THE GENES AND MESSENGER RNA FOR THE MYOSIN HEAVY CHAIN

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To my mother and the memory of my father.

I declare that the work described in this thesis is my own.

SUMMARY

A fraction of polysomes was isolated from chick embryonic leg skeletal muscle, which synthesized solely the myosin heavy chain in a homologous cell-free translation system. Furthermore, the mRNA isolated from these polysomes was shown to synthesize exclusively the myosin heavy chain in a heterologous cell-free translation system. This myosin heavy chain mRNA (MHC mRNA) was used as a template for the synthesis of complementary DNA (MHC cDNA). The kinetics of hybridization reaction between the MHC cDNA and an excess of its template mRNA revealed the presence of two different mRNA sequences coding for the chick embryonic myosin heavy chain. The reannealing of MHC cDNA to an excess of chicken DNA indicated the presence of two genes for the two MHC mRNAs, suggesting little reiteration for the chick embryonic myosin heavy chain genes.

The MHC cDNA was used as a probe in *in situ* and *in vitro* hybridization experiments, designed to detect and quantitate the MHC mRNA in individual cells during myogenesis *in vitro*. The presence of MHC mRNA in postmitotic mononucleated cells suggested that cell fusion is not necessary to trigger off the transcription of MHC mRNA.

Finally, a comparison between the purified MHC mRNA and 26S mRNA (considered as the myosin heavy chain mRNA) showed that although 26S mRNA contained MHC mRNA, there were many other sequences in it, which coded for other proteins.

CONTENTS

INTRODUCTION

During the development of higher organisms cells undergo morphological and biochemical differentiation. The differences between cell types are undoubtedly due to regulation of gene expression, one of the manifestations of which is the synthesis of different proteins.

Differentiation of muscle cells (myogenesis) is associated with distinct steps, which are both morphologically recognized and biochemically characterized by the synthesis of specific proteins. Recent research on myogenesis has attempted to resolve questions such as the nature of the extracellular factors affecting differentiation, the role of the final mitosis before differentiation in the switching off and the switching on of genes necessary for the overt differentiation, and the transcriptional and translational control mechanisms.

Of the proteins characteristic of muscle cells the myosin heavy chain is the most abundant, comprising 30% of the total protein (Sender, 1971). Because of this, its accumulation in muscle cells has been taken as indicative of differentiation. Different forms of the myosin heavy chain have been detected in physiologically different muscle, embryonic skeletal muscle and non-myogenic cells (for review see John, 1976,a). These differences have raised the possibility that several structural genes exist for the myosin heavy chain, which are differentially expressed during development.

Analyses of the proteins are not sufficient to make deductions about the nature of genes. For several proteins post-translational modifications are known to occur which could give a false impression of the number of genes. Direct studies of the genes and their mRNA

transcriptional products are necessary in order to resolve questions concerning the number of genes or the mechanisms that affect their expression.

A discovery that has given considerable impetus to studies of gene expression and regulation is the transcription of eukaryotic messenger RNAs (mRNAs) into complementary DNA (cDNA) molecules (Ross et al., 1972; Verma et al., 1972). Such cDNA copies have been successfully used in molecular hybridization studies, designed to investigate gene frequencies, mRNA complexity and metabolism and gene isolation (Sullivan et al, 1973; Harris et al., 1975; Bishop et al. 1974,b; Efstratiadis et al., 1976). Furthermore, the use of cDNA probes in in situ hybridization experiments has allowed the detection and distribution of minute amounts of an mRNA sequence in cytological preparations of individual cells at different stages of differentiation (Harrison et al., 1974,b; Conkie et al., 1974).

In the present investigation a cDNA probe to the myosin heavy chain mRNA (MHC mRNA) has been used in a study of the mRNAs and genes for the myosin heavy chain.

Myosin Structure

The structure of myosin has been the subject of many reviews (e.g. Gergely, 1975). It is established that myosin is a long assymetrical molecule with a molecular weight of about 470.000 daltons. It is composed of two large polypeptides "heavy" chains (molecular weight 200.000 each) and a variable number of small polypeptide "light" chains with molecular weights of 16.000-28.000. The myosin molecule is polarized having two globular heads attached at one end of a long

highly α-helical rod-like tail. The tail is about 140 nm long and is composed entirely of the two heavy chains. Each globular head consists of the folded end of one of the heavy chains with associated light chains. The heavy chains run parallel to one another in the rod with their N-terminal regions in the globular head structures and the C-terminal ends at the opposite tail end of the molecule.

Digestion by trypsin cleaves the molecule approximately 90 nm from the tip of the tail to give two fragments: heavy meromyosin (HMM) consisting of the heads attached to a shortened tail, and light meromyosin (LMM) consisting of the remainder of the tail. A low papain concentration cleaves the molecule into two fragments from the heads, called S_1 fragments, and a rod segment, about 135 nm in length, corresponding to the tail.

Differentiation of myosin

The muscle tissue of the body is classified according to physiological functions into (1) skeletal, (2) cardiac and (3) smooth muscle. Skeletal muscle fibres are further distinguished into fast-twitch and slow-twitch. Biochemical investigations of the myosins obtained from muscles with different physiological properties have indicated that myosin exist in different isoenzymatic forms.

Myosins isolated from fast and slow skeletal muscle exhibited differences with respect to ATPase activity, stability to acid and alkaline ph, susceptibility to tryptic digestion and light chain pattern (Barany et al., 1965; Sreter et al., 1966; Maddox and Perry, 1966; Lowey and Risbey, 1971). Cardiac and slow muscle, although similar in respect to some of these parameters, differed in their

light chains and tryptic peptides (Sarkar et al., 1971; Sreter et al., 1975). Furthermore, a number of reports on embryonic myosin, which may differ from the corresponding adult in enzymatic properties, subunit composition and fine structure, suggested a differentiation of the myosin molecule during development (John, 1974, 1976,a; Trayer and Perry, 1966; Sreter et al., 1975; Obinata et al., 1976).

In addition to the physiologically distinct myosins, myosin-like proteins have been found in a variety of non-myogenic cells (Pollard and Weihing, 1974). Although they share with muscle-myosins, some properties like size, molecular weight, ATPase activity and cross-linking with actin filaments, studies on their subunit composition and antigenicity indicated that they constitute a distinct class of myosin, called "cytoplasmic" myosin (Adelstein and Conti, 1972; Ostlund et al. 1974; Willingham et al., 1974; Weber and Groeschel-Stewart, 1974).

Heterogeneity of the heavy chains among all these different types of myosin has been established. Structural analysis of various myosin heavy chains by limited proteolytic digestion or chemical cleavage showed major differences between skeletal and cardiac myosins (Burridge and Bray, 1975). Furthermore, SDS gel electrophoresis revealed molecular weight differences in the heavy chains of muscle and cytoplasmic myosins (Burridge and Bray, 1975). Electron microscopy of negatively stained light meromyosin (LMM) paracrystals, showed that slow and cardiac myosin heavy chains exhibited a pattern different from that seen with fast muscle heavy chain, while the embryonic preparation was distinct from either slow or fast (Nakamura et al., 1971; Sreter et al., 1975). As a result of amino acid sequencing studies on peptides of heavy chains, Huszar (1972) reported the presence of three

different heavy chains in the embryonic, cardiac and fast myosins of rabbits. Immunodiffusion and fluorescent-antibody techniques indicated that the heavy chains of fast, slow and cardiac myosins were immunologically quite distinct (Masaki, 1974). Fluorescent-labelled antibody against skeletal light meromyosin did not bind to non-myogenic cells, suggesting that the myosin heavy chain in these cells was different from the one synthesized in fully differentiated muscle fibres (Chi et al., 1975,a,b).

Furthermore, differences even among the heavy chains of a distinct myosin population have been indicated by iso-electric focussing and electrophoresis in dilute gels (Florini and Brivio, 1969; Florini et al., 1971; Bechtel et al., 1971; Hale and Beecher, 1971; Burridge and Bray, 1975). Masaki and Yoshizaki (1974) using fluorescent antibody techniques observed that myotubes in early chick embryos, simultaneously synthesized heavy chains for fast, slow and cardiac myosins,but that in the adult each heavy chain was present exclusively in the expected muscle fibre. Analysis of the N-terminal peptide of rabbit fast myosin heavy chain by chymotryptic digestion, revealed the presence of two different sequences,indicating the existence of two types of heavy chains (Starr and Offer, 1973).

All the above mentioned observations suggest that myosin exists in isoenzymatic forms, containing different heavy chains. However, it has not been established whether the different heavy chains are coded by distinct mRNA sequences or whether they arise as the result of a post-translational modification of a single molecule. Since differences on the primary structure of the heavy chains are unlikely to be due to a post-translational mechanism, it is most probable that

the former assumption is correct, and that there are several different myosin heavy chain genes, differentially expressed during development. In the present work an attempt was made to determine directly the number of different myosin heavy chain mRNA sequences and the number of their respective genes.

Myosin Synthesis during Myogenesis *in vitro*

Muscle differentiation is an asynchronous process *in vivo*, while myogenesis in tissue culture can be highly synchronous, permitting frequent observations and experimental manipulations. Furthermore, the structural resemblence between muscle fibres formed *in vivo* and *in vitro* and the spontaneous contractibility in myogenic cell cultures, have been considered as an indication that the tissue culture system is a reliable model for the study of myogenesis.

The development of myogenic cultures occurs in three main phases: (1) cell proliferation, (2) intense cell fusion and (3) myotube formation. The transition from proliferation to the formation of multinucleated myotubes is associated with distinct changes in synthetic activities, such as cessation of DNA synthesis (Okæaki and Holtzer, 1966), decrease in RNA synthesis (Marchok and Wolff, 1968; Nguyen Thi Man and Cole, 1972), altered pattern of DNA transcription into RNA (Nguyen Thi Man and Cole, 1974), synthesis of contractile proteins and changes in the activities of enzymes (Coleman and Coleman, 1968). Based on these observations the concept has arisen that cell fusion is not only an important event in early myogenesis but that it somehow triggers off the expression of genes, responsible for later stages of differentiation. This view has created a lot of controversy in the

literature since at least some events characteristic of terminal differentiation, such as appearance of actin thin and myosin thick filaments, enzymatic transition and membrane differentiation, were shown to be independent of myogenic fusion (Holtzer et al., 1975; Tarikas and Schubert, 1974; Turner et al., 1976; Prives and Paterson, 1974).

