. (1951), using bovine serum albumin as the protein standard (Sigma).

**Molecular weight determinations.**

The apparent molecular weight of myosin heavy chains was determined by relative mobilities upon the same 5% or 7.5% acrylamide-SDS gel with the following proteins as molecular weight standards: rabbit skeletal muscle myosin (212,000), *E. coli* β-galactosidase (130,000),
and rabbit skeletal muscle phosphorylase b (92,000). The relative molecular weight of the heavy chain of embryonic and adult myosin was determined to be about 210,000.

**Amino acid analysis.**

Samples were hydrolyzed in 6N HCl in vacuo at 110°C for 18, 24 or 48 hours. Hydrolysates were analyzed on a Beckman amino acid analyzer.

**Partial tryptic digestion of immunoprecipitated myosin and purified myosin.**

The crude actomyosin fraction (see preparation of skeletal myosin) was precipitated at low ionic strength (9 volumes of distilled water containing 2 mM EDTA) and pelleted by centrifuging at 40,000 x g for 10 minutes. The pellet was dissolved at a concentration of less than 1 mg/ml of protein in a buffer containing 10 mM sodium phosphate (pH 7.8), 0.3 M sodium phosphate and iodinated as described above. The reaction products were then dialyzed for twelve hours against a buffer containing 0.5 M NaCl, 0.015 M Tris-HCl (pH 7.5) and 0.1 M PMSF. The dialyzed proteins were then lyophilized and resuspended in less than 1 ml of the above dialysis buffer. Myosin was then precipitated with anti-myosin antibody (see above) and the precipitate was washed by centrifugation through a discontinuous sucrose gradient (see above). The myosin-antibody complex was then electrophoresed on a SDS-acrylamide gel and, after staining with Coomassie Blue, the SDS-acrylamide gel was exposed to X-ray film. After autoradiography, the putative myosin bands were cut out from the gels and the myosin was eluted from gel slices by electrophoresis in a Pasteur pipette (see section on preparation of
anti-myosin antibody). The contents of the dialysis bag were then precipitated with 10% trichloroacetic acid to remove most of the SDS (Weber and Osborne, 1975). This procedure was repeated twice. Finally, the myosin was dissolved at a concentration of 1 mg/ml in 0.6 M NaCl, 0.015 M Tris-HCl (pH 7.4), 0.1% B-mercaptoethanol and mixed with trypsin at 0.005 the weight of myosin (Burridge and Bray, 1975). The digestion was allowed to proceed at 37°C for 30 minutes and was stopped by boiling in SDS gel sample buffer (see section on electrophoresis). Non-denaturing conditions were used for proteolysis because under these conditions trypsin produces fragments large enough to be separated by electrophoresis which are distinctive for a large number of myosins (Biro et al., 1972). Of course, it is impossible to specify whether tryptic peptides of myosins arise from primary, secondary or tertiary structures of myosin. Electrophoresis was carried out in 10% SDS-acrylamide. Column- and gel-purified myosin was iodinated and trypsinized in the same way as immunoprecipitated myosin and was run on the same SDS-acrylamide gels for comparisons.
RESULTS A

Characterization of anti-myosin antibody.

Anti-myosin immunoglobulins were prepared from serum of rabbits immunized with electrophoretically purified myosin. In these experiments, it is important that the antibody monospecifically recognize only the heavy chain of myosin because any other cross-reacting muscle proteins might be synthesized at different developmental stages. To ascertain that the immunogen which runs on SDS-acrylamide gels with a molecular weight of 210,000 daltons is indeed myosin, the following tests were performed: 1) The ATPase activity of a chemically purified myosin preparation was measured. 2) The amino acid composition of the putative myosin was compared with that of another vertebrate. 3) The reaction of purified myosin and crude muscle extract was compared by immunodiffusion (Clausen, 1969). 4) Crude muscle extracts were labelled in vitro with $^{125}\text{I}$ using lactoperoxidase (Palacios et al., 1972) and precipitated with anti-myosin antibody. The precipitated antigen/antibody complex was run on SDS-acrylamide gels and the radioactive protein compared to authentic myosin bands obtained on SDS-acrylamide gels after immunoprecipitation and the resulting peptides compared to those derived from purified myosin.

The assay for ATPase activity in 0.6 M KCl and various ions was used to follow the purification of myosin from adult frogs. The relative ATPase activities of adult frog skeletal myosin (column-purified) and skeletal muscle myosins obtained from other sources (literature
values) at 25°C (pH 7.5) in the presence of various ions are summarized in Table 1. In the presence of EDTA, *Xenopus* myosin ATPase was higher than that of rabbit red muscle but lower than that of rabbit white muscle. Similar results were obtained in the presence of Ca$^{2+}$. These results indicate that the myosin was probably extracted from muscles which contained a mixture of red and white muscle fibres. In the presence of Mg$^{2+}$ no ATPase activity was detectable, indicating that actin was not co-purified with the myosin. It can be seen in Fig. 1 that column-purified myosin contained little detectable actin. At 2 mM ATP the *Xenopus* muscle enzyme had an activity double that at 1 mM ATP. On the basis of the enzyme activity studies, the myosin heavy chain band identified on SDS gels appears to be skeletal muscle myosin.

Table 2 summarizes the results of two amino acid analyses performed on the heavy chains of muscle myosin isolated from SDS gels. The results indicate that the amino acid composition of skeletal myosin from *Xenopus* is similar to the amino acid composition from rabbit skeletal muscle.

The reaction of the anti-myosin antibody with purified or with crude muscle extracts gave precipitation lines of identity, and no spur lines were observed as shown in Fig. 2. By this criterion, the anti-myosin antibody is monospecific for the heavy myosin chain. Furthermore, fluorescein-labelled anti-myosin antibody reacts specifically with glycerinated muscle slices, while non-immune serum shows no such reaction (Fig. 3). This indicates that the anti-myosin antibody recognizes antigenic determinants present on the gel-purified myosin, on the high salt-extracted myosin and on fixed tissue slices.

In another test for monospecificity, adult frog muscle protein
was extracted in 0.6 M NaCl and labelled \textit{in vitro} with $^{125}{\text{I}}$ using the procedure of Palacios \textit{et al.} (1972), and then was incubated with non-immune, or anti-myosin antibody. After pelleting of the myosin-anti-myosin complexes, the precipitates were analyzed by SDS-acrylamide electrophoresis, followed by X-ray autoradiography as can be seen in Fig. 4. Precipitation of the muscle proteins with anti-myosin antibody resulted in the appearance of radioactive myosin on the SDS gels and no other protein bands except occasionally low molecular weight species (which may represent degraded proteins) migrating with the Bromophenol Blue front were observed on the gels. When increasing amounts of the muscle extract were added to the antibody reaction mixture, the resulting immunoprecipitates were not contaminated with new proteins. No labelled proteins appeared on gels when non-immune sera were used. Fig. 5 shows an X-ray autoradiograph of a one-dimensional electrophoretic tryptic peptide map of pure myosin and antibody-precipitated myosin labelled with $^{125}{\text{I}}$. It appears that the labelled tryptic fragments of antibody-precipitated myosin co-electrophorese with the labelled tryptic fragments derived from pure myosin. It is not possible to obtain completely identical peptide maps since from one experiment to another the specific activity of trypsin may vary and there may be subtle changes in incubation temperature.

\textbf{Quantitative precipitation of myosin.}

A precipitin curve was used to confirm the results of the Ouchterlony immunodiffusion tests and the analysis of immunoprecipitates in SDS-acrylamide gels.

The precipitin curve obtained with gel-purified $^{125}{\text{I}}$-myosin
and anti-myosin is shown in Fig. 6. The curve shows a classical precipitin curve with one peak, indicating that only one antigenic species is present (Clausen, 1969). Addition of increasing amounts of radioactive myosin to a constant amount of anti-myosin antibody (1 mg) results in the formation of an antibody excess region (below 75 μg), an equivalence zone at 75 μg, and finally a region of antigen excess in which the immunoprecipitate becomes progressively soluble. As explained in Clausen (1969), rabbit antibodies when present in excess will quantitatively precipitate an antigen (especially a large one such as myosin). In the equivalence zone, all free antibody and antigen form a complex, which is readily precipitable. As more antigen is added, the complex becomes eventually soluble because excess antigen forms smaller aggregates which are not readily precipitated.

According to Fig. 6, quantities of myosin less than 75 μg were quantitatively precipitated when added to a constant amount of anti-myosin antibody. For example, when the amount of radioactivity precipitated by anti-myosin antibody was compared to the amount precipitated by ice-cold 20% trichloroacetic acid (TCA), the results agreed within 10% for amounts of myosin in the nanogram range. It was possible, using this radioimmunoassay, to reliably detect quantities of myosin equal to 0.05-0.1 ng.

Since the antigen-antibody reaction is affected by various physico-chemical events, several additional controls were performed on the myosin anti-myosin reaction. When [125I]-myosin was mixed with a high salt extract of Xenopus liver cells under the same conditions as described in the legend to Fig. 6, an immunoprecipitation curve identical to the one in Fig. 6 was obtained. When a high salt extract
of tadpole tails was iodinated and reacted with anti-myosin antibody, again a curve identical to the one in Fig. 6 was obtained. This result indicates that embryonic muscle and adult muscle myosins share similar antigenic determinants.

The effect of reaction volume on the precipitation of myosin was determined by diluting 1 mg of antibody and 75 µg of $^{125}$I-myosin with various amounts of reaction buffer. Reduced precipitation occurred when the reaction volume was increased beyond 1.0 ml. The presence of 0.75 M sucrose, a compound used in the isolation of polysomes, in the reaction mixture reduced the amount of $^{125}$I-myosin precipitated at the equivalence point to 88% of the value in its absence.

The anti-myosin antibody thus appears to monospecifically react with the heavy chain of skeletal muscle myosin and is able to precipitate myosin from crude muscle extracts. By these criteria the anti-myosin antibody can be used to precipitate myosin-synthesizing polysomes and to detect low levels of skeletal myosin protein.

Isolation and analysis of embryonic polysomes.