The timing of myosin synthesis during the in vitro skeletal myogenesis has been especially used as an indication as to whether the synthesis of proteins, thought to represent the differentiated state, is causally related to cell fusion. Studies on the rate of myosin synthesis in chick or rat myogenic cultures indicated that myosin synthesis begun at fusion and increased linearly with the main fusion burst (Paterson and Strohman, 1972; Yaffe and Dym, 1972). Addition of Actinomycin D, at a concentration which inhibited RNA synthesis, just before or at the onset of fusion, did not prevent the synthesis of myosin in the multinucleated myotubes (Yaffe and Dym, 1972). Furthermore, detection of myosin heavy chain mRNA in the cytoplasm of dividing myoblasts, in the absence of myosin synthesis in the prefusion period, suggested that myosin heavy chain mRNA existed in a non-translated form before fusion (Buckingham et al., 1974, 1976).

However some recent experiments seem to have established the presence and synthesis of myosin in at least some unfused myogenic cells. Reporter (1974) found that lysolecithin-treated myogenic rat cells, which did not undergo extensive fusion, synthesized myosin at the accelerated rates, typical of standard myotube cultures. Rubinstein et al. (1974) observed that replicating myoblasts as well as embryonic fibroblasts and chondroplasts synthesized myosin. Moss and Strohman (1976) and Vertel and Fischman (1976) reported that the fusion arrested myoblasts,

in primary chick cultures, were capable of accumulating myosin heavy chain at a rate comparable to that of fused cells. Similarly, Emerson and Beckner (1975) showed myosin synthesis within mononucleated quail muscle cells, provided such cells were inactive in DNA synthesis. Finally, Young et al. (1975) analyzed the polysomal population of embryonic chick muscle cultures and observed that mononucleated myoblasts contained polysomes capable of synthesizing myosin heavy chain in an in vitro protein synthesizing system, suggesting that myosin heavy chain mRNA was attached to the polysomes even in mononucleated myoblasts.

Chi et al. (1975a,b) examined the myosins synthesized during the successive stages of myogenesis and found that myosin synthesized in non-myogenic cells and replicating myoblasts differed from the one synthesized in post-mitotic mononucleated myoblasts and in myotubes, at least as far as the light chain pattern and the antigenicity of heavy chain were concerned. Their results raised the question of whether different heavy chain genes were expressed in replicating myoblasts and in myotubes.

In the present investigation cells in different stages of differentiation in tissue culture were examined for the presence of myosin heavy chain mRNA by in vitro and in situ hybridization. In situ hybridization is a new approach which allows the identification of the individual cells which accumulate myosin heavy chain mRNA.

Myosin biosynthesis-Myosin heavy chain mRNA (MHC mRNA)

Considerable information exists about the biosynthesis of myosin. Heywood et al. (1967) showed that the large subunit of myosin was synthesized on polysomes whose size indicated that the mRNA was

monocistronic. The polysomes,on which myosin heavy chain was synthesized,were separated by sucrose density gradient. Electron microscopy observations revealed that they consisted of 50 - 60 ribosomes and because of their large size formed a distinct peak at the bottom of a linear sucrose gradient (Heywood et al., 1967).

The large MHC polysomes from leg muscle of chick embryos had been used as a source for the isolation of MHC mRNA, which was shown to sediment at 26S on a linear sucrose gradient but migrated slower than 28S on polyacrylamide gel (Heywood and Nwagwu, 1969; Sarkar et al., 1973; Morris et al., 1973). Mondal et al. (1974) reported the presence of polyadenylic acid segment, about 170 nucleotides long, attached to the 3' end of MHC mRNA while Przybyla and Strohman (1974) suggested that MHC mRNA, at least from myogenic cultures, contained polyadenylic acid tracts smaller than 50 nucleotides.

MHC mRNA was reported to exist in a non-polysomal form, associated with proteins in a cytoplasmic ribonucleoprotein particle (mRNP) (Heywood et al., 1975a). The presence of mRNP supported arguments about the existence of a translational control mechanism operating at least in muscle cells (Heywood et al, 1974, 1975b; Bester et al., 1975).

However, it is not clear whether the large polysomes,used in the previous reports as starting material for the extraction of MHC mRNA, were supporting the cell-free synthesis of myosin heavy chain solely (Heywood et al., 1967; Heywood and Rich, 1968). A number of myofibrillar proteins exist, the molecular weights of which are large enough to suggest that they may be synthesized on the same size of polysomes as myosin heavy chain, e.g. B protein (180,000) and C protein (150,000)

reported by Starr and Offer (1971), and filamin (250,000) reported by Wang et al (1975b). Thus MHC mRNA extracted from large polysomes and sedimenting as 26S on sucrose gradient could be easily contaminated with other mRNAs. Furthermore when cDNA transcribed from 26S mRNA, considered as MHC mRNA, was back hybridized to its template mRNA, the kinetics of the hybridization reaction indicated that the RNA preparation contained a vast number of different mRNA sequences (Robbins and Heywood, 1976; Buckingham et al., 1974). Although the number of different heavy chains is still unknown, the number of mRNA sequences calculated from these hybridization experiments was higher than should be reasonably expected for MHC mRNAs, suggesting a contamination of the MHC mRNA.

An essential prerequisite of the present investigation was to prepare a MHC mRNA which synthesized solely the myosin heavy chain.

DNA-RNA hybridization

The transcription of mRNA into cDNA by the viral RNA-dependent DNA polymerase has increased the applications of nucleic acid hybridization. Two of the most widely used applications of cDNA are:
(1) hybridization in RNA excess and (2) hybridization in DNA excess.
(1) Hybridization of cDNA back to a vast excess of the template mRNA has been used for measuring the complexity of RNA populations. Under conditions of large RNA excess over the cDNA, the reaction has pseudo-first order kinetics and the rate of hybridization is substantially independent of the ratio of RNA to cDNA. The rate of hybridization reaction can be conveniently characterized by the parameter Rot ½ (product of the initial RNA concentration and time at which the reaction

is half completed). With homogeneous RNA populations the reaction shows a single transition and the parameter Rot ½ is proportional to the complexity of the RNA species in the reaction, complexity being defined as the number of nucleotides (or their M.W.), necessary to code for the unit of information in the RNA population. In the case of heterogeneous RNA populations, where not all the RNAs are in the same concentration, the rate of hybridization reflects not only the complexity but also the frequency of the differing RNA sequences within the population. In this case, the cDNA-RNA reaction will not have a single transition, but two or more, the early one being attributed to the RNA species that are present in greater amounts than the others. In theory, the base sequence complexity of an mRNA population may be determined by a comparison of Rot ½ for the reaction between mRNA and its cDNA with the Rot ½ obtained with a kinetic standard (i.e. an mRNA with known complexity). Analyses of cDNA-mRNA reaction in different eucaryotic systems have permitted a determination of the number of different sequences present as mRNA and of the relative abundance of these sequences within the mRNA population (Bishop et al., 1975; Birnie et al., 1974; Axel et al., 1976). Furthermore, cDNA-RNA hybridization has been used for measuring the cellular concentration of specific mRNAs (Harris et al., 1975).

(2) Hybridization of cDNA to DNA, in DNA excess, has been used for measuring the reiteration frequency of specific DNA sequences. The reaction follows essentially the same rules as have been described for RNA-DNA hybridization in DNA excess (Melli et al., 1971). The use of cDNA in this reaction has certain advantages over the use of RNA

molecules,since cDNA is more stable at the annealing temperature than RNA, it survives better the long annealing times that are required, and the reaction is not subject to the same number of corrections as if RNA was used. To measure the reiteration of genes coding for a certain mRNA species,its cDNA is hybridized with a large excess of total DNA and the Cot ½ value of the reaction is compared to the Cot ½ value for the unique sequences. (Cot ½ is defined as the product of the initial DNA concentration and time at which the reaction is half completed). Such hybridization studies have shown that most of the mRNA in a variety of cells is transcribed from the unique fraction of the DNA, and have established that the genes coding for specific proteins, such as globin or ovalbumin, are definitely not present more than a few times in the genome (Bishop & Rosbash, 1973; Harrison et al., 1972; Sullivan et al., 1973). Until now only the genes for histones and feather keratins have been found to belong in the moderately repetitive DNA (Scott and Wells, 1976; Kemp, 1975).

The technique of in situ hybridization is an extention of the conventional DNA-RNA hybridization,which allows the detection and localization of specific nucleic-acid sequences in cytological preparations (John et al., 1969; Gall and Pardue, 1969). DNA or RNA are denatured in their intracellular location, radioactive nucleic acid probes are added, hybridization reaction is allowed to take place at the appropriate ionic strength and temperature,and the excess of radioactive material is removed. The radioactive hybrid molecules are detected by autoradiography,as silver grains superimposed on cellular components in which hybrid formation has occured.

Naturally synthesized DNA and RNA species or *in vitro* transcribed RNA molecules have been used as sensitive probes for chromosomal mapping of DNA sequences, identification of RNA synthesizing chromosomal sites or detection of viral nucleic acids in eucaryotic systems (Hennig, 1973; Jones, 1972; Moar *et al.*, 1975; Couskens and May, 1974). Recently, Harrison *et al.* (1973), using globin cDNA as a probe,succeeded in localising globin mRNA in the cytoplasm of red blood cells and established that the *in situ* hybridization permits detection of specific mRNA sequences at the single cell level. The process of globin mRNA synthesis during erythropbiesis was studied by the same technique (Harrison *et al.*, 1974b).

Purpose and Main Findings of the Present Investigation

In the present investigation,cDNA to highly purified MHC mRNA has been used as a probe to determine (1) the number of MHC mRNAs in chick embryonic skeletal muscle, (2) whether there is a family of MHC genes in the genome and their reiteration frequency and (3) at what stage during myogenesis in tissue culture MHC mRNA appears.

A fraction of the large MHC synthesizing polysomes from leg muscle of chick embryos was isolated and shown by translation in a homologous system to direct the synthesis of MHC only. RNA was isolated from this polysomal fraction and mRNA was further purified by oligo(dt) cellulose chromatography. The purified mRNA was shown to be MHC mRNA by translation in a heterologous cell free system,which established that myosin heavy chain synthesis did not result from a stimulation of endogenous mRNA. The cell-free system derived from wheat germ (Roberts and Paterson, 1973) was used,since it has low levels of

proteases and nucleases, low endogenous protein synthesis, it can be rapidly prepared from easily available starting material and it has been successfully used for the translation of eucaryotic mRNAs (Rosen et al., 1975; Gozes et al, 1975). In the present investigation by using the modification suggested by Harwood et al. (1975), the MHC mRNA was translated for the first time with high efficiency in the wheat germ system.