Typical polysome profiles from stages 12, 16/17 and stage 20 *X. laevis* embryos are shown in Figs. 7a, b and c. The polysomes were isolated in the presence of sodium deoxycholate, NP-40, 0.3 M KCl and 0.2M Tris-HCl. The salt and the detergents increase the yield of embryonic polysomes (Woodland, 1974). The high salt concentration (0.3 M KCl) in the homogenization buffer has two effects. First, it appears that high salt dissociates *X. laevis* embryonic monosomes which are not associated with mRNA (Woodland, 1974). Indeed, Fig. 7 shows that the polysome pattern of stages 12 and 16/17 embryos is dominated by subunits.
Secondly, since myosin is soluble at high ionic strength, the use of a high salt concentration should increase the yield of myosin polysomes (Sarkar, 1976).

The 60S subunit and the 80S monosome are not well separated in these profiles because the gradients are overloaded in order to increase the yield of polysomes (the arrow in each panel of Fig. 7 indicates the position of the monosome peak). Whether overloading of gradients causes polysome aggregation and sedimentation to the bottom of centrifuge tubes will be discussed when data on the immunoprecipitation of polysomes is presented below.

When the polysome homogenizing buffer was made 0.13% v/v in diethylpyrocarbonate (DEP), identical profiles were obtained, suggesting that degradation of polysomes did not occur. The DEP was added to the homogenizing buffer to inactivate nucleases that might cause polysome degradation. When stage 20 homogenates were treated with RNase, the sedimentation pattern shifted to that of subunits (Fig. 8). Treatment of stage 12 and stage 16/17 homogenates with RNase produced no change in the sedimentation pattern. The evidence presented here indicates that the area of the gradients present in Fig. 7a, b and c between fractions 6 and 12 represents polysomes. Furthermore, isolated fractions of these regions of the polysomal gradients incorporated $[^3H]$-leucine when added to the 100,000 x g supernatant from a wheat germ cell-free protein synthesizing system, again suggesting the polysomal nature of this region.

**Binding of anti-myosin antibody to embryonic polysomes.**

The anti-myosin antibody described in the preceding section was
used to determine at which stage of embryonic development skeletal-like myosin is first synthesized on polysomes. Polysomes were isolated from different embryonic stages and separated on sucrose density gradients by centrifugation as described in Materials and Methods. After centrifugation, each polysome fraction was reacted with $^{125}$I-labelled antibody and the precipitates were washed by centrifugation through a discontinuous sucrose gradient (see Materials and Methods).

The results of binding of labelled antibody to polysomes from different stages of embryonic development are shown in Fig. 7a, b and c. At stages 12 (Fig. 7a) and 16/17 (Fig. 7b) radioactivity is present at a low level throughout the gradients and represents non-specific binding. Fig. 7c shows that at stage 20 the myosin antibody is binding with rapidly sedimenting polysomes. The width of the putative myosin peak observed in Fig. 7c may be attributed to variable ribosome spacing, incomplete loading onto the mRNA and minor aggregation (Latham and Darnell, 1965). The fact that the peak spreads into the area of the polysome profile which has polysome sizes of less than 30S may be due to degradation. To demonstrate that this antibody binding is stage-specific, two further tests were used to assess the presence or absence of myosin in embryos. In the first test, the reaction of high salt extracts of stage 12, stage 16/17, stage 20 and stage 26 embryos with anti-myosin were compared by immunodiffusion (Fig. 9). Only extracts of stage 20 and stage 26 embryos gave precipitin lines. In the second test, crude extracts of the above stages were analyzed by SDS-gel electrophoresis (Fig. 10). Again, these gel patterns indicated that detectable myosin appeared only in extracts of stage 20 and stage 26 embryos. In a few cases a band with a molecular weight of 210,000
appeared on SDS-acrylamide gels from extracts of stages 1-5 and stages 9-13 embryos (Brock, unpublished observations). Since these bands could not be immunoprecipitated, we assume that they represent non-muscle myosin or some other protein.

**Polysome reaction control experiments.**

A number of control experiments were performed to test the specificity of the anti-myosin antibody-polysome binding reaction. For example, when polysome preparations from stage 20 or 26 embryos were first reacted with cold anti-myosin antibody, this resulted in the abolishment of the $^{125}\text{I}$-labelled anti-myosin binding. This indicates that the sites for binding anti-myosin antibody on the myosin molecule can be saturated. Other controls also indicated that sera from non-immunized rabbits or anti-ovalbumin antibody from rabbits do not inhibit the binding of labelled anti-myosin antibody to the polysomes. Furthermore, labelled anti-ovalbumin antibody does not bind to stage 20 polysomes.

Labelled anti-myosin antibody does not bind to *Xenopus* liver polysomes when they are mixed 1:1 with the 100,000 x g supernatant from stage 20 embryos (Fig. 11). Furthermore, another control, shown in Fig. 12, indicates that exogenously supplied myosin does not appear to contaminate polysomes when it is added to the embryo supernatant prior to sucrose gradient fractionation. In this situation, the free myosin remains in the supernatant near the top of the gradient and reacts with the anti-myosin antibody. And finally, treatment of stage 20 embryo homogenates with RNase prior to sedimentation results in the polysomal profile shown in Fig. 8. As would be expected, labelled anti-myosin
binds in this case to the monosome and supernatant regions of the gradient.

These results indicate that myosin-synthesizing polysomes are first observed around stage 20 of *Xenopus* embryonic development. This implies that myosin is synthesized at stage 20. All controls performed indicate that the anti-myosin antibody is monospecific for skeletal myosin, that it binds specifically to polysomes synthesizing myosin, and that it does not bind to non-skeletal muscle myosin polysomes.
TABLE 1

ATPase activity of *Xenopus laevis* skeletal myosin.\(^a\)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Myosin-specific ATPase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X. laevis</td>
</tr>
<tr>
<td></td>
<td>WM</td>
</tr>
<tr>
<td>1. 1 mM EDTA</td>
<td>2.0</td>
</tr>
<tr>
<td>0.5M KCl</td>
<td></td>
</tr>
<tr>
<td>1 mM ATP</td>
<td></td>
</tr>
<tr>
<td>2. 1 mM EDTA</td>
<td>3.9</td>
</tr>
<tr>
<td>0.5M KCl</td>
<td></td>
</tr>
<tr>
<td>2 mM ATP</td>
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</tr>
<tr>
<td>3. 10 mM Ca(^{2+})</td>
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</tr>
<tr>
<td>0.5M KCl</td>
<td></td>
</tr>
<tr>
<td>2 mM ATP</td>
<td></td>
</tr>
<tr>
<td>4. 5 mM Mg(^{2+})</td>
<td>0</td>
</tr>
<tr>
<td>2 mM ATP</td>
<td></td>
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</table>

\(^a\)All reaction mixtures contained 0.2 mg of protein/ml. Reactions were initiated by the addition of ATP and incubated for 15 min at 25°C. Phosphate hydrolysis was measured as described in Materials and Methods. A and specific activities are reported as μmoles Pi/mg of protein/min. The results are average values of two experiments.

\(^b\)Data taken from Sreter et al. (1966). White muscle (WM), red muscle (RM), cardiac muscle (CM).
TABLE 2

Amino acid composition of myosin heavy chains prepared from adult *Xenopus laevis* skeletal muscle.\(^a\)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Xenopus adult myosin</th>
<th>Rabbit skeletal(^b)</th>
<th>Rabbit skeletal(^c)</th>
</tr>
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<tbody>
<tr>
<td>lysine</td>
<td>90</td>
<td>92</td>
<td>93</td>
</tr>
<tr>
<td>histidine</td>
<td>17</td>
<td>16</td>
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<tr>
<td>threonine</td>
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<tr>
<td>serine</td>
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<td>glutamate</td>
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<tr>
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</tr>
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<td>alanine</td>
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<td>78</td>
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</tr>
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<td>21</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>29</td>
<td>29</td>
<td>28</td>
</tr>
</tbody>
</table>

\(^a\)Expressed as moles per \(10^5\) g of myosin. Data are averages of amino acid hydrolyses at 1, 24, 48 and 72 hours.

\(^b\)Data taken from a list compiled by Tonomura (1973).

\(^c\)Data taken from Huszar and Elzinger (1972).

\(^d\)The value for methionine was not corrected for losses during hydrolysis.
Figure la. Electrophoretic separation on a 10% SDS-acrylamide gel of myosin fractions purified by ammonium sulfate precipitation. This gel shows considerable contamination of myosin with actin from various muscle sources. Slots 1, 2 and 3: myosin from thigh muscles of adult *Xenopus laevis* after one, two and three ammonium sulfate precipitations (30 µg of protein in each slot). Slots 4 and 5: myosin from back muscles of adult *X. laevis* after one and two ammonium sulfate precipitations (about 15 µg of protein in each slot). Slot 6: myosin obtained from the tails of pre-metamorphosed *X. laevis* tadpoles and precipitated twice with ammonium sulfate (about 10 µg of protein in the slot). It appears from this gel that the myosin heavy chains have similar molecular weights.
Figure 1b. Electrophoretic separation on an 8% SDS-acrylamide gel of column-purified myosin fractions. This gel shows that column-purified myosin contains little actin contamination. Arrow denotes position of myosin. The negative of the gel was printed on high contrast paper to eliminate background staining. Slot 1: 60 μg of column-purified (c.p.) myosin from the thigh and back muscles of adult X. laevis. Slot 2: 25 μg of myosin as in Slot 1. Slot 3: 15 μg of myosin as in Slot 1. Slot 4: myosin from tails of pre-metamorphosed tadpoles. Slot 5: mixture of c.p. myosin and tadpole myosin.
Figure 2. Serological identity of gel-purified myosin and crude myosin from high salt extracts of adult *X. laevis* muscle. Photograph of an Ouchterlony double immunodiffusion plate stained with Amido Black 10B. The gel is made of 1% agarose prepared in 0.4 M KCl, 0.03 M PO<sub>4</sub> buffer (pH 7.3). The plate was developed for 48 hours in the cold (4°C), then washed overnight in 0.4 M KCl, 0.03 M PO<sub>4</sub> buffer (pH 7.3) at room temperature before staining. The centre well contained 15 µl of anti-myosin antibody (about 15 µg of protein). Well 1 contained about 20 µg of a high salt extract of adult *X. laevis* liver. Wells 2, 3 and 4 contained electrophoretically-purified myosin (protein concentration about 0.3 µg, 0.6 µg, 1.2 µg respectively). Wells 5 and 6 contained crude myosin (total protein concentration about 2.5 µg and 15.0 µg respectively). In separate diffusion plates, serum from non-immune rabbits did not form a precipitin line with gel-purified myosin or crude myosin.
Figure 3. Phase contrast and indirect immunofluorescence micrographs of the same field of muscle from the thigh muscle of adult Xenopus frogs. Glycerinated muscle was prepared as described by Pepe (1967). The labelling of anti-myosin antibody with fluorescein isothiocyanate was performed as described by Pepe (1966). A Reichert Zetopan microscope with an ultraviolet source and appropriate filters was used for fluorescent microscopy. Non-immune serum did not give a reaction with another piece of muscle. X 400.
Figure 4. Autoradiogram of $^{125}\text{I}^{-}$-labelled pure myosin and $^{125}\text{I}^{-}$-labelled crude muscle extract precipitated with anti-myosin antibody. Slot 1 contains myosin precipitated by antibody. Slot 2 contains iodinated myosin electrophoretically purified. The proteins were analyzed on a SDS-polyacrylamide slab gel (5% monomer).
Myosin
Figure 5. Autoradiogram of iodinated tryptic fragments of electrophoretically purified myosin and antibody precipitated myosin. Slot 1 contains iodinated myosin digested with trypsin. Slot 2 contains myosin precipitated by antibody, labelled with $^{125}$I and digested with trypsin. The peptide fragments were analyzed on a SDS-acrylamide slab gel (10% monomer).
molecular weight $\times 10^{-3}$

100

2

1
Figure 6. Precipitation of myosin by anti-myosin antibody. $^{125}$I-myosin heavy chain (9 x 10$^6$ cpm per mg), anti-myosin antibody (containing about 40 A$_{280}$ units per ml) and antibody from non-immunized rabbits (control) (about 20 A$_{280}$ units per ml) were prepared as described in Materials and Methods A. Each reaction tube contained about 1 mg of anti-myosin antibody, $^{125}$I-myosin in a total volume of 200 µl. The tubes were incubated for 12 hours at 4°C and processed as described in Materials and Methods A. Each point on the graph represents an average of 4 different experiments which agreed within 10%.

The control antibody obtained from a non-immunized rabbit precipitated between 0.2 to 0.3% of the $^{125}$I-myosin. Line codes: (o---o), precipitation with anti-myosin antibody; (••••), precipitation with non-immune serum (control).
Figure 7. Binding of $^{125}\text{I}$-labelled anti-myosin antibody to polysomes from various developmental stages of *Xenopus laevis* embryos. The gradients were 20-50% sucrose as described in Materials and Methods. (7a) Polysomes from stage 12 embryos (medium yolk plug stage). (7b) Polysomes from stage 16/17 embryos (neural fold stage). (7c) Polysomes from stage 20 embryos (fused neural folds stage). Line codes: ( ), optical density at 254 nm; (o——o), $^{125}\text{I}$-labelled antibody bound to polysomes. The position of the ribosome monosome is indicated by the arrows and was determined by comparison to *Xenopus* liver polysomes centrifuged under the same conditions (see Materials and Methods).
Figure 8. Binding of $[^{125}\text{I}]$-labelled antibody to stage 20 polysomes treated with RNase prior to centrifugation. Prior to sucrose gradient sedimentation stage 20 homogenates were treated with RNase. Line codes: (——), optical density at 254 nm; (o—o), $[^{125}\text{I}]$-labelled antibody bound to polysomes.
Figure 9. Serological analysis of myosins from stage 16/17, stage 20 and stage 26 embryos. This is a photograph of an Ouchterlony double immunodiffusion plate stained with Amido Black 10B. For details of how the plate was processed see Fig. 2. Centre well contained about 15 μg of anti-myosin antibody. Well 1 contained a high salt extract of *X. laevis* liver. Well 2 contained about 30 μg of protein from a high salt extract of stage 16/17 embryos. Wells 3 and 4 contained a high salt extract from stage 20 embryos (about 75 μg and 15 μg of protein respectively). Well 5 contained about 20 μg of a high salt extract of stage 26 embryos. Well 6 contained about 60 μg of crude myosin from a high salt extract of adult *X. laevis* muscle. In a separate experiment, low salt extracts of stage 12 and 16/17 embryos did not form a precipitin line when reacted with anti-myosin antibody.
Figure 10a. Electrophoretic separation on a 10% SDS-acrylamide gel of myosin extracts of stage 20/22 and stage 26/27 embryos. About 3000 embryos of each stage were processed for myosin preparation as described in Materials and Methods A up to and including an (NH₄)SO₄ precipitation (38%-45%). Slot 1: high salt extract of adult *Xenopus laevis* myosin. Slot 2: low salt homogenization extract of stage 20 embryos. Slot 3: high salt insoluble proteins from stage 20 embryos. Slot 4: ammonium sulfate-precipitated fraction of stage 20 embryos (about 20 µg of protein). Slot 4: ammonium sulfate-precipitated fraction of stage 26 embryos (about 20 µg of protein).
Figure 10b. Electrophoretic separation on a 7% SDS-acrylamide gel of high salt extracts of stage 16/17 embryos and pre-metamorphosis tadpole tails. About 5000 stage 16/17 embryos were processed for myosin isolation (see Materials and Methods A and Fig. 10a). Several tadpole tails were processed in a similar way. Slot 1: low salt extract from stage 16/17 embryos (about 40 µg of protein). Slot 2: ammonium sulfate fraction (38–48%) of tadpole tail extracts. Slot 3: high salt extract from stage 16/17 embryos.
Figure 11. Binding of \[^{125}\text{I}^\]\text{-anti-myosin} to \text{Xenopus} liver polysomes mixed 1:1 with the 100,000 x g supernatant of stage 20 polysomes. Polysomes from adult \text{Xenopus} liver were mixed with the 100,000 x g supernatant from stage 20 embryos prior to sucrose gradient sedimentation. After centrifugation, fractions were collected and reacted with \[^{125}\text{I}^\]\text{-anti-myosin} as in Materials and Methods sections. Line codes: (——), absorbance profile at 254 nm; (o——o), \[^{125}\text{I}^\]\text{-labelled} antibody bound to polysomes. The arrows indicate the position of the monosome and the 30-some peaks.
Figure 12. Binding of $^{125}$I-anti-myosin to stage 20 polysomes isolated in the presence of myosin heavy chains. Purified myosin heavy chains were added to stage 20 homogenates prior to sucrose gradient sedimentation. Line codes: (---), absorbance profile at 254 nm; (o--o), $^{125}$I-labelled antibody bound to polysomes.
MATERIALS AND METHODS B

Embryonic staging.

The embryos used for isolation of polysomes and RNA were staged according to the Normal Table of *Xenopus laevis* (Daudin) by Nieuwkoop and Faber (1967). Embryos were staged after removing the jelly coat and vitelline membrane. As soon as 50-100 embryos were staged, they were washed in sterile Holtfreter's medium and frozen in plastic vials in a mixture of dry ice and ethanol.

It is not possible to estimate how synchronous the embryos were at each stage. However, it is possible to eliminate certain stages from particular batches. Stage 12 embryos morphologically display a medium yolk plug and are about 13 1/4 hours old. This batch in all likelihood consisted of stage 11 1/2 embryos (12 1/2 hours old), stage 12 embryos and stage 12 1/2 embryos (14 1/2 hours old). Earlier stages (stage 11) and later stages (stage 13) would not contaminate this batch because they do not display a yolk plug, which is easily seen under the light microscope.

Stage 16/17 embryos are characterized by a readily distinguishable neural fold and are 18 1/4 to 18 3/4 hours old. It is easy to separate stage 15 embryos from stage 16 embryos because the neural folds are differently shaped. However, since it is not possible to control ambient temperature and physiological differences during staging, some stage 15 embryos (17 1/2 hours old) might be included in stage 16 batches. Stage 17 and stage 18 embryos can easily be distinguished because the neural folds are well separated posteriorly at stage 17 but are very
close to each other at stage 18. However, stage 17 embryos may contain stage 18 embryos because up to an hour can elapse during the staging of even small quantities of embryos. Earlier stages (stage 14) or later stages (stage 19) would not contaminate these batches because they can be easily spotted. Therefore, batches of stage 16/17 embryos may span a time from 17 1/2 hours (stage 15) to 19 3/4 hours (stage 18). However, most of the embryos should be at stage 17.

At stage 20 (21 3/4 hours old) the neural folds are fused, the suture is still present, the future sucker is characteristic and the two eye anlagen are becoming dumb-bell shaped. Therefore, this batch should not contain any earlier stages. Presence of stage 21 embryos (22 1/2 hours old) cannot be ruled out because of time factors involved in staging. However, since stage 21 embryos are easily distinguished from stage 20 embryos, contamination should be minimal.

Therefore, it appears that stage 20 embryos were the most accurately staged. Batches of stage 12 embryos most likely span 2 hours of development, a period mainly involved with the formation of ecto-, meso- and endodermal layers. Batches of stage 16/17 embryos spanned the longest developmental time period (2 1/4 hours), a time period characterized by neural plate formation and the segregation of three to four somites on each of the embryos. These staging problems do not invalidate the findings reported in this study.