The criterion for the identity of the synthesized protein, in both cell-free systems, as myosin heavy chain was the comigration of the radioactive polypeptide with the 200.000 molecular weight myosin heavy chain marker on SDS-polyacrylamide gels. However, additional characterisation experiments, reported in the Appendix and performed with the collaboration of Dr H.A. John, confirmed the identification of the in vitro synthesized protein as myosin heavy chain.

The isolated MHC mRNA was transcribed into a cDNA molecule, which was subsequently used in hybridization studies.

Hybridization of MHC cDNA in MHC mRNA excess was used for estimating the complexity of the embryonic MHC mRNA, and revealed the presence of at least two different mRNA sequences in the 14 day skeletal muscle of embryonic chick.

Hybridization of MHC cDNA with an excess of total chicken DNA was used for determining the reiteration frequency of embryonic MHC genes. The results suggested a limited number, most likely two, for the chick embryonic MHC genes.

In situ and in vitro (RNA excess) hybridization of MHC cDNA with the RNA of myogenic cells from different stages of myogenesis in tissue

culture, allowed a study on the appearance of MHC mRNA during myogenesis.

Finally, because 26S mRNA is now widely used in investigations as MHC mRNA and because of doubts over its purity, 26S mRNA was isolated from chick embryos leg muscles, translated in a heterologous cell-free system and hybridized with its cDNA. The results showed that 26S mRNA preparation contained MHC mRNA along with a large number of other mRNAs.

MATERIALS AND METHODS

Abbreviations

Ara-C	Cytosine Arabinoside
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleotides
EDTA	Ethylenediaminetetra-acetic acid sodium salt
EGTA	Ethyleneglycerol bis (b-amino ethy ether) -N'-N'-tetraacetic acid
GTP	Guanosine triphosphate
HAP	Hydroxylapatite
HC	Heavy chain
MHC	Myosin heavy chain
MEM	Minimal essential medium
Oligo(dt)	Oligo-thymidylic acid
Poly(A)	Poly-adenylic acid
Poly(U)	Poly-uridylic acid
Poly(A) + RNA	RNA containing a Poly(A) segment
Poly(A) minus RNA	RNA lacking a Poly(A) segment
PPO	2-5-diphenyloxazole
POPOP	1, 4 bis-2-(4-methyl-5-phenyloxazole)-benzene
RNA	Ribonucleic acid
mRNA	Messenger RNA
rRNA	Ribosomal RNA

tRNA	Transfer RNA
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate
TCA	Trichloroacetic acid
TRIS	Trishydroxymethylaminomethane

Buffers and Solutions

M Buffer	0.25M KCl
	0.01M $MgCl_2$
	0.01M Tris HCl, pH 7.4
Kirby's Solution	1%Na-triisopropylnaphtalene sulphonate
	6% Na-4-amino salicilate
	1% Na Cl
	6% n-Butanol
	0.5% SDS
Phenol Solution	500 gr phenol = 550 ml
	water-saturated phenol
	550 ml chloroform
	70 ml m-cresol
	0.5 gr 8-hydroxyquinoline
Binding Buffer	0.4M NaCl
	1mM EDTA
	10mM Tris pH 7.6
	0.1% SDS
Elution Buffer	1mM EDTA
	10mM Tris pH 7.6
	0.1% SDS

Column Buffer	0.3M NaCl
	0.01M Na acetate pH 5.0
SSC	150mM NaCl
	15mM Na citrate
PB	Equimolar phosphate buffer
PEB	1.2M PB + 5mM EDTA
KB3	0.15M Na Acetate pH 4.5
	0.003M $ZnSO_4$
	0.5M NaCl
	0.26N CH_3COOH
Dulbecco A	200 mg/lt $K_2H_2PO_4$
	8×10^3 mg/lt NaCl
	200 mg/lt KCl
	1150 mg/lt Na_2HPO_4

Counting Fluids

Toluene-PPO-POPOP	3 gm PPO and 0.3 gm POPOP per litre of Toluene

Materials

Fertilized unincubated chicken eggs were obtained from Marshal Ltd., Newbridge, Scotland.

Rabbit globin mRNA was obtained from Searle Res. Lab., High Wycombe, England.

Wheat germ was a gift from Dr B. Paterson (University of Edinburgh)

Avian myeloblastosis virus (AMV) reverse transcriptase was a gift from Dr J.W. Beard (Duke University).

Oligo(dt) was obtained from Collaborative Res. Inc., Waltham, Massachusetts, USA.

^{3}H-adenosine triphosphate, ^{3}H-leucine and ^{3}H-deoxycytidine triphosphate were obtained from the Radiochemical Centre, Amersham, England.

DNase, RNase, S_1 nuclease, creatine kinase, creatine phosphate, ATP, GTP, dNTPs, amino acids, spermine, Dextran sulphate, Triton X-100, Tris, SDS, HEPES, EGTA, Ara-C were obtained from Sigma Biochem. Inc., London, England.

Sephadex was obtained from Pharmacia, Uppsala, Sweden.

MEM and Horse serum were obtained from Gibco-Biocult, Glasgow, Scotland.

Dulbecco Saline was obtained from Oxoit Ltd., London, England.

Trypsin was obtained from Difco Lab., Detroit, Michigan, USA.

Actinomycin D was obtained from Cal Biochem, California, USA.

HAP was obtained from Bio-Rad Labor. California, USA.

PPO and POPOP were obtained from Packard Instrument Company Inc., Illinois, USA.

Aquasol was obtained from New England Nuclear, West Germany.

All the other chemicals were obtained from British Drug Houses Ltd.

All the solutions and the glassware used during polysome or RNA preparation, translation and hybridization were either sterilized or autocleaved.

Polysomes Preparation

Polysomes from leg muscles of 14-day old chick embryos were prepared and fractionated according to the procedure described by Heywood et al. (1967), with some modifications.

Leg muscle was homogenized in an equal volume of ice-cold M Buffer

containing 50 µg Dextran sulphate per ml, as an RNase inhibitor. The post-mitochondrial cytoplasmic supernatant obtained by centrifugation of the homogenate at 10.000 g for 10 min was layered on 36 ml of a 15 - 40% linear sucrose gradient. To avoid sedimentation of MHC synthesizing polysomes to the bottom of the tube, each gradient had a 1 ml cushion of 60% sucrose. The cytoplasmic supernatant from 10 embryos was divided and layered onto six 37 ml gradients which were centrifuged for 2 hr at 25.000 rpm at 4°C, using a Beckman SW 27 rotor in an L5 centrifuge. Fractions were collected from the bottom of the tube and the optical density at 254 nm was recorded continuously in an ISCO spectophotometer.

The SW 3 x 23 and SW 6 x 15 rotors of MSE 50 and MSE 65 centrifuges respectively, were found inadequate for the preparation of reasonable polysome profiles.

For preparation of unfractionated total polysomes, 4.5 ml of post-mitochondrial cytoplasmic supernatant were layered on 4.5 ml of 20% sucrose in M Buffer, and centrifuged in an MSE 50 centrifuge, 10 x 10 rotor, for 2 hr at 100.000 g at 4°C (Lee and Brawerman, 1971).

Either total or fractionated polysomes were collected from the respective regions of a polysomal sucrose gradient and were pelleted by centrifugation for 16 hr at 100.000 g at 4°C. All polysomal pellets were usually rinsed with ice-cold distilled H_2O, to remove traces of sucrose, and kept at -70°C for up to one week without significant loss of activity.

RNA extraction from embryonic chick leg muscle.

(a) Cytoplasmic RNA

The RNA from the cytoplasmic fraction was extracted by a modification of Kirby's method (Kirby, 1965; Parish and Kirby, 1966).

The tissue was homogenized at 0°C with a Dounce homogenizer in 1 volume of ice-cold M Buffer containing 50µg Dextram Sulphate per ml. The homogenate was centrifuged for 10 min at 10.000 g to remove unbroken tissue, nuclei and mitochondria. To the cytoplasmic fraction 1 volume of Kirby's solution and 2 volumes of Phenol solution were added and the mixture was vigorously shaken for 20 min at 22°C. Centrifugation for 10 min at 10.000 g separated phenolic from aqueous phase. The aqueous phase was removed and the phenolic phase was re-extracted with equal volume of cold distilled H_2O. The first and second aqueous phases were combined and re-extracted three more times with fresh phenol solution and finally with chloroform to remove any remaining phenol. The final aqueous phase was removed, made 0.2M in sodium acetate pH 5 and the RNA was precipitated by the addition of three volumes of cold ethanol and stored at -20°C for at least 12 hours.

(b) Polysomal RNA

For the extraction of polysomal RNA a combination of the procedures described by Lee et al. (1971) and Perry et al. (1972) was used. The use of alkaline pH reduces loss of Poly(A) containing RNA and the addition of chloroform in the phenol solution guards against removal of Poly(A) ends from the mRNA.

Polysomal pellets were resuspended in 0.01 M Tris pH 9.0, 0.5% SLS and were disrupted by pipetting. An equal volume of phenol : chloroform : iso-amyl alcohol (50 : 50 : 1) mixture was added and the

suspension was shaken at 22°C for 15 min. After separation of the phases by centrifugation at 10.000 g for 10 min at 4°C the upper phase (aqueous) was collected and the phenolic phase was re-extracted with 0.01 M Tris, pH 9. The combined aqueous phases were extracted two or three times with fresh phenol : chloroform : iso-amyl alcohol solution and finally with chloroform. RNA was precipitated at -20°C, after the addition of sodium acetate and three volumes of cold ethanol.

Precipitated RNA was collected by centrifugation at 25.000 x g for 30 min at -10°C.

Oligo(dt) cellulose fractionation of RNA

Two grades (T_2 and T_3) of oligo(dt) cellulose (containing covalently bound oligodeoxythymidylic acid residues) were tested. The T_2 grade had low binding capacity. The T_3 grade had 2 - 5 fold greater binding capacity and less non-specific binding than the T_2 grade. The binding and elution method of Aviv and Leder (1972) was used, with some modifications in the composition of the buffers. That is, EDTA was added and NaCl was used instead of KCl, so that SDS could be included to inhibit any RNase.

The RNA sample was dissolved in a small volume of a high ionic strength buffer (binding buffer) and was layered on a 500 mg oligo(dt) cellulose column (grade T_3). Under these conditions the Poly(A) + RNA was hybridized to the oligo(dt), while the Poly(A) minus RNA was excluded by washing the column to a background optical density with the binding buffer. The Poly(A) +RNA was then eluted from the column by washing it with small volumes of low anionic strength buffer (elution buffer). It had been a common observation that non-specific types of

binding occured when oligo(dt) cellulose was used to analyze or to prepare poly(A) + RNA, the most common being contamination with rRNA (Wang et al., 1975a; Bantle et al., 1976). A second fractionation of the Poly(A) + RNA on the same column reduced considerably the degree of contamination.

RNA from either Poly(A) minus or Poly(A) + fractions was precipitated with three volumes of cold ethanol at -20°C, after adjusting the salt concentrations to 0.2M. The purified mRNA was either kept in ethanol at -20°C or it was dissolved in a small volume of sterile distilled H_2O and kept at -70°C until further use. Storing the RNA at -70°C for at least 3 weeks did not cause any significant loss of activity.

Separation of RNA on Sucrose Gradient.

RNA fractionated by oligo(dt) was analyzed on 17 ml, 15 - 30% sucrose gradient in 0.02M sodium acetate, 0.5% SDS, 0.005M EDTA, 0.04 M Tris-HCl pH 7.8. Centrifugation was for 16 hr at 25.000 rpm at 22°C in the SW 27 rotor of a Beckman L5 model. The gradients were collected with an ISCO fraction collector. The absorbancy at 254 nm was continuously monitored with an ISCO flow analyzer and recorded with a Bryans recorder.

Hybridization of ^{3}H-labelled Poly(U) along sucrose gradient

A small aliquot from each gradient fraction was hybridized directly with ^{3}H-labelled Poly(U) (specific activity 2.5 x 10^5 cpm/µg) in Poly(U) excess (Bishop et al., 1974a). Hybridization was carried out in 2 x SSC for 15 min at 37°C. The samples were then chilled,

diluted 20 times with cold 2 x SSC and treated with 20µg/ml of RNase in ice for 20 min. The samples were TCA precipitated, collected on millipore filters and counted.

Estimation of the Poly(A)-containing RNA by titration with radioactive Poly(U)

The mRNA concentration of all Poly(A) + RNA preparations used in hybridization or translational experiments was estimated by hybridization to ^{3}H-Poly(U) (Bishop et al., 1974a). Two different dilutions of the RNA sample were hybridized with an excess of ^{3}H-labelled Poly(U), in 2 x SSC. The same dilutions of a Poly(A) solution of known concentration were also hybridized with an excess of the ^{3}H-labelled Poly(U), in order to be used as calibration standard. Hybridization, RNase treatment and measurement of radioactivity was as described before. Controls without added RNA but with ^{3}H-Poly(U) and RNase were subtracted from the sample's counts.

Cell-free system for translation of Polysomes

Polysomes were translated in vitro by the method described by Heywood et al. (1967). S-150 enzyme fraction was prepared according to Heywood and Nwagwu (1969). Unfractionated total polysomes, pelleted through 20% sucrose, were used to establish the optimum conditions for protein synthesis.

Polysomal pellets were gently resuspended with a glass rod at 0°C in 1 ml of the translational mixture containing: 0.15M KCl, 0.01M $NgCl_2$, 6mM 2-mercaptoethanol, 0.01M Tris (ph 7.4), 2mM ATP, 0.5mM GTP, 50 µg of creatine kinase, 10mM creatine phosphate, 5 µM of all amino acids excluding leucine, 15 µCi of ^{3}H-leucine (50 Ci/mMol), 0.6 mg of

S-150 fraction. Addition of t-RNA to the protein synthesis assay was omitted since it did not improve incorporation. Incubation was for 50 min at 37°C and the reaction was terminated by the addition of 50 µg of RNase.

To assay for total radioactivity incorporated into protein, aliquots of up to 50 µl were pipetted immediately on to discs of Whatman 3MM paper. The discs were dried, placed on to 10% TCA containing 1000-fold excess of unlabelled leucine for 30 min, transferred to 5% TCA, boiled at 90°C for 15 min, rinsed with cold 5% TCA and dried by sequential rinsing in ethanol, ethanol-ether (3:1 v/v) and ether (Parish, 1972). Radioactivity was determined by liquid scintillation counting with 10 ml of Toluene-PPO-POPOP scintillant.

The incubation mixture was freshly prepared from stock solutions, kept as suggested by Dr C. Darnbrough (personal communication). Creatine kinase was stored separately in 50% glycerol,while ATP, GTP and creatine phosphate were kept together as a mixed solution. Mercaphoethanol was included in the amino acids solution.

Incorporation of amino acids proceeded linearly for 40 min and then levelled off (Fig. 1). When aurintricarboxylic acid (ATA), a selective inhibitor of initiation (Mathews, 1971),was added to the incubation mixture, most of the radioactive incorporation was ATA insensitive (Fig. 1), indicating that initiation did not take place. Thus, only completion of polypeptide chains, that had been initiated before isolation of the polysomes, occured in the translational system.

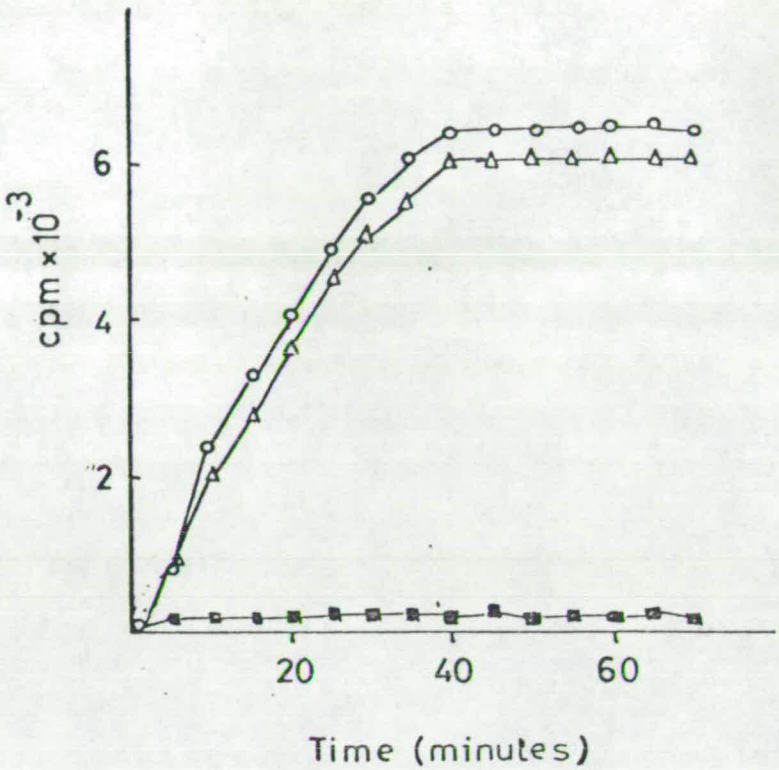

Figure 1.

Time course of cell-free protein synthesis directed by 0.25 mg of chick leg muscle polysomes. 50 µl samples were withdrawn from an 1 ml incubation mixture at the indicated time intervals and TCA insoluble radioactivity was determined.

o——o——o Complete system

Δ——Δ——Δ Complete system + 1 mM Aurintricarborylic acid (which inhibits initiation)

■——■——■ Complete system minus polysomes.

RNA translation in the wheat germ cell-free system.

The wheat germ translational system of Robert and Paterson (1973) was used to test the RNA samples for functional mRNAs. Fresh untoasted wheat germ was obtained as a gift from Dr B. Paterson. The 30,000 g supernatant fluid (S-30) was prepared as described, except that the wheat germ was ground before adding buffer (Marcu and Dudock, 1974). The preparation was stored as frozen spheres at -70°C; only slight loss of activity was observed over a period of 4 months.

Standard protein synthesis assays contained in a final volume of 50 µl: 20 µl of S-30, 20 mM Hepes (pH 7.6), 2 mM dithiothreitol, 1 mM ATP, 200 µM GTP, 8 mM creatine phosphate, 40 µg/ml creatine kinase, 2.8 mM Magnesium Acetate, 25 µM unlabelled amino acids excluding leucine, 5 µCi ^{3}H-leucine (50 Ci/mMol) 60 µM spermine (free base) and KCl and RNA as indicated in the figure legends. Reactions were incubated for 90 min at 25°C and treated with RNase as described by Roberts and Paterson (1973). To test for total TCA insoluble radioactivity aliquots of 5 µl were spotted on to 2.5 mm discs of Whatman 3MM paper which were processed as previously described. (Page 25).