RNA extraction.

a) Magnesium precipitation of polysomes. Polysomes and ribonucleoprotein particles from embryo homogenates were precipitated with magnesium essentially as described by Palmiter (1974). All buffers
were sterilized with DEP. All glassware was heat-sterilized and treated with a solution of dichlorodimethylsilane. All procedures were performed at 4°C. To each batch of embryos (3000), which had been stored at -70°C, Buffer A (0.2 M Hepes, pH 7.5; 25 mM NaCl, 10 mM MgCl₂, 1.0% Triton X-100, 0.5% sodium deoxycholate) was added. The embryos were homogenized in a Dounce homogenizer (Kontes Glass Co.) with 10-20 strokes of a loose pestle. The homogenate was centrifuged for 5 minutes at 10,000 x g; the supernatant was decanted into a beaker and an equal volume of Solution B was added (200 mM MgCl₂, 2.0% Triton X-100, 1 mg/ml heparin). The pellet from the previous centrifugation was re-extracted with Buffer A, centrifuged and the second supernatant combined with the first. The mixture was kept on ice for 1 hour. Subsequently, the mixture was layered slowly over one-third volume of Buffer C (25 mM NaCl, 5 mM MgCl₂, 20 mM Hepes, pH 7.5, and 1.0 M sucrose) and spun for 10 minutes at 27,000 x g max. The supernatants were decanted rapidly from the tubes and any lipid or sucrose adhering to the walls was wiped with tissue paper. The pellet was taken up in 20 mM Hepes (pH 7.6), then extracted with phenol and chloroform as described by Palmiter (1974). Phenol (Fisher reagent grade) was routinely made 0.1% in 8-hydroxyquinoline. Chloroform was routinely redistilled. After the organic extractions, the RNA was precipitated with 2 volumes of ethanol at -20°C. The RNA pellet was then washed twice with 2 ml of 3 M sodium acetate (pH 6.0). The RNA was then dissolved in 0.1 M potassium acetate (pH 7.0), precipitated with ethanol, and collected by centrifugation; the pellet was dried and dissolved in 20 mM Hepes (pH 7.4) at a concentration of 1.0 mg/ml.
b) Guanidinium hydrochloride extraction of RNA. This extraction procedure was developed by W. Rutter (University of California, San Francisco) using a modification of the method described by Cox (1968). Embryos were homogenized as in the previous section in Buffer A and then centrifuged. The supernatant and the pellet were made 4 M in guanidine-HCl (Sigma) by the addition of 6 M GuCl; 2 M potassium acetate was added to achieve a pH of 5.0. The two mixtures were homogenized 20 times in the Dounce homogenizer with a loose pestle at -20°C (salt/ice). The homogenization mixture containing the pellet was centrifuged at 5,000 x g for 5 minutes. Then, 1/2 volume of -20°C 95% ethanol was added to the separate supernatants and the mixtures were precipitated at -20°C overnight. The individual precipitates were centrifuged at 10,000 x g for 5 minutes and the precipitates were made 6 M in GuCl and 0.1 M in potassium acetate (pH 5.0). The mixtures were then precipitated with 1/2 volume of -20°C 95% ethanol and precipitated for 12 hours at -20°C. The precipitates were centrifuged at 5,000 x g for 10 minutes. The precipitates were combined and subsequently dissolved in 20 mM Hepes (pH 7.4) and extracted with organic solvents according to Palmiter (1974).

All RNA preparations had $A_{260}/A_{280}$ ratios in the 2.10 range. The yields of RNA extracted by both methods varied for each batch of embryos. Generally, with the Mg$^{2+}$ method, the yield was 11.2 mg of RNA/3000 embryos; for the GuCl method the yield was 10.6 mg of RNA/3000 embryos. For comparison, when RNA was isolated by the hot phenol method of McCarthy and Hoyer (1964), the yields were as high as those obtained by the Mg$^{2+}$ precipitation method. However, the RNA extracted by hot phenol was not as active in directing protein synthesis in the wheat germ cell-free system. RNA concentrations were estimated by assuming
that one $A_{260}$ unit of RNA in the absence of Mg$^{2+}$ was equal to 50 µg of RNA. For some experiments, RNA concentrations were determined by the orcinol reaction (Merchant et al., 1969).

**Oligo(dT)-cellulose binding of mRNAs.**

RNA extracted from embryos was separated from ribosomal and other RNAs not containing poly(A) by oligo(dT)-cellulose chromatography. The oligo(dT)-cellulose was purchased from Collaborative Research (Type T-3, lot 534-35). The samples were processed batchwise (Haines and Palmiter, 1974; Strohman et al., 1977) permitting elution of small amounts of message in very small volumes. The oligo(dT)-cellulose was sterilized with 0.05% DEP. "Fines" were removed by extensive washing with 0.5 M KCl, 20 mM Hepes (pH 7.5). The oligo(dT)-cellulose was then washed with binding buffer (0.5 M KCl, 20 mM Hepes (pH 7.5), 1.0% SDS) until an $A_{260}$ of 0 was obtained. Binding of control preparations of poly(A) is > 95% complete under these conditions (R. Reeves, personal communication). To prevent aggregation of RNA, the RNA-ethanol precipitates were dissolved in double-distilled water and heated to 65°C for 10 minutes (Strohman et al., 1977). The RNA mixture was diluted with an equal volume of 2X binding buffer before mixing with oligo(dT)-cellulose. However, the same amount of binding was obtained if a concentration not higher than 2-3 mg/ml of RNA/200 mg oligo(dT)-cellulose was used. RNA in 0.2 ml of binding buffer was mixed with 0.2 ml of oligo(dT)-cellulose and incubated at room temperature for 30 minutes. The mixture was centrifuged and the cellulose was washed with binding buffer until no UV-absorbing material was eluted. The bound material was eluted with several washes of 20 mM Hepes buffer (pH 7.5). The
cellulose was then regenerated by washing with 0.1 N KOH and subsequently with binding buffer. The bound and flow-through RNA fractions were then adjusted to 0.1 M with potassium acetate (pH 7.0), precipitated overnight with ethanol and collected by centrifugation; the RNA pellets were dried and dissolved in 20 mM Hepes (pH 7.5).

Under these conditions, 3000 stage 12 embryos yielded 54.6 µg of poly(A)$^+$ RNA (bound); 3000 stage 16/17 embryos yielded 61.0 µg of poly(A)$^+$ RNA and 3000 embryos at stage 20 yielded 63.4 µg of poly(A)$^+$ RNA.

Preparation of wheat germ lysates.

A crude wheat germ extract was prepared according to the method of Roberts and Paterson (1973) with minor modifications. The extraction buffer consisted of 20 mM Hepes (pH 7.6; adjusted with KOH), 100 mM KOAc, 2 mM CaCl$_2\cdot$2H$_2$O, 1 mM Mg(0Ac)$_2$ and 1 mM DTT. The wheat germ was a gift of General Mills, Vallejo, California. The column elution buffer contained 20 mM Hepes (pH 7.6), 120 mM KOAc, 5 mM Mg(0Ac)$_2$ and 1 mM DTT. After desalting on a Sephadex G-25 column, the wheat germ homogenate (the S-30 preparation) was centrifuged at 0°C at 30,000 x g for 10 minutes. This step reduces the protein concentration of the S-30 without affecting its translational activity. The S-30 extract was stored in polypropylene microfuge tubes (Brinkman, Inc.) at -70°C until needed. All embryo RNA translations reported here were performed with the same S-30 wheat germ preparation.

Cell-free protein synthesis.

$^{14}$C-leucine (270 µCi/mole) was from New England Nuclear.
ATP, GTP, creatine phosphate and creatine phosphokinase were from Sigma, and were prepared and stored as a master mix in small aliquots at -70°C.

The unlabelled amino acids were from Sigma and were neutralized before adding them to the master mix. The complete cell-free system in a final volume of 50 µl contained: 15-20 µl of wheat germ extract, 20 mM Hepes (pH 7.4; adjusted with KOH), 2 mM DTT, 8 mM creatine phosphate, 50 units/ml of creatine phosphokinase, 25 µM GTP, 150 mM KOAc, 3.0 mM Mg(OAc)₂, 25 µM of all 19 unlabelled amino acids and 0.25 µCi of [¹⁴C]-leucine (270 µCi/umole). Assays were incubated for 60 to 90 minutes at 30°C. The reactions were stopped by addition of an equal volume of 0.1 N KOH and were then incubated for 2 minutes at 37°C to deacylate the tRNAs. The mixtures were then precipitated with 20% ice-cold trichloroacetic acid (made 1 mM in "cold" leucine) and collected onto Whatman GF/A filters (previously moistened with cold 5% TCA). Assay tubes were rinsed two times with 20% TCA and the filters were rinsed one more time with ice-cold 20% TCA. Filters were then washed with 95% ethanol and ethyl ether and counted in Aquasol in a Nuclear Chicago Isocap scintillation counter.

Antibody precipitation of cell-free products.

For antibody preparation, 50 µl wheat germ lysate assays were terminated by addition of a cold solution of 10% Triton X-100 and sodium deoxycholate in 5 M KCl. Subsequently, 100 µg of unlabelled gel-purified myosin heavy chain was added, followed by 5 µl of 0.1 M "cold" leucine containing 0.2 mM PMSF. Since these mixtures always contained a precipitate, they were centrifuged in Beckman microfuge tubes at full speed for 15 minutes. The pellets and the supernatants
were then processed for antibody precipitation.

The pellets were resuspended in a low ionic strength buffer (20 mM Hepes (pH 7.5), 1 mM MgCl₂ and 2% Triton X-100 and sodium deoxycholate). The mixture was reacted overnight with 30-60 μg of anti-myosin or non-immune antibody. The resulting precipitates were freed of non-specifically adsorbed material by sedimentation through a discontinuous, detergent-containing sucrose gradient in microfuge tubes as previously described. The tubes were then frozen and the tips were cut off and the precipitated radioactivity counted as previously. No radioactivity above background levels of non-specific precipitation was detected in the pellets.

The supernatants from the wheat germ incubation lysates (see above) were diluted with 9 volumes of a "cold" buffer solution (1 mM Hepes (pH 7.5), 1 mM EDTA) and left standing for 12 hours at 0°C. The precipitated myosin was collected by centrifugation at 12,000 x g for 15 minutes and dissolved in 20 mM Hepes (pH 7.5), 1 mM MgCl₂, 1% Triton X-100 and sodium deoxycholate. Finally, 30-60 μg of anti-myosin antibody or non-immune serum were added. The mixtures were incubated overnight at 0°C and the precipitates were processed as above.