Purified rabbit globin mRNA (obtained from Searle) was used for testing the activity of the wheat germ cell-free system. The incorporation of ^{3}H-leucine into TCA-precipitable material was stimulated 100 fold or more on the addition of globin mRNA at an optimum concentration of 20 µg/ml. The optimal concentration of KCl was found to be 96 mM and spermine seemed to enhance amino acid incorporation, as it has been reported before (Gozes et al., 1975). Under these optimal conditions and at 25°C, incorporation was at a linear rate for 60 min and continued for at least 90 min (Fig. 2). Furthermore, electrophoretic

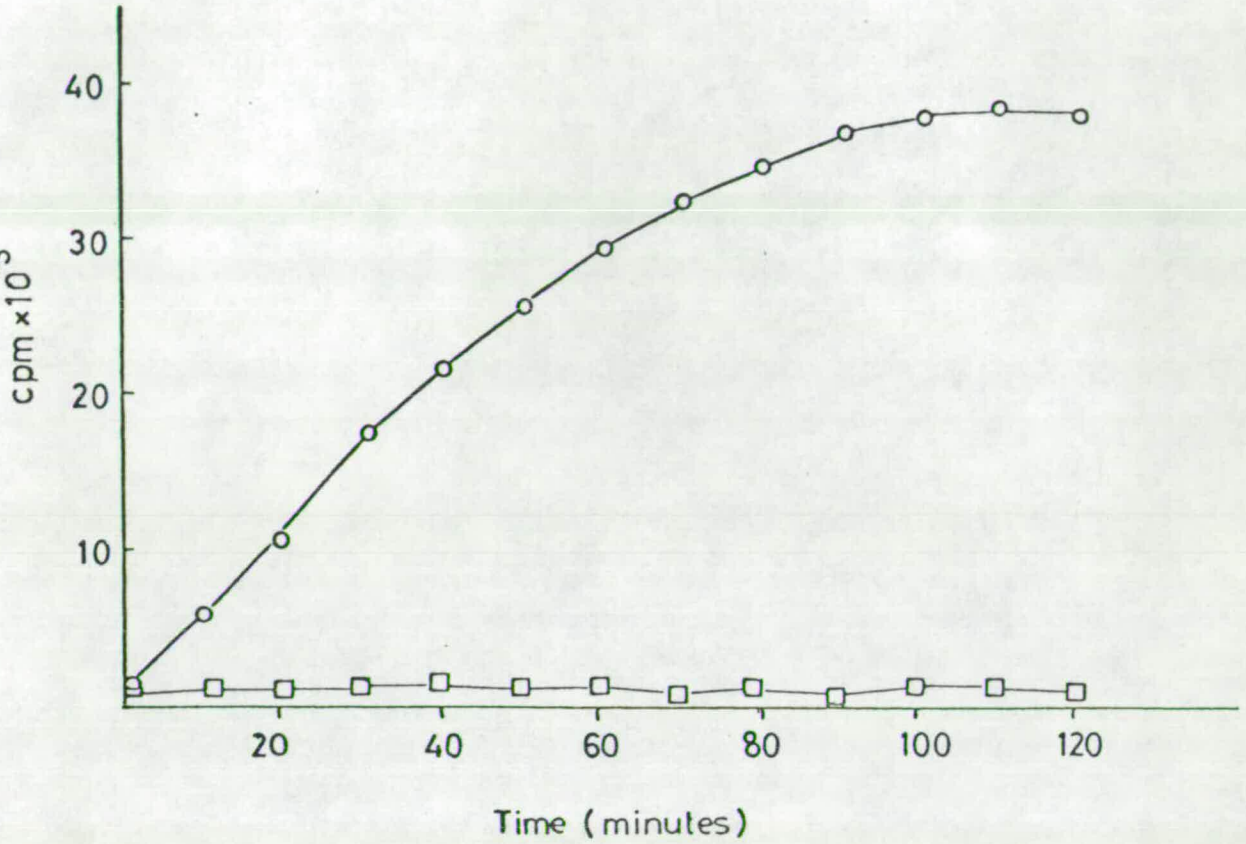

Figure 2.

Time course of protein synthesis in the wheat germ cell-free system. The incubation mixture (50 µl) contained 96 mM KCl. At the indicated time intervals 2.5 µl aliquots were withdrawn and TCA insoluble radioactivity was measured.

o—o—o Complete system with 1 µg rabbit globin mRNA.

□—□—□ Complete system without added RNA.

analysis of the translational products synthesized upon the addition of rabbit globin mRNA showed that all the radioactivity migrated as a single peak which co-electrophoresed with marker globin.

SDS-Polyacrylamide Gel Analysis of the *in vitro* synthesized product

50µl samples of the translational incubation mixtures (after RNase treatment) was added to 100 µl of 12 M urea, 0.05 M sodium phosphate, pH 7.0, 1.5% SDS, 1.5% b-meracapto-ethanol, containing 50 µg of chicken myosin as a marker and electrophesed as described by John (1974). The gels were stained in Coomassie Brilliant Blue (John, 1974) and were sectioned into 1 mm slices. Each slice was dissolved by incubation in 0.5 ml 30% H_2O_2 at 50°C for 12 - 16 hr. 10 ml of Aquasol scintillation medium were added and the radioactivity was determined in a Packard liquid scintillation counter.

Enzymatic synthesis of cDNA

The procedure that was used was based on the one described by Verma *et al.* (1972) and Kacian *et al.* (1972). The 200µl standard reaction mixture consisted of the following components: 50 mM Tris-HCl (pH 8.3), 20 mM dithiothreitol, 6 mM $MgCl_2$, 60 mM NaCl, 550 µM each of dGTP, dATP, TTP, 75 µl of ^{3}H-dCTP (21 Ci/µMol), 0.1 µg oligothymidylic acid $(pT)_{10}$, 21 µg of Actinomycin D, 15 µl reverse transcriptase from AMV, 2 - 6 µg of mRNA.

The reaction was begun by the addition of enzyme to the ice-cold reaction mixture and was continued at 37°C for 1 hr. At the beginning and the end of the reaction aliquots were precipitated with TCA and counted for calculation of yield. After incubation the reaction mixture was made 1% in SDS and incubated for 2 min at 37°C. 0.3 ml of 0.5 N

NaOH were added and the tube was placed for 5 min in a boiling water bath for hydrolysis of the template mRNA. The solution was chilled on ice and the alkali was neutralized by the addition of 0.3 ml of 1 N NaH_2PO_4. 1/10 volume of 10 x Column Buffer was added and the mixture was passed through a column of Sephadex G-60 and Chelex X-100 (1.5 x 2.7 cm). The excluded peak of radioactivity eluted with Column Buffer was pooled, made 0.2 M Sodium acetate pH 5 and ethanol precipitated after addition of carrier t-RNA from HeLa cells. The precipitated cDNA was collected by centrifugation, dissolved in Column Buffer and kept at $-20^{\circ}C$.

Centrifugation of cDNA on alkaline sucrose gradient

To estimate the size of the cDNA an aliquot of it was made 0.2 M NaOH - 0.5% SLS, layered on a 16 ml 5 - 20% linear sucrose gradient in the same buffer and centrifuged for 41 hours at 24,000 rpm in a Beckman L-5 SW 27 rotor at $24^{\circ}C$. Unlabelled sonicated mouse DNA of known size was run on the same gradient. Gradients were fractionated, read for optical density at 260 nm in a Unicam spectophotometer and assayed for radioactivity in Aquasol, after fractions had been neutralized with HCl.

^{3}H-DNA preparation from fibroblast cultures

Chick fibroblast cultures (see Page 33) were labelled with 10 µCi/ml of ^{3}H-Thymidine (20 ci/mMol) for 46 hrs. DNA was prepared by Marmur's (1961) procedure,with the inclusion of repeated phenol-chloroform, RNase and Pronase treatments.

Cells were scraped into Dulbecco A, centrifuged at 500 g for 5

min and the pellets were resuspended in cold saline-EDTA buffer (0.1 M EDTA - 0.15 M saline chloride). SDS and pronase were added to final concentrations of 1% and 400 µg/ml respectively, and the mixture was incubated at 37°C for 2 hr. The cell lysate was made 1 M in sodium perchlorate, 1/10 volume of saturated Tris was added and the mixture was extracted for 30 min at 20°C with an equal volume of phenol : chloroform (1 : 1). After centrifugation at 10.000 xg for 10 min, 2 volumes of absolute ethanol were added on the top aqueous phase and the crude DNA was spooled, washed through an alcohol series, air dried and dissolved in 0.1 x SSC. Solid NaCl was added to a concentration of 0.1M and the DNA preparation was subjected to RNase treatment (50 µg/ml) at 37°C for 2 hrs. Protease was added to a final concentration of 400 µg/ml and digestion was carried out at 37°C for 3 hrs. The DNA was extracted with phenol : chloroform (1 : 1) as above and 1/10 volume of acetate EDTA (3 M Na Acetate, 0.001 M EDTA pH 7.0) and 0.7 volumes of isopropanol were added to the aqueous phase. The DNA was spooled out of solution, dehydrated, air dried and dissolved in 0.1 x SSC.

The specific activity of the extracted DNA was estimated to be 1.2×10^5 cpm/µg.

DNA Renaturation

Chicken DNA and ^{3}H-DNA from chick fibroblasts, used in hybridization, were sheared in a French Press in 0.1 x SSC (Rosen et al., 1973). The solution was then made 0.15 M in Na^+, the DNA fragments were precipitated with ethanol and passed through a column of sephadex SP-50 and Chelex 100 equilibrated with column buffer (Bishop et al., 1974b).

The fragments obtained in this way had an approximate length (single stranded) of 450 - 550 nucleotides.

The sheared DNA mixture in 0.012 M PB (Bishop et al., 1974b) was heated for 5 min in a boiling water bath, in order to achieve complete denaturation. A control sample was withdrawn and diluted at least 20-fold in ice-cold 0.06 M PEB. The reaction mixture was then transferred to a 70°C water bath and after 30 sec (to allow the temperature to equilibrate) the salt concentration was adjusted to 0.24 M PB. (Liquid paraffin was layered on the mixture to avoid evaporation). Samples were withdrawn at different times and diluted in ice-cold 0.06 M PEB. The extent of renaturation was measured by resistance to the enzyme S_1 nuclease, which digests single stranded nucleic acid (Sutton, 1971).

Isolation of non-repetitive DNA sequences

Non-repetitive DNA was purified by annealing sheared ^{3}H-labelled DNA from chick fibroblasts to a Cot of 200 (Rosen et al., 1973). Hybridization was stopped by placing the tube in ice. The hybridization mixture was made 0.12 M PB and was loaded on a 0.3 ml HAP column equilibrated in 0.12 M PB at 66°C. Single stranded DNA was eluted with six 0.6 ml washes of 0.12 M PB and double stranded DNA was eluted with five 0.6 ml washes of 0.4 M PB. Radioactivity was measured in the fractions by counting 2µl of each in 5 ml Aquasol.

The single stranded DNA was extensively dialyzed against Column Buffer / and subsequently precipitated with ethanol. Finally the DNA was resuspended in a small volume of Column Buffer / and used for hybridization with total chicken DNA.

cDNA-DNA Hybridization.

cDNA prepared using MHC mRNA was annealed to sheared chicken DNA in DNA excess. The reaction mixture contained 20 mg/ml of DNA and trace amounts of ^{3}H-labelled cDNA. The sample was heated in a boiling water bath for 5 min to denature the DNA. The subsequent procedure was as described for DNA renaturation (Bishop et al., 1974b).

cDNA-RNA Hybridization

cDNA was annealed to an excess of RNA according to the procedure of Bishop et al.,(1974b).

The reaction mixture in 0.012 M PB was heated for 3 min in a boiling water bath and a control sample was taken and diluted at least 20-fold in ice-cold stop mixture (0.06 M PEB, 120 µg/ml of rat denatured sheared DNA). The reaction was then transferred to a 70°C bath and after 30 sec the salt concentration was adjusted to 0.24 M PEB. Aliquots were withdrawn at different times and diluted in ice-cold stop mixture. The extent of cDNA annealing was measured by resistance to the enzyme S_1 nuclease.

Rot/Cot represents the product of initial RNA/DNA concentration and time of the reaction. They are expressed as the product of moles nucleotides per liter and the annealing time in seconds. For calculations of Rot/Cot values nucleotides were assumed to have an average molecular weight of 320.

For estimating the theoretically expected Rot ½ of an mRNA, the equation:

$$\frac{\text{Rot } ½ \text{ mRNA}}{\text{Complexity mRNA}} = \frac{\text{Rot } ½ \text{ RNA standard}}{\text{Complexity RNA standard}}$$

was used (Shapiro and Schimke, 1975).

Assay of hybrid with S_1 nuclease.

Hybridization samples were rapidly frozen and stored at -20°C. All the samples for a given experiment were treated at the same time with S_1 nuclease (Bishop et al., 1974b). 1/5 volume of KB3 was added to the samples and each one was divided into two equal aliquots,one of which was treated with 100 units of S_1 nuclease. A final volume of 0.45 ml contained 0.048 M PEB, 0.03 M Na Acetate pH 4.5, 6×10^{-4} M $ZnSO_4$, 0.02 M NaCl and 0.052 N Acetic acid. After incubation at 45°C for 40 min, the samples were placed in ice and TCA precipitated. The hybrids were collected on Millipore filters,which were dried and counted with 10 ml of Toluene-PPO-POPOP scintillant.

The ratio of the nuclease-treated sample to the untreated one was expressed as the percentage of the cDNA or ^{3}H-DNA hybridized in each sample. These values, after subtracting the S_1 resistant background levels, determined immediately after the denaturation step, were plotted as a function of Rot/Cot.

Cell Cultures

Myogenic cultures were prepared according to the technique described by Könisberg (1963) and Coleman and Coleman (1968), with some modifications routinely used in this laboratory.

12-day old chick embryo leg muscles were dissected free of skin and bone, minced briefly and incubated in trypsin (0.25% solution in Dulbecco A) for 1 hr at 20°C. The incubation mixture was centrifuged for 5 min at 500 g and the pelleted cells were resuspended in complete medium with repeated pipetting. A "pre-plating" step for diminishing the fibroblast contamination of the myogenic culture was carried out

as described by O'Neil and Stockdale (1972). The cells were counted in hemocytometer, diluted to the final nominal concentration for plating and plated on 9 cm Sterilin Plastic Petri dishes which have been treated with gelatin. The gelatin solution (0.1% in dist. H_2O) was autocleaved and 10 ml were used for each dish. Dishes stood for at least 2 hr at $4^{\circ}C$, excess gelatin was taken off and the dishes were washed with Dulbecco A. Cells were usually plated at a concentration of 0.8 - 1 x 10^6 cells/dish in MEM supplemented with sodium bicarbonate and antibiotics (Penicillin and Streptomycin), containing horse serum and embryo extract in the ratio 88 : 10 : 2. (MEM is a Hank's Base solution supplemented with non-essential amino acids and glutamine). Cultures were incubated at $37^{\circ}C$, in 5% CO_2. The medium was routinely changed at day 2 and 4.

For the *in situ* hybridization experiments cells were grown on slides which have been acid-washed and gelatinized.

Fibroblast cultures were prepared by several passages from overgrown chick myotube cultures.

For myosin heavy chain, DNA, RNA and protein estimation, cells from one Petri dish were collected in a small volume of ice-cold Dulbecco A (by scraping with a rubber policeman), centrifuged for 5 min at 500 g, resuspended in fresh Dulbecco A and pelleted for 10 min at 10.000 g. The cell pellets were kept at $-20^{\circ}C$ until analysed. RNA was estimated using Schmidt's and Thannhauser's procedure (1945), DNA was measured using Burton's modification (1956) of the Dische diphenylamine reaction (1955) and proteins by the method of Lowry *et al.* (1951).

Myosin heavy chain estimation in tissue cultures

The cell pellets were homogenized in 1 ml 8 M urea, 1.0% SDS, 1% b-mercaptoethanol, 0.01 M Sodium phosphate pH 7.0, using a Dounce homogenizer. After incubation at 37°C for 5 min the homogenate was drawn vigorously through a 21 gauge needle 5 times and then centrifuged for 10 min at 10.000 g at 20°C (Patrinou, John and Jones, in preparation). Aliquots (50 µl) of the supernatant were electrophoresed as described previously (John, 1974). Gels, stained in Coomassie Blue, were scanned on a Joyce-loebl densitometer using red filter 204. The areas under the myosin heavy chain peaks were cut out and weighed as described by Potter (1974).

RNA extraction from cultures

After removing the medium, the Petri dishes were rinsed twice with ice-cold M buffer and cells were scraped from the surface of the dish with a rubber policeman, in a small volume of M buffer. The cell suspension was concentrated by centrifugation at 500 xg for 5 min and the cell pellets were resuspended in M buffer containing 0.5% Triton X-100 (Morse et al., 1971) and 50 µg/ml Dextran sulphate. Cells were lyzed by gentle homogenisation in a loose fitting Dounce homogenizer. All the above steps were carried out on ice. An equal volume of Parish-Kirby solution and two volumes of phenol solution were added to the lysate and total RNA was extracted as described for the cytoplasmic RNA preparation from chick leg muscles (Kirby, 1965).

In situ hybridization

The procedure used for the in situ hybridization was based on the one described by Harrisson et al. (1973). Cells grown on slides were fixed in ethanol : acetic acid (3 : 1) for 30 min at $0^{\circ}C$ and were kept at $-20^{\circ}C$ until further use. Denaturation was carried out in 0.2 N HCl for 20 min at $20^{\circ}C$ and was followed by dehydration though 50%, 70%,90% and 100% ethanol. cDNA was dissolved in 2 x SSC (18.000 cpm/µl, 1.5 x 10^{7} cpm/µg) and 3µl of cDNA was applied to each slide, covered with an acid washed coverslip and sealed with dilute Cow Gum solution in petroleum ether. Incubation was at $68^{\circ}C$ for 10 hours. Slides were uncovered, washed in 3 changes of 2 x SSC at $20^{\circ}C$, incubated in 2 x SSC at $55^{\circ}C$ for 2 hrs to remove unhybridized cDNA and dehydrated through ethanol solutions. For autoradiography gel emulsion diluted 1 : 1 with distilled H_2O was used. Exposure was for 5 - 8 weeks. Slides were developed in Kodak D19B developer for 4 min, fixed for 10 min and stained in 5% solution of Giemsa R66 in Buffer pH 6.8 for 2 hr, according to the procedure described by Guinness (1973).

RESULTS

Chick embryonic MHC mRNA and MHC genes

(a) Isolation of MHC Synthesizing Polysomes

The starting material for the preparation of MHC mRNA was the large polysome class that had previously been shown to synthesize myosin heavy chain (Heywood et al., 1967; Heywood and Rich, 1968; Sarkar and Cooke, 1970). The profile of polysomes prepared from 14 day old embryonic chick leg muscles (Fig. 3) showed a small peak at the bottom of the gradient, representing the large myosin heavy chain synthesizing polysomes.

The chick leg muscle polysomes, isolated in this way, were tested for their protein synthetic activity in a cell-free system (Heywood et al., 1967), defined as the homologous system, in which some factors (S-150 enzymes) were isolated from embryonic chick leg muscles. When the translational products of total polysomes (Fractions 1 - 30, Fig. 3) were analyzed, their electrophoretic pattern (Fig. 4) indicated the synthesis of polypeptide chains in the M.W. range of 16,000 - 200,000. Two of the radioactive polypeptides co-migrated with marker myosin heavy chain and actin. When the polysomes in the rapidly sedimenting peak (Fractions 1 - 6, Fig. 3) were used in the homologous cell free system, several radioactive polypeptides were synthesized, which migrated a short distance into the gel and one of which had the same mobility as myosin heavy chain marker (Fig. 5). A similar observation has been reported by Heywood and Rich (1968), but in this report the radioactivity migrating faster than myosin heavy chain was attributed to nascent polypeptide chains present on

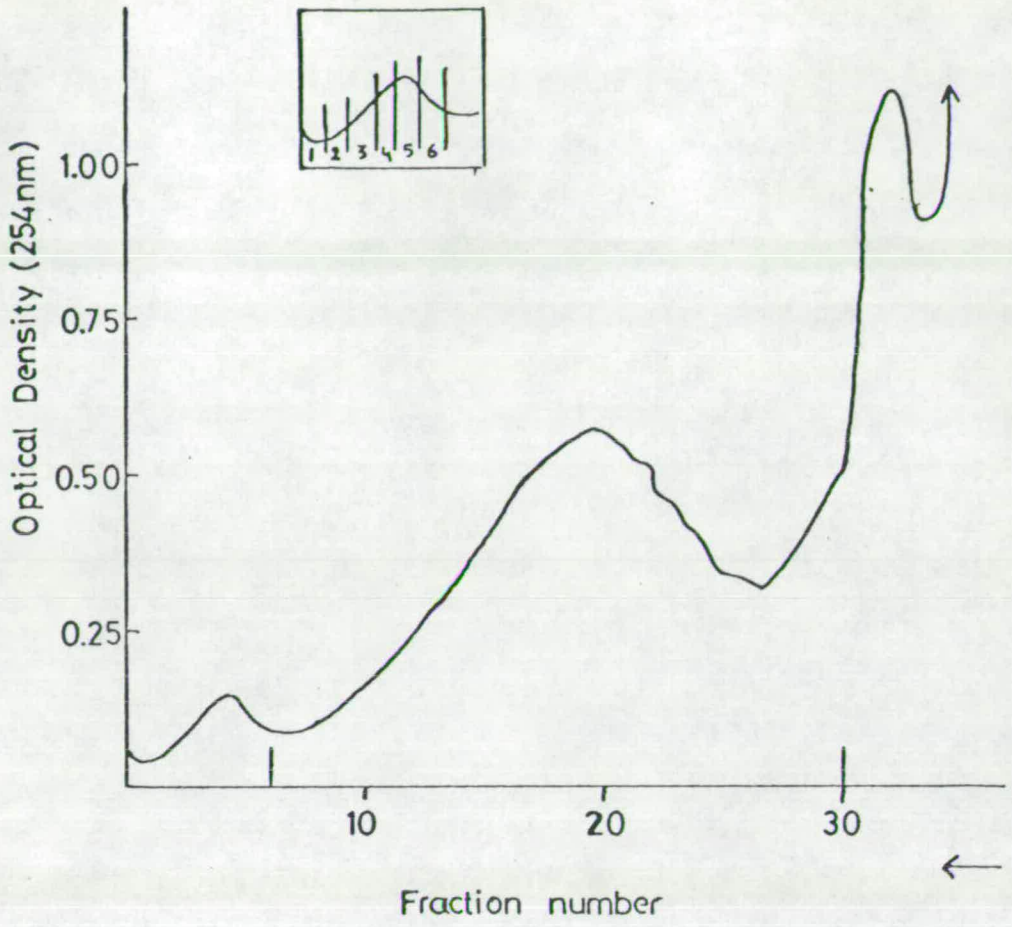

Figure 3.