**Tryptic digestion of cell-free translation products.**

The antigen-antibody precipitates resulting from the reaction of anti-myosin antibody and the cell-free wheat germ incubates (see previous section) were electrophoresed on SDS-acrylamide gels (when precipitates resulting from the reaction of non-immune rabbit sera with wheat germ incubates were electrophoresed, no radioactive myosin band was detected on the gels). The bands migrating in the 210,000 dalton
range were cut out and processed as described in Results A under myosin purification. The products were pooled and resuspended in 0.6 M NaCl, 0.015 M Tris-HCl (pH 7.4), 0.1% β-mercaptoethanol (see Results A under the heading Tryptic digestion) and mixed with 2 μg of trypsin. This mixture was digested for 60 minutes at 37°C and the reaction was stopped by the addition of 4 μg of trypsin inhibitor and 20 μl of Laemmli's SDS sample buffer followed by boiling for 4 minutes. The mixture was then centrifuged in a Beckman microfuge for 1 minute and dialyzed for a few hours against Laemmli's SDS sample buffer (see Results A). About 70 μl of this mixture containing about 1397 cpm was electrophoresed (see Results A) on a 10% SDS-acrylamide gel and stained with Coomassie Blue. The position of the bands was determined by measuring with a ruler and then the gel was frozen and sliced into 2 mm slices. Each slice was placed in a counting vial and digested with 100 μl of 30% H₂O₂ overnight at 50°C. After adding 300 μl of Protosol (Nuclear Chicago) solubilizer and 4 ml of Aquasol scintillation fluid, the radioactivity was determined in a scintillation counter. About 87% of applied radioactivity was recovered.
Isolation and fractionation of RNA.

RNA was isolated from stage 12, stage 16/17 and stage 20 Xenopus embryos. Batches of Xenopus embryos were treated in two different ways as a first step towards isolation of RNA.

In the magnesium precipitation technique (Palmiter, 1974), polysomes, mRNP particles and high molecular weight RNAs were precipitated by 0.1 M Mg$^{2+}$ from the post-mitochondrial supernatant. In the guanidinium hydrochloride technique, the post-mitochondrial supernatant was made 4 M in guanidinium chloride and nucleic acids were precipitated by the addition of 1/2 volume of 95% ethanol. In both types of experiment, RNA was extracted from the precipitates by the phenol:chloroform procedure as described by Palmiter (1974). This comparative approach, using two different RNA extraction procedures, should minimize the possibility that a negative result in protein translation is due to RNA degradation or loss during isolation.

Table 3 shows that similar amounts of RNA are obtained after extraction of nucleic acid from Mg$^{2+}$ precipitates or from guanidinium-ethanol precipitates. Results from cell-free translation assays, to be described later, show that both procedures yield mRNAs with similar translation characteristics. As shown in Table 4, the approximate amount of total RNA and polysomal RNA in Xenopus embryos has been estimated from measurements of RNA content (Brown and Littna, 1964) and of polysome content (Woodland, 1974). According to this data, the total
RNA content of Xenopus embryos is about 12-13 mg/3000 embryos. Therefore, the yields shown in Table 3 indicate that the magnesium precipitation and guanidinium chloride methods gave good yields of RNA.

Total RNA obtained by either method was further fractionated by oligo(dT)-cellulose chromatography. Table 5 shows that most of the total RNA did not bind to the oligo(dT)-cellulose. The concentration of the RNA that did bind to the cellulose was in the same range as the amount found in polysomes (Table 3; and Woodland, 1974). As a test of the specificity of binding to oligo(dT)-cellulose, [3H]-uridine-labelled rRNA from Xenopus oocytes (isolated by Dr. R. Reeves) was mixed with the cellulose. The labelled rRNA was not retained on the cellulose after two passages. To test for aggregation, RNA from stage 20 embryos was heated to 65°C for 10 minutes, rapidly cooled and subjected to oligo(dT) fractionation. The yields obtained were the same as those shown in Table 5. It therefore appears that, under the conditions used, poly(A)+ mRNA does not form aggregates with rRNA. It is assumed, but not directly proven, that poly(A)+ and poly(A)- RNAs do not form aggregates either. Also, it was found that it was unnecessary to add tRNA to the binding buffer in order to prevent non-specific absorption of RNA to the oligo(dT)-cellulose since a control experiment indicated that the yields of poly(A)+ RNA from stage 20 embryos were identical in the presence and absence of tRNA.

We therefore conclude from these results that both the magnesium precipitation and guanidinium chloride methods are quantitative means of obtaining total RNAs from Xenopus embryos. The oligo(dT)-chromatography of total cellular RNA results in a poly(A)+ RNA fraction which is probably not appreciably contaminated with rRNA. This
cellulose-bound fraction is probably composed of poly(A)$^+$ mRNA derived from polysomal and mRNP particles. We cannot rule out the possibility that some poly(A)$^-$ RNA contaminates the poly(A)$^+$ fraction or that some mRNAs with short poly(A)-tails (Rosbash et al., 1977) were not bound to the oligo(dT)-cellulose column.

**Cell-free translation of Xenopus mRNA.**

Messenger RNA fractions were translated in a wheat germ cell-free protein-synthesizing system using $[^{14}\text{C}]$-leucine as the radioactive protein precursor. Incorporation of this amino acid into proteins was measured by precipitation of the cell-free extracts with 20% cold TCA or with antibody against myosin as described in Materials and Methods.

A final concentration of about 3.5 mM magnesium is optimum for the cell-free translation of a variety of mRNAs (Efron and Marcus, 1973; Davies and Kaesberg, 1974; Roberts and Paterson, 1973). Table 6 shows that in the presence of 100 mM KOAc, the Mg$^{2+}$ optimum for stage 20 poly(A)$^+$ RNA is 3.0 mM. Table 7 indicates that at 3.0 mM Mg$^{2+}$, high counts in immunoprecipitates are obtained when the K$^+$ concentration is 156 mM. Although at this K$^+$ concentration the total cell-free incorporation was found to be less than that at optimum K$^+$ concentration, for the wheat germ system more radioactive counts were immunoprecipitated by antibody at 156 mM KOAc. This result implies that either 156 mM K$^+$ is optimum for myosin mRNA or that the fidelity of translation of myosin mRNA is improved at this concentration of potassium.

Table 8 shows the dependence of protein synthesis on the amount of mRNA present. Protein synthesis is seen to be linearly dependent on
the amount of added mRNA up to 10.0 pg per incubate. Above this mRNA concentration, incorporation of the $[^{14}\text{C}]$-label reaches a plateau.

Table 9 shows a time-course study of the cell-free reaction with poly(A)$^+$ mRNA. It appears that after 90 minutes of incorporation the synthetic reaction reaches a plateau.

For some RNAs, addition of spermine to the wheat germ cell-free system increases the amount of isotope incorporation (Atkins et al., 1975; Roberts et al., 1975; Hunter et al., 1977). In this study, addition of 40 mM spermine had an inhibitory effect on the translation of poly(A)$^+$ and poly(A)$^-$ RNA from stage 20 embryos (incorporation was reduced about two-fold). A similar effect has been reported for silk moth chorion mRNA translation in the wheat germ system (Efstratiadis and Kafatos, 1976). This result may be explained in several ways: 1) Certain eukaryotic mRNA may be efficiently translated without polyamines; 2) There may be a sufficient amount of polyamines in the wheat germ used in this study which were not removed by gel filtration (by virtue of attachment of ribosomes or some other cellular factors); 3) The high magnesium and potassium concentration used in this study may replace the necessity for the use of polyamines; and 4) The polyamine used in this study may have been degraded during storage.

Cell-free translation analysis of appearance of myosin mRNA during Xenopus embryogenesis.

Messenger RNA isolated from various stages of Xenopus embryogenesis was translated and assayed for myosin synthesis by immunoprecipitation as described in Materials and Methods. The identification of the heavy chain of skeletal myosin as one of the
products synthesized in vitro under the influence of added mRNA was achieved by immunoprecipitation with anti-myosin antibody shown to be specific in section A of the Results. Furthermore, the protein synthesized in vitro was digested with trypsin and the labelled peptides were shown to co-migrate with heavy chain myosin peptides.

Using the anti-myosin antibody we could show (Table 10) that myosin mRNA appears at the late neural-fold stage (stages 16/17) of Xenopus development. This is approximately 3 hours before the appearance of nascent myosin on polysomes at stage 20, as shown in section A of the Results. Table 10 shows that between 0.3-2.8% of the radioactivity incorporated into polypeptides in the cell-free system in vitro under the influence of stage 16/17 RNA is found in immunoprecipitates. The total incorporation into synthesized protein varied from one RNA preparation to another as can be seen in Table 10 (stage 16/17). This result may be due to the fact that incorporation is not strictly quantitative in the wheat germ system or that the mRNA preparations differ from batch to batch of embryos. This second possibility may result from a number of factors: 1) Different batches of embryos are not of exactly the same size (light microscopy observations); 2) There was minor inaccuracy in embryo staging (to be discussed in the following section); and 3) Possibly biochemical changes do not necessarily correlate exactly with morphological changes (Macklin and Wojtkowski, 1973). However, it can be seen in Table 10 that at stage 16/17, the number of counts in the immunoprecipitates is about the same for all of the different mRNA preparations. Therefore, it seems more likely that subtle differences in the wheat germ incubation mixtures may be responsible for differences in total incorporation.
It might be argued that myosin mRNA from stage 12 embryos is inactivated by some factor(s) which cannot be removed by the fractionating procedures used in this study. To test if this factor(s) is capable of inhibiting stage 20 myosin mRNA the following mixing experiment was performed. A batch of 1500 stage 12 embryos and 1500 stage 20 embryos were mixed and homogenized. Poly(A)-terminated RNA was isolated as previously described. Table 11 shows that poly(A)$^+$ mRNA obtained from these mixed stage 12 and stage 20 embryos is translated into immunoprecipitable myosin. Therefore, stage 12 cell homogenates cannot degrade stage 20 mRNA and do not contain an excess of the putative inhibitors, which could completely prevent the translation of stage 20 myosin mRNA in the wheat germ cell-free system. If it is assumed that in the mixing experiment there is approximately 5 pg of poly(A)$^+$ from stage 20 embryos in each wheat germ incubation mixture, then the synthesis of myosin represents about 7% of total incorporation (see legend to Table 11). This calculation suggests that there is no inhibition of translation of stage 20 myosin mRNA and also, no activation of untranslatable stage 12 myosin mRNA by some form of activator from stage 20 cells. If there is any untranslatable myosin mRNA in stage 12 embryos, it cannot be detected by our methods because of too low concentrations. Other models which might explain these results will be presented in the discussion.