Sucrose gradient profile of chick embryonic skeletal muscle polysomes. Sedimentation was from right to left in a 15-40% gradient. Centrifugation was for 2 hrs at 25,000 rpm at 4°C in a Beckman L-5 SW 27 rotor.

INSET: Fractionation of the large polysome peak.

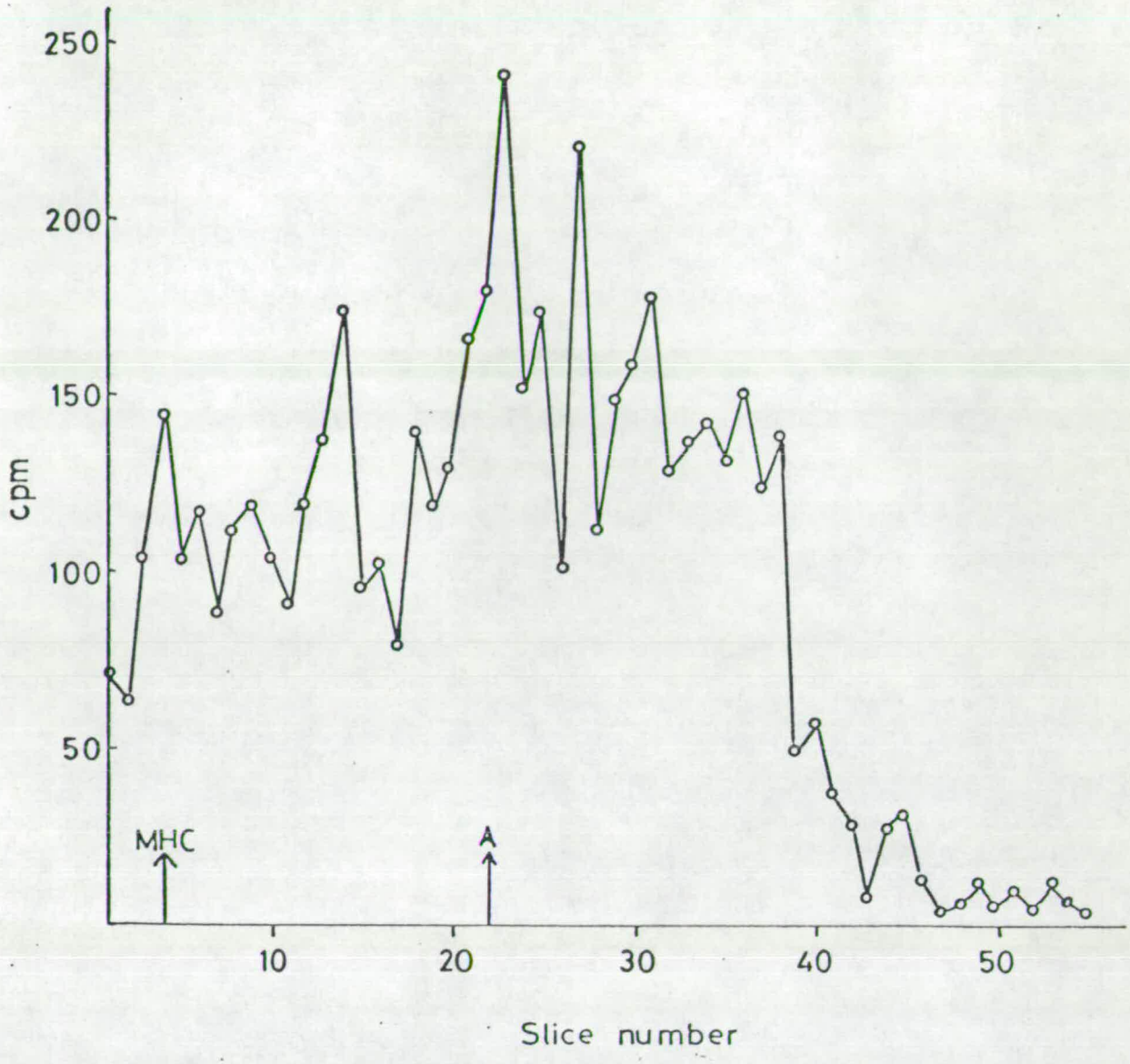

Figure 4

Direct analysis on SDS-polyacrylamide gel of the translation products of total polysomes (fractions 1-30, Fig. 3) in vitro. Gel contained 10% acrylamide and 0.402% bisacrylamide. Migration is to the right. The arrows indicate the position of myosin heavy chain (MHC) and actin (A) markers run on the same gel.

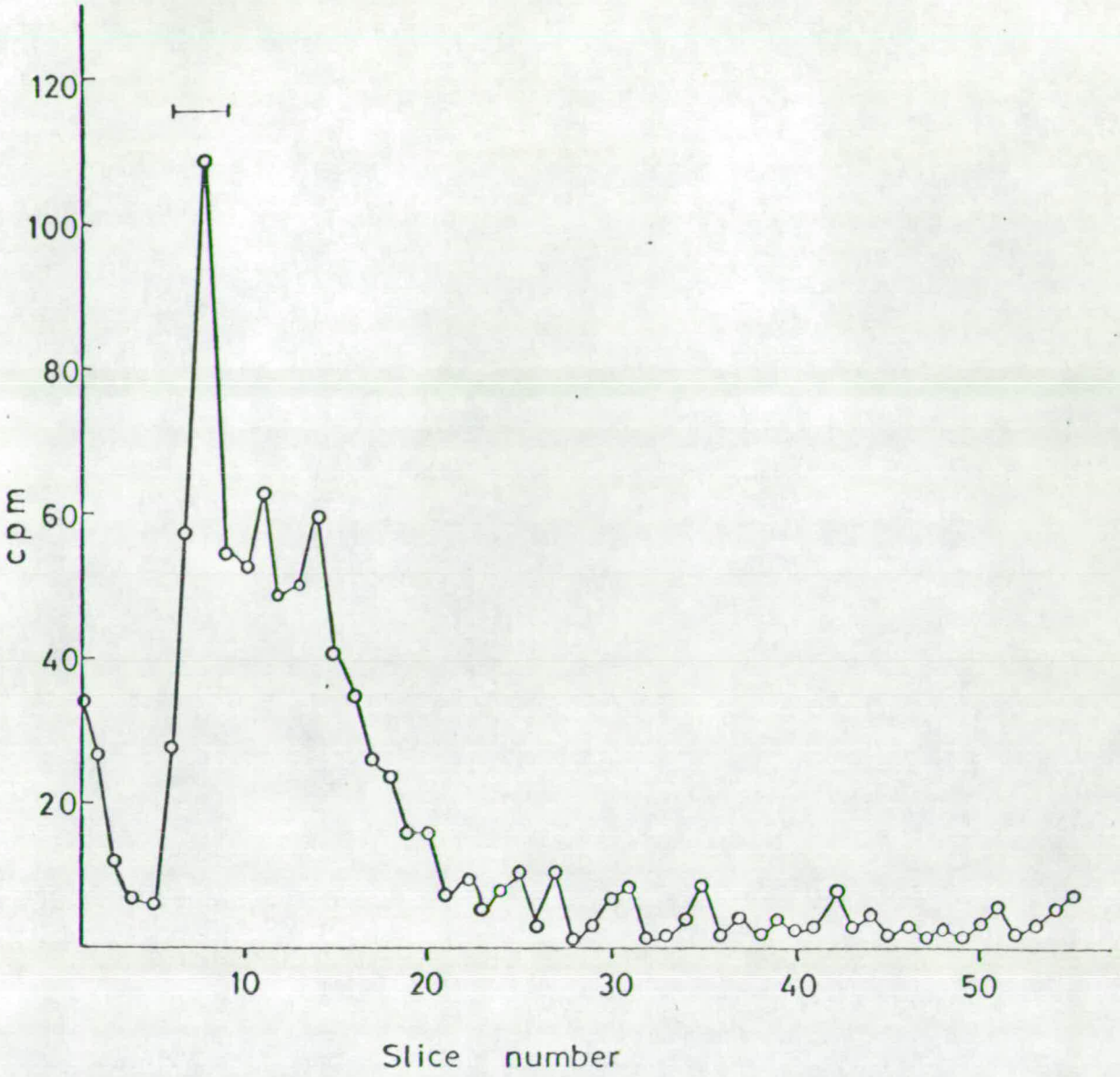

Figure 5.

Direct analysis on SDS-polyacrylamide gel of the translation products of large polysomes (fractions 1-6, Fig. 3) *in vitro*. Gel contained 10% acrylamide and 0.134% bisacrylamide. Migration is to the right. The position and width of myosin heavy chain marker band, run on the same gel, is indicated by horizontal bar.

the polysomes. It is believed that this is not the case since even after centrifugation of the translational mixture at 150,000 g for 2 hrs, in order to pellet the polysomes and the attached radioactive polypeptide chains, radioactive proteins smaller than myosin heavy chain could still be detected in the supernatant.