The immunoprecipitates of the products synthesized in vitro under the influence of stage 20 mRNA were mixed with carrier authentic myosin and displayed by SDS-acrylamide electrophoresis (gels not shown). The Coomassie Blue bands co-migrating with authentic heavy chain of skeletal muscle myosin were cut out and the proteins eluted and counted.
for radioactivity. Between 90-95% of \(^{14}\)C-leucine radioactivity was found in the "carrier" myosin bands that were cut out from the gels. The radioactivity that did not migrate with the heavy chain of myosin may be due to incomplete chains of myosin.

As discussed in Results A, nascent peptide chains on polysomes are efficiently isolated by immunoprecipitation. The fact that about 90-95% of the antibody-precipitated cell-free product consisted of completed myosin chains can be explained in several ways. High concentrations of magnesium (0.1 M), guanidinium chloride (4M) and heparin (1 mg/ml) are known to inhibit ribonucleases (Cox, 1967; Rhoads et al., 1973; Palmiter, 1973). Therefore, it is likely that myosin mRNA was not degraded significantly during isolation due to the presence of these reagents. For this reason the amount of low molecular weight peptides due to the translation of degraded myosin mRNA would be greatly reduced. Although the wheat germ system contains endogenous RNases, the high potassium acetate concentration (156 mM) used in the translation stabilizes polysomes and it is possible that the rate of nucleolytic attack on polysome-associated mRNA is slower than on unbound mRNA (Hunter et al., 1977).

Although the reasons outlined above do not preclude the possibility that the myosin antibody can precipitate prematurely terminated polypeptides or incomplete polypeptide chains still attached to polysomes (Darnbrough and Ford, 1976), this is unlikely because the wheat germ system in this study was optimized for the synthesis of high molecular weight proteins (specifically myosin). As described in a previous section, the optimal conditions for the synthesis of myosin were not coincident with the optimal conditions for formation of
acid-precipitable material. After this study was completed, Patrinou-Georgoulas and John (1977) compared myosin mRNA translation at 96 mM KCl and 160 mM KCl (2.0 mM Mg^{2+}) in the wheat germ system. They found that approximately 12 times more myosin was synthesized in the presence of 160 mM KCl than in the presence of 96 mM KCl. On the other hand, they found that incorporation into total protein, determined as acid-insoluble radioactivity, was greatly reduced in the presence of 160 mM KCl. Furthermore, these workers removed quantitatively the low molecular weight radioactive peptides, synthesized in the presence of 160 mM KCl, by precipitating myosin at low ionic strength. The low molecular weight peptides were probably mostly composed of non-myosin proteins since they appeared to run on SDS-acrylamide gels with a molecular weight of 30,000 or less. If there were any incomplete myosin chains present, they would most certainly consist of heavy meromyosin chains (HMM) (MW -110,000 or less) which are synthesized from the 5' end of the mRNA molecule and are soluble at low ionic strength (Sarkar, 1976).

In the present study, myosin was precipitated from wheat germ lysates at low ionic strength (see Materials and Methods; Sarkar, 1976). Therefore, the fact that in this study the myosin antibody precipitated protein with a molecular weight of approximately 210,000 daltons is due to the fact that stringent measures were taken to eliminate nascent myosin chains and proteolytic cleavage of complete myosin molecules. The wheat germ system was optimized for magnesium and potassium concentrations optimal for myosin mRNA translation. This probably resulted in a better fidelity of translation of myosin mRNA. Precautions were taken to reduce degradation of myosin mRNA by including inhibitors
of ribonucleases in the isolation procedure. The in vitro product was precipitated at low ionic strength prior to antibody precipitation. This resulted in enrichment of the preparation with completed myosin molecules. Also, for antibody precipitation (see p. 70) an attempt was made to inhibit proteolysis of myosin synthesized in the cell-free system by treating the wheat germ lysates with Triton X-100, PMSF and sodium sulphite. The neutrally charged detergent Triton X-100 partially denatures some proteins and consequently, may inhibit proteolytic activity. It is commonly used in antigen-antibody reactions (Palacios et al., 1972; Strohman et al., 1977). The protease inhibitor PMSF specifically inhibits serine proteases (Glazer, 1975). It is not known if serine-like proteases are present in the wheat germ cell-free system. In some preparations, sodium sulfite (2.5 mM) was also used and did not affect the amounts of myosin precipitated by antibody. Sodium sulfite sulfonates the disulfide bonds of some proteins and inhibits their enzymatic activity (Glazer, 1975). No experiment was carried out to determine if the presence of PMSF and/or sodium sulfite in the wheat germ system during incubation would increase the yield of myosin synthesis. Finally, since the in vitro reaction was incubated for about 90 minutes, it is possible that a large percentage of nascent peptide chains were allowed to proceed to completion.

Analysis of tryptic digestion fragments of radioactive myosin, precipitated by antibody, in the presence of cold carrier myosin, was consistent with the presence of radioactive myosin heavy chain (Fig. 13). Approximately 9 prominent radioactive peaks coincide with visible fragments derived from the carrier myosin heavy chain. If the antibody-precipitated protein had contained peptides other than skeletal heavy chain myosin, it would be expected that some radioactive fragments
would electrophorese independently of the visible fragments of the purified carrier myosin.

The results reported here support the conclusion that translatable myosin mRNA first makes its appearance during *Xenopus* development at the late neurula stage (stage 16/17).
TABLE 3

Yields of RNA prepared from *Xenopus* embryos by different isolation procedures.\(^a\)

<table>
<thead>
<tr>
<th>Stage (^b)</th>
<th>Isolation procedure(^c)</th>
<th>Number of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg(^{2+}) (mg)</td>
<td>GuCl (mg)</td>
</tr>
<tr>
<td>12</td>
<td>10.8</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>11.4</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>10.3</td>
<td>10.7</td>
</tr>
<tr>
<td>16/17</td>
<td>11.2</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>11.0</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>10.7</td>
<td>10.5</td>
</tr>
<tr>
<td>20</td>
<td>10.9</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>11.1</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>10.4</td>
<td>10.5</td>
</tr>
</tbody>
</table>

\(^a\) Assumption used in calculations: 20 \(A_{260}\) units of RNA in absence of Mg\(^{2+}\) = 1 mg.

\(^b\) Nieuwkoop and Faber (1967); stages of development.

\(^c\) RNA from *Xenopus* embryos was prepared using the Mg\(^{2+}\) or guanidinium chloride procedure and extracted with phenol:chloroform.
TABLE 4

Quantities of total and polysomal mRNA in *Xenopus* embryos at stages 12, 16/17 and 20.\(^a\)

<table>
<thead>
<tr>
<th>Stages</th>
<th>DNA content per embryo(^b) (ng)</th>
<th>No. of cells(^c)</th>
<th>Total RNA(^d) per embryo (ng)</th>
<th>Polysomal mRNA per embryo(^e) (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>300</td>
<td>$4.8 \times 10^4$</td>
<td>4000</td>
<td>26</td>
</tr>
<tr>
<td>16/17</td>
<td>400</td>
<td>$6.4 \times 10^4$</td>
<td>4000</td>
<td>17</td>
</tr>
<tr>
<td>20</td>
<td>500</td>
<td>$7.9 \times 10^4$</td>
<td>4000</td>
<td>25</td>
</tr>
</tbody>
</table>

\(^a\)These values are approximate.
\(^b\)Values for DNA content were taken from David (1965).
\(^c\)Values for No. of cells were calculated on the basis of 6.3 pg of DNA per diploid cell (David, 1965).
\(^d\)Taken from Brown and Littna (1964).
\(^e\)Calculated on the basis that 90% of the total RNA is ribosomal (Galau, 1974), 16.2% of the ribosomes are in polysomes at stage 12, 10.7% of the ribosomes are in polysomes at stage 16/17 and 15.6% of the ribosomes are in polysomes at stage 20 (Woodland, 1974). Finally, 4.5% of polysomal RNA is mRNA (Galau, 1974).
TABLE 5

Purification of poly(A)$^+$ RNA from **Xenopus** embryos by binding to oligo(dT)-cellulose.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Purification step</th>
<th>Total RNA applied to oligo(dT)-cellulose (µg)</th>
<th>Amount (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>Experiment I: 2\textsuperscript{nd} oligo(dT)-cellulose Flow-through Bound</td>
<td>10,800</td>
<td>9,234 56.6</td>
</tr>
<tr>
<td></td>
<td>Experiment II: 2\textsuperscript{nd} oligo(dT)-cellulose Flow-through Bound</td>
<td>11,400</td>
<td>10,032 57.4</td>
</tr>
<tr>
<td>16/17</td>
<td>Experiment I: 2\textsuperscript{nd} oligo(dT)-cellulose Flow-through Bound</td>
<td>11,200</td>
<td>9,576 54.4</td>
</tr>
<tr>
<td></td>
<td>Experiment II: 2\textsuperscript{nd} oligo(dT)-cellulose Flow-through Bound</td>
<td>10,700</td>
<td>9,341 56.0</td>
</tr>
<tr>
<td>20</td>
<td>Experiment I: 2\textsuperscript{nd} oligo(dT)-cellulose Flow-through Bound</td>
<td>10,900</td>
<td>8,829 58.4</td>
</tr>
<tr>
<td></td>
<td>Experiment II: 2\textsuperscript{nd} oligo(dT)-cellulose Flow-through Bound</td>
<td>11,100</td>
<td>8,891 58.8</td>
</tr>
</tbody>
</table>
TABLE 6

Effect of magnesium on total protein synthesis.\(^a\)

<table>
<thead>
<tr>
<th>Magnesium concentration (mM)</th>
<th>Total cpm(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>600 ± 48</td>
</tr>
<tr>
<td>2.5</td>
<td>23,528 ± 1,011</td>
</tr>
<tr>
<td>3.0</td>
<td>53,939 ± 3,236</td>
</tr>
<tr>
<td>3.5</td>
<td>49,018 ± 1,715</td>
</tr>
<tr>
<td>4.0</td>
<td>35,222 ± 2,465</td>
</tr>
<tr>
<td>4.5</td>
<td>30,000 ± 1,200</td>
</tr>
</tbody>
</table>

\(^a\) All incubations were carried out for 90 min at 30⁰C with 10 µg of stage 20 poly(A)\(^+\) RNA per 50 µl of assay.

\(^b\) At a K\(^+\) concentration of 100 mM with all other components as described in Materials and Methods.

\(^c\) Background radioactivity (no RNA controls) was about 370 cpm. This value has been subtracted.
TABLE 7

Effect of potassium acetate on total protein synthesis and myosin synthesis.\(^a\)

<table>
<thead>
<tr>
<th>Potassium concentration (mM)(^b)</th>
<th>Total cpm(^c)</th>
<th>Cpm in immunoprecipitates(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>23,528 ± 1,411</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td>47,056 ± 2,729</td>
<td>843 ± 93</td>
</tr>
<tr>
<td>100</td>
<td>58,821 ± 3,529</td>
<td>900 ± 90</td>
</tr>
<tr>
<td>120</td>
<td>48,000</td>
<td></td>
</tr>
<tr>
<td>156</td>
<td>27,647 ± 1,990</td>
<td>2,109 ± 147</td>
</tr>
<tr>
<td>160</td>
<td>20,587 ± 905</td>
<td>1,600 ± 133</td>
</tr>
<tr>
<td>170</td>
<td>14,705 ± 1,029</td>
<td>767 ± 75</td>
</tr>
</tbody>
</table>

\(^a\) All incubations were carried out as described in Table 6.
\(^b\) At a Mg\(^{2+}\) concentration of 3.0 mM.
\(^c\) Background radioactivity has been subtracted (see Table 6).
\(^d\) Immunoprecipitates were prepared as described in Materials and Methods. The background radioactivity (non-immune serum added to wheat germ incubates) amounted to about 65 cpm and was subtracted.
TABLE 8

Dependence of total protein synthesis on added stage 20 poly(A)$^+$ Xenopus RNA.\textsuperscript{a}

<table>
<thead>
<tr>
<th>RNA ((\mu)g)</th>
<th>Total cpm\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3</td>
<td>4,300 ± 430</td>
</tr>
<tr>
<td>2.5</td>
<td>9,462 ± 870</td>
</tr>
<tr>
<td>5.0</td>
<td>17,844 ± 1,070</td>
</tr>
<tr>
<td>7.5</td>
<td>25,200 ± 1,260</td>
</tr>
<tr>
<td>10.0</td>
<td>26,910 ± 1,345</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All incubations were carried out for 90 min at 30°C in a total volume of 50 \(\mu\)l. All components were at the concentrations described in Materials and Methods.

\textsuperscript{b} Background radioactivity was subtracted.
TABLE 9

Time course of total protein synthesis and myosin synthesis in the wheat germ protein-synthesizing system.\(^a\)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Total cpm(^b)</th>
<th>Cpm in immunoprecipitates(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1,942 ± 161</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3,950 ± 355</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>6,200 ± 558</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>7,768 ± 567</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>12,760</td>
<td>1,060 ± 117</td>
</tr>
<tr>
<td>40</td>
<td>15,475 ± 1,238</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>18,061 ± 957</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>25,800 ± 1,135</td>
<td>2,425 ± 194</td>
</tr>
<tr>
<td>70</td>
<td>26,210 ± 1,310</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>27,000 ± 1,620</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>29,411 ± 1,765</td>
<td>2,646 ± 218</td>
</tr>
</tbody>
</table>

\(^a\) All incubations were carried out at 30°C with 10 µg of stage 20 poly(A)\(^+\) RNA per 50 µl of assay. The K\(^+\) concentration was 156 mM, the Mg\(^2+\) concentration was 3.0 mM. All other components as described in Materials and Methods.

\(^b\) Radioactivity in background samples (no RNA) was subtracted.

\(^c\) Radioactivity in samples precipitated with non-immune serum was subtracted.
### TABLE 10

**Immunoprecipitation of the cell-free reaction product with anti-myosin antibody.**

<table>
<thead>
<tr>
<th>mRNA source</th>
<th>Incorporation of $^{14}$C-leucine into Total cell-free product</th>
<th>Myosin</th>
<th>100 B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Stage 12$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9,183</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10,664</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>10,207</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stage 16/17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>148,404</td>
<td>510</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>157,277</td>
<td>503</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>24,607</td>
<td>670</td>
<td>2.7</td>
</tr>
<tr>
<td>4</td>
<td>23,661</td>
<td>661</td>
<td>2.8</td>
</tr>
<tr>
<td>Stage 20$^c$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>58,294</td>
<td>3,363</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>27,893</td>
<td>2,769</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>26,470</td>
<td>2,382</td>
<td>9</td>
</tr>
</tbody>
</table>

$^a$Wheat germ cell-free protein synthesis was carried out as described in Materials and Methods with 10 µg poly(A)$^+$ RNA for each experiment. Blank values (no RNA added to the wheat germ system) as well as values obtained with control serum were subtracted.

$^b$Each experiment represents mRNA isolated from a different batch of embryos. Three other experiments were performed using one-half as much RNA (5 µg) as in the previous experiments. Total incorporation was 6089 cpm and myosin synthesis was undetectable.

$^c$Each experiment represents mRNA isolated from a different batch of embryos. For other experiments using stage 20 poly(A)$^+$ RNA see Tables 7, 8 and 9.
TABLE 11

Immunoprecipitation of the cell-free reaction product with anti-myosin antibody directed by poly(A)$^+$ and poly(A)$^-$$^-$ RNA from mixed stage 12 and stage 20 embryos.$^a$

<table>
<thead>
<tr>
<th>mRNA source</th>
<th>Incorporation of [$^{14}$C]-leucine into</th>
<th>100 B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cell-free product</td>
<td>Myosin</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Stage 12 + stage 20$^b$ poly(A)$^+$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>21,601</td>
<td>1,166</td>
</tr>
<tr>
<td>2</td>
<td>20,000</td>
<td>1,120</td>
</tr>
</tbody>
</table>
| Stage 12 + stage 20$^c$ poly(A)$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^-$$^{-}
Figure 13. Analysis by electrophoresis on a 10% SDS-acrylamide gel of tryptic fragments of translation products precipitated with anti-myosin antibody.

Radioactive myosin heavy chains in the presence of cold carrier-purified myosin heavy chains were obtained by immunoprecipitation as described in Materials and Methods. The myosin heavy chains were digested with trypsin and electrophoresed on 10% SDS-acrylamide gels. Photograph represents Coomassie Blue stained gel. Top profile represents densitometric scan of the photograph of the gel. Bottom profile represents radioactive counts in 2 mm slices of the original gel.
DISCUSSION

Our experiments demonstrate that myosin mRNA is synthesized in a translatable form at about the time the first somites are segregated in *Xenopus* embryos. Myosin is first synthesized when about 6-7 somites have segregated in the embryos. The time lag between myosin mRNA synthesis and myosin appearance is about 1 1/2 hours. The myosin synthesized in *Xenopus* embryos cross-reacts with adult myosins. This may mean that embryonic myosin shares similar antigens with adult myosins or that both embryonic and adult myosins are being synthesized in early development.

We have attempted in this study to answer the question of when myosin mRNA is first produced in development. An answer to this question then raises the most important issue: namely, what events produce the genetic machinery that allows only a particular cell to produce myosin mRNA and myosin protein (Holtzer and Sanger, 1972)? In what follows we discuss the reliability of the techniques we used to answer the first question.

**Antigen and antibody characterizations.**

An antibody against the heavy chain of skeletal muscle myosin was prepared from adult *Xenopus* muscles to probe the developmental appearance of myosin mRNA and myosin itself. The muscle, to be used as immunogen, was dissected from the back and superficial thigh muscles. The muscles of the back in amphibians develop from the myotomes
(Balinsky, 1975). The limb musculature of amphibians probably develops from mesodermal cells that originate in the myotomes (Balinsky, 1975). Therefore, the myosin molecule isolated from adult tissue should have some antigenic determinants that are similar to embryonic myosin. Furthermore, the immunogen used in this study was isolated from denaturing SDS-acrylamide gels and consequently most if not all of its antigenic determinants should have been exposed. We have shown that the antibody generated under these conditions reacts with adult and stage 20 embryonic myosin. This does not imply that adult and embryonic myosin are identical but that they share at least a partial set of antigenic determinants. Sreter et al. (1972) showed that myosin isolated from young chick myotubes has the same ATPase activity as adult fast muscle myosin (FM). These facts and others implied that embryonic myosin (EM) is similar to adult FM. Trayer and Perry (1966) on the other hand, found that embryonic avian and mammalian myosin resemble slow adult muscle myosin (SM) as judged by immunological tests, ATPase activity and the lack of 3-methylhistidine. Subsequently, Sreter et al. (1975) found that the ATPase activity of myosin prepared from muscles of 3-4 week rabbit embryos is similar to the activity of FM. They also found some similarity between the tryptic fragments generated from EM, SM and FM.

However, contractile proteins have been shown to exist in non-myogenic cells including cleaving sea urchin embryos (Schroeder, 1973; for a review see Pollard and Weihing, 1974). It thus appears that Xenopus non-muscle myosin and muscle myosin are the products of different genes and do not share many common antigenic determinants. In agreement with this conclusion, Holtzer and co-workers (Chi et al., 1975) have
used immunodiffusion to precipitate myosin extracted from presumptive chick myoblasts, fibroblasts, nerve cells, smooth muscle cells and gut epithelial cells. These workers also found that only myosin from myoblasts, myotubes and muscle cells were precipitated by antibody against skeletal myosin. Similarly, Pollard et al. (1976) have demonstrated that anti-human skeletal myosin antibodies do not precipitate myosins from human uteri or platelets. Burridge and Bray (1975) have analyzed non-muscle myosins and muscle myosins from various chicken tissues by cyanlation and proteolytic cleavage. If the cleavage patterns reflect differences in primary sequences, then six different myosins exist in the chicken, one of them being skeletal muscle myosin. Interestingly, chick embryonic muscle myosin gave a pattern similar to adult leg. Thus, it appears that myosin is synthesized in many cell types but that only myoblasts synthesize skeletal-like myosin, which itself may be heterogeneous. Furthermore, skeletal-type myosin can be detected in vivo in chick embryos using antibodies against myosin, at stage 13-15 (50-55 hours after fertilization) for the myoblasts of the brachial myotomes (Holtzer et al., 1957; Masaki and Yoshizaki, 1974). These studies imply that myosin synthesis is linked to somite segregation in the chick.