In an attempt to obtain a polysomal fraction which would direct the synthesis of solely the heavy chain, individual fractions comprising the peak (1-6) were analyzed separately. Fractions from 6 identical gradients were pooled prior to pelleting, the polysomes were pelleted and translated in the homologous cell-free systems. The electrophoretic analysis of the synthesized products is shown in Fig. 6. Lower concentration of bis-acrylamide were used in the gels (0.134%) to allow better resolution of high M.W. proteins. Fraction 1,which contained the 60% sucrose cushion, only slightly stimulated incorporation of radioactivity into TCA-precipitable material and virtually no radioactivity was found on the gel. Fraction 2 showed only one major peak with the same mobility as heavy chain marker and which was identified as synthesized myosin heavy chain (see Appendix). The synthesis of heavy chain could be detected throughout the translational products of Fractions 3 - 6. However, other radioactive polypeptides, in addition to heavy chain, were synthesized under the direction of polysomes from Fractions 3 - 6, which may be attributed to large myofibrillar proteins that have been described previously (Starr and Offer, 1971, B and C proteins, or Etlinger et al., 1975, M-line protein, Z, or a-actinin). Furthermore, several large proteins existed in 14 day old chick embryo leg muscle,as revealed by electrophoretic analysis of total protein (Appendix).

Legend to Figure 6.

Direct analysis on SDS-polyacrylamide gel of the translation products of large polysome fractions (Insert, Fig. 3) *in vitro*. Gels contained 10% acrylamide and 0.134% bisacrylamide. Migration is to the right. The position and width of the myosin heavy chain marker band, run on the same gel, is indicated by horizontal bar. Only the top halves of the gels are shown.

(a) Polysomes from fraction 1

(b) Polysomes from fraction 2

(c) Polysomes from fraction 3

(d) Polysomes from fraction 4

(e) Polysomes from fraction 5

(f) Polysomes from fraction 6

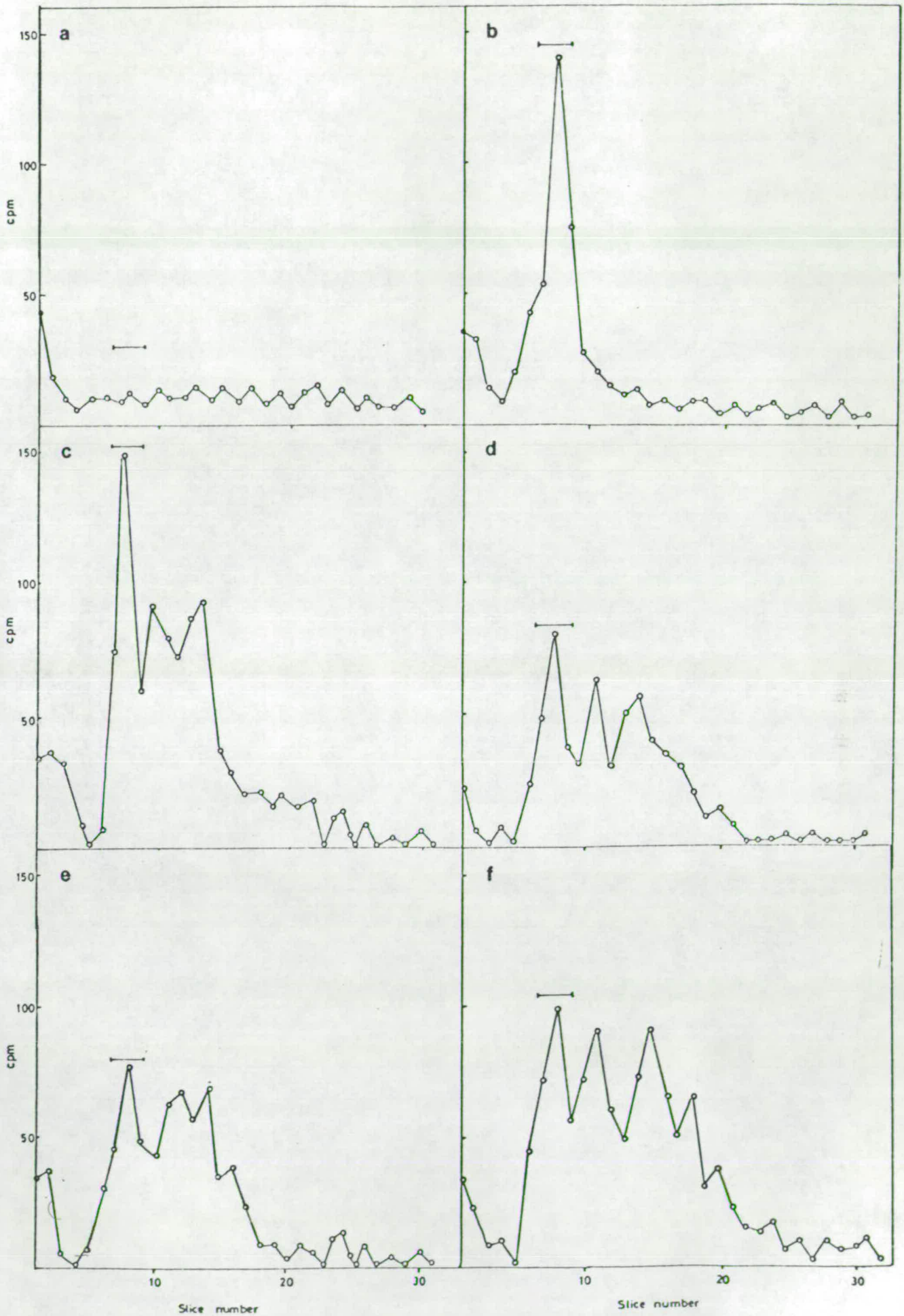

Figure 6.

The synthesis of proteins with different M.W. on the same polysomal fraction has been reported before and it has been explained on the grounds of a broad sedimentation of the large polysomes (Heywood and Rich, 1968; Heywood and Nwagwy, 1968).

The results of the translation of the different polysomal fractions suggested that the large polysomes, forming a peak at the bottom of a sucrose gradient, contained the polysomes which synthesize myosin heavy chain and other large polypeptides. Only the fraction on the heavy side of the peak (Fraction 2, Fig. 3) synthesized the heavy chain exclusively. In all the remaining fractions heavy chain synthesis occured along with the synthesis of other large polypeptides. Almost all the radioactive polypeptide synthesized by fraction 2 polysomes co-electrophoresed with MHC, suggesting thus that this was an almost pure fraction of MHC synthesizing polysomes. The heavy chain synthesized in Fractions 3 - 6 had the same M.W. as the one synthesized in Fraction 2.

(b) Extraction and purification of MHC mRNA

The fraction of large polysomes from the bottom of the gradient (Fraction 2, Fig. 3), which synthesized only the myosin heavy chain, was used as a source for MHC mRNA. The same fraction from 30 polysomal gradients was pooled, the polysomes were pelleted by an overnight centrifugation at 100,000 g, at 2°C and the polysomal pellets were used for RNA extraction.

Mondal et al. (1974) have shown that MHC mRNA contained a polyadenylic acid segment of about 170 nucleotides, thus suggesting the possibility of purifying the message by hybridization to oligo(dt),

immobilized on cellulose. When RNA extracted from the subfraction of the large polysomes was separated from rRNA by two cycles on oligo(dt) cellulose column, the amount of Poly(A) + RNA that was finally eluted represented 1.2% of the applied polysomal RNA. This poly(A) + RNA fraction presumably represented the MHC mRNA since the starting polysomal material was exclusively active in the synthesis of myosin heavy chain.

In order to check whether all the RNA eluted from the oligo(dt) column at low ionic strength was poly(A) + RNA, a small aliquot of it was hybridized with an excess of ^{3}H-Poly(U). Assuming that the poly(A) segment in the MHC mRNA represented 3% of the message molecule (Mondal et al., 1974) a 15% contamination of the MHC mRNA preparation with Poly(A) minus RNA (presumably rRNA) was estimated. Fifty,14-day old chick embryos gave a yield of 6 µg MHC mRNA as estimated by ^{3}H-Poly(U) hybridization.

(c) In vitro translation of MHC mRNA in the wheat germ cell-free system

The purified mRNA was further characterized by analyzing the in vitro synthesized products,after its translation in a heterologous cell-free system. The use of a heterologous system versus a homologous one eliminated the possibility that the isolated mRNA only stimulated the endogenous mRNA for MHC (Hunt and Wilkinson, 1967). By this second translation it was also shown that the extraction and purification procedure did not result in degradation or inactivation of the mRNA.

Addition of MHC mRNA to the wheat germ cell-free system strongly stimulated ^{3}H-leucine incorporation in TCA-insoluble material. In order to test if the purified mRNA directed the synthesis of myosin heavy chain, the radioactive products were directly analyzed on SDS-Polyacrylamide gel. When translation was carried out at the optimum KCl concentration (96 mM) for incorporation of total acid insoluble counts, a very low radioactive peak was found comigrating with marker heavy chain, while the bulk of the radioactivity migrated quite fast failing to form any definite peaks (Fig. 7a).

Harwood et al. (1975) and Benveniste et al. (1976) have reported a similar result on the translation of collagen mRNA in the wheat germ system and have suggested that concentrations of K^{+} higher than that for optimal protein synthesis was required to support the synthesis of large polypeptides. MHC mRNA was translated in the presence of 160 mM KCl and Fig. 7b shows a direct analysis of the incubation mixture on SDS-Polyacrylamide gel. A major radioactive single peak could be seen which co-migrated with marker myosin heavy chain band. The presence of low M.W. labelled components on the gel could be attributed to premature terminated polypeptides or even incomplete polypeptide chains still attached on the polysomes since, the total incubation mixture without prior removal of polysomes was applied on the gel. A similar observation has also been reported by other investigators (Prives et al., 1974; Danbrough and Ford, 1976). Only the radioactive protein, comigrating with myosin heavy chain marker, remained after one precipitation at low ionic strength (Fig. 7c).

However the results clearly indicated that the purified mRNA

Legend to Figure 7.

Analysis on SDS-polyacrylamide gels of the translational products of 0.9 µg MHC mRNA in the wheat germ cell-free system. Gels contained 10% acrylamide and 0.134% bisacrylamide. Migration is to the right. The position and width of the myosin heavy chain marker band, run on the same gel, is indicated by horizontal bar.

a - b : Direct analysis of the translation products after incubation in a KCl concentration of 96 mM and 160 mM respectively.

c : Analysis of the translation products after incubation in 160 mM KCl followed by one precipitation with carrier myosin at low ionic strength.