These results all suggest that, although different myosin genes may be turned on during development, a large degree of similarity exists at the level of the primary sequence and, perhaps, even at higher folding levels of different myosins. Therefore, since the antibody used in this study was probably generated by a microheterogeneous immunogen composed of different myosin heavy chains, we feel that its cross-reactivity with embryonic myosin is not an artifact or even surprising.
Another point needs to be stressed concerning the preparation of the antigen for immunological work. The myosin used in this study was isolated from SDS-acrylamide gels. Care was taken not to include in the gel slices proteins that migrate with slightly higher or lower mobilities than the myosin heavy chain. Offer (1976) has shown that C-protein, which is a component of myofibrils and frequently contaminates so-called pure myosin preparations, is a potent immunogen which is capable of eliciting a large amount of antibody. It is therefore important to remove all traces of so-called "contaminating proteins" during the preparation of an antigen since it is known that the appearance of these proteins during development is closely linked to the appearance of myosin (Fischman, 1970). We cannot rule out the possibility that the SDS gel bands used as antigens contain proteins other than myosin heavy chains. However, any significant contamination of the myosin preparation with minor proteins is unlikely because the proteins would have to display similar chemical and electrophoretic properties to myosin, in order to have been included in the antigen preparation.

Isolation of myosin mRNA.

The studies reported here on the appearance of myosin mRNA suggest that the message is synthesized at low levels during the late neurula stage (16/17). The mRNA is not detectable at the medium yolk plug stage (stage 12) using the wheat germ cell-free system and immunoprecipitation.

The possibility that Xenopus embryonic RNA and specifically myosin mRNA was degraded during RNA isolation cannot be ruled out completely but is unlikely. As discussed in Results B, RNA was isolated
in the presence of reagents which inactivate ribonucleases. Furthermore, RNA isolated from stage 12 and stage 20 embryos mixed together was capable of challenging the wheat germ cell-free system to synthesize myosin as determined by immunoprecipitation. This experiment showed that myosin mRNA is not inactivated by some substance present in stage 12 embryos and that myosin mRNA is not preferentially degraded in homogenates of stage 12 embryos. However, it is possible that an inhibitory factor in pre-neurula embryos binds only to nucleotide regions present on non-polysomal RNA molecules. In this case, stage 20 myosin mRNA would not be inhibited since it most likely is present on polysomes. Such a model has been proposed for myosin mRNA stabilization in chick myogenesis (Heywood and Kennedy, 1976). However, conclusive evidence is lacking for this model and the purity of the putative inhibitor has not been established.

Weber et al. (1977) have found that at high potassium concentrations, unmethylated Vaccinia mRNAs are translated at 15-20% of the efficiency of methylated RNAs in reticulocyte and wheat germ cell-free systems. It is possible that the lack of this modified 5' terminus may prevent translation of stage 12 myosin mRNA under our conditions (see also Furuichi et al., 1976). However, evidence to date indicates that lack of a methylated 5' terminus on a message reduces the efficiency of binding of an mRNA to the 40S ribosomal subunit but does not inhibit its translation (Kaesberg, 1976). If myosin mRNA is synthesized at stage 12 or earlier in Xenopus embryos, it probably can be translated but cannot be detected by our methods because of low concentrations.

It could be argued then that prior to stage 16/17 in Xenopus
embryos, myosin mRNA is present at such a low concentration that, in
the absence of a specific Xenopus myosin mRNA regulatory factor, it is
not translated efficiently enough to be detected in a wheat germ
cell-free system. Heywood and co-workers (Rourke and Heywood, 1972;
Heywood and Kennedy, 1974; Heywood et al., 1974) have reported that
chick myosin mRNA is more efficiently translated in a reticulocyte
cell-free system in the presence of muscle initiation factors (IF-3),
which make a specific appearance in chick myogenesis. However, many
mRNAs have been translated in heterologous cell-free systems and no
absolute requirement for specific initiation factors has been
demonstrated (Bloemendal, 1972). For example, myosin mRNA has been
translated in heterologous rabbit reticulocyte and wheat germ systems
without specific requirements (Mondal et al., 1974; Strohman et al.,
1977; Paterson and Bishop, 1977). No doubt both in vivo and in vitro
many factors (such as initiation factors) and many conditions (such as
ribosome concentration and mRNA purity) are responsible for translational
efficiency. However, so far, various studies show that modifications
of eukaryotic mRNAs and the presence of various translation factors
play a facilitatory rather than an obligatory role in in vitro
translations.

One area related to the translation of mRNA, which recent
structural studies have left unresolved, but which is an area of
importance to differentiation, is the question of mRNA turnover.
Buckingham et al. (1974; 1976) have demonstrated that dividing
presumptive muscle cells contain a 26S RNA that has a half-life of 10
hours. As the presumptive muscle cells undergo differentiation, the
half-life of this 26S RNA increases. After fusion of muscle cells, this
26S RNA is found on polysomes. However, to date, the protein product of this 26S RNA in pre-fusion and post-fusion has not been identified. The 26S RNA in pre-fusion cells may code for some non-muscle myosins or other muscle cell proteins. It is possible that prior to neurulation in *Xenopus* embryos, the precursor of myosin mRNA has a short half-life and cannot be isolated undegraded or in large amounts. Furthermore, because of its instability it may not be translated. This possibility cannot be eliminated. However, if failure to detect myosin mRNA was exclusively due to rapid intranuclear breakdown of newly-synthesized myosin mRNA, the half-life of the newly-synthesized message would probably have to be much less than 10 hours in order not to be detected in a cell-free system.

The results of Buckingham et al. (1974; 1976) do not agree with the results of Strohman and co-workers (Hughes et al., 1977). In this study the rates of synthesis of whole cell RNA and myosin 26S RNA from chick embryonic breast muscle cells were measured by analyzing both the specific activity of cellular precursor UTP pools and the rates of incorporation of \( \text{[^3H]-uridine} \) into these RNAs. It was found that the overall rate of synthesis of whole cell RNA remained relatively constant throughout myotube development in culture, but the rate of 26S mRNA synthesis was 2.5-fold higher after cell fusion. This evidence strongly supports the conclusion that the increased synthesis of muscle myosin during myogenesis is regulated by the concentration of mRNA in the cytoplasm rather than by activation of a pre-existing mRNA population— that is, myogenesis is probably regulated by transcriptional rather than by translational control mechanisms (Paterson and Bishop, 1977).

Finally, to prove that the absence of myosin mRNA in stage 12
embryos does not represent the limit of sensitivity of the wheat germ assay systems, two more controls should be run in the future. First, it should be ascertained what the smallest amount of myosin mRNA molecules that can be detected in the wheat germ system using anti-myosin antibody actually is. Secondly, when small amounts of myosin mRNA are translated in the wheat germ system, it should be determined whether there is a systematic loss of myosin mRNA or newly-synthesized myosin (degradation of a constant number of molecules) which would create an artificial absence of myosin or myosin mRNA upon analysis (Palmiter, 1974).

Conclusions.

The data presented in this study are among the first information, in *X. laevis* embryos, on the synthesis of a structural protein related to a specific phenotype. Recently, Reeves (1977) showed that *Xenopus* tadpoles can be induced to synthesize keratin mRNA by exogenous thyroid hormone treatment. Ribonucleic acid isolated from uninduced tadpoles did not synthesize keratin. These results imply that little keratin mRNA is present in uninduced tadpoles. Therefore, it appears that the synthesis of myosin in *Xenopus* somites is controlled by the same kind of mechanisms regulating the synthesis of hormonally-induced proteins (keratin in *X. laevis* embryos, vitellogenin in *X. laevis* livers, ovalbumin in the chick oviduct).

Other proteins that have been studied in *X. laevis* embryos are histones, tubulins and actins. These proteins make their appearance at various times before and after fertilization of the embryos (Schroeder, 1973; Pestell, 1975; Adamson and Woodland, 1976; Brock and Reeves, in press; Woodland, unpublished results). Since all of these
proteins are needed by cells at all times, it is not surprising that their mRNAs are synthesized before and after fertilization.

It contrast to our studies, Perlman et al. (1977) have reported the presence of tadpole and adult hemoglobin mRNA in *Xenopus* oocytes. The synthesis of this message may represent "leakage" of globin gene transcription (implying that no biological system may be turned off completely), since the amounts detected are relatively low if one takes into account the large size of *Xenopus* oocytes (Perlman et al., 1977).

On the other hand, it is possible that in the case of cell-specific proteins (such as myosin, hemoglobin, etc.), the respective messages may be synthesized at low levels prior to fertilization. The rates of synthesis of these messages may also be controlled by morphogenetic determinants synthesized early in oogenesis. Assuming such a situation, one can postulate that as development proceeds, myosin mRNA is synthesized at low rates (and possibly even myosin itself) and then sequestered in those cells that will give rise to somites. The function of such a hypothetically stored message (and/or its protein) would be to tide the presumptive muscle cells over until they begin new transcription of myosin mRNAs and to play an important role in this transcription. However, until some direct experimental evidence is advanced to support such a hypothetical situation for muscle differentiation, the results of the work reported here and from other laboratories (Holtzer et al., 1975; Strohman et al., 1977; Paterson and Bishop, 1977) indicate that such a situation is not the general case for muscle differentiation in vertebrates. Rather, it seems that the appearance of skeletal myosin during muscle differentiation is under some form of transcriptional control. The question that remains is
what causes a cell to synthesize myosin mRNA.
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ADDENDA

p. 103:

p. 106: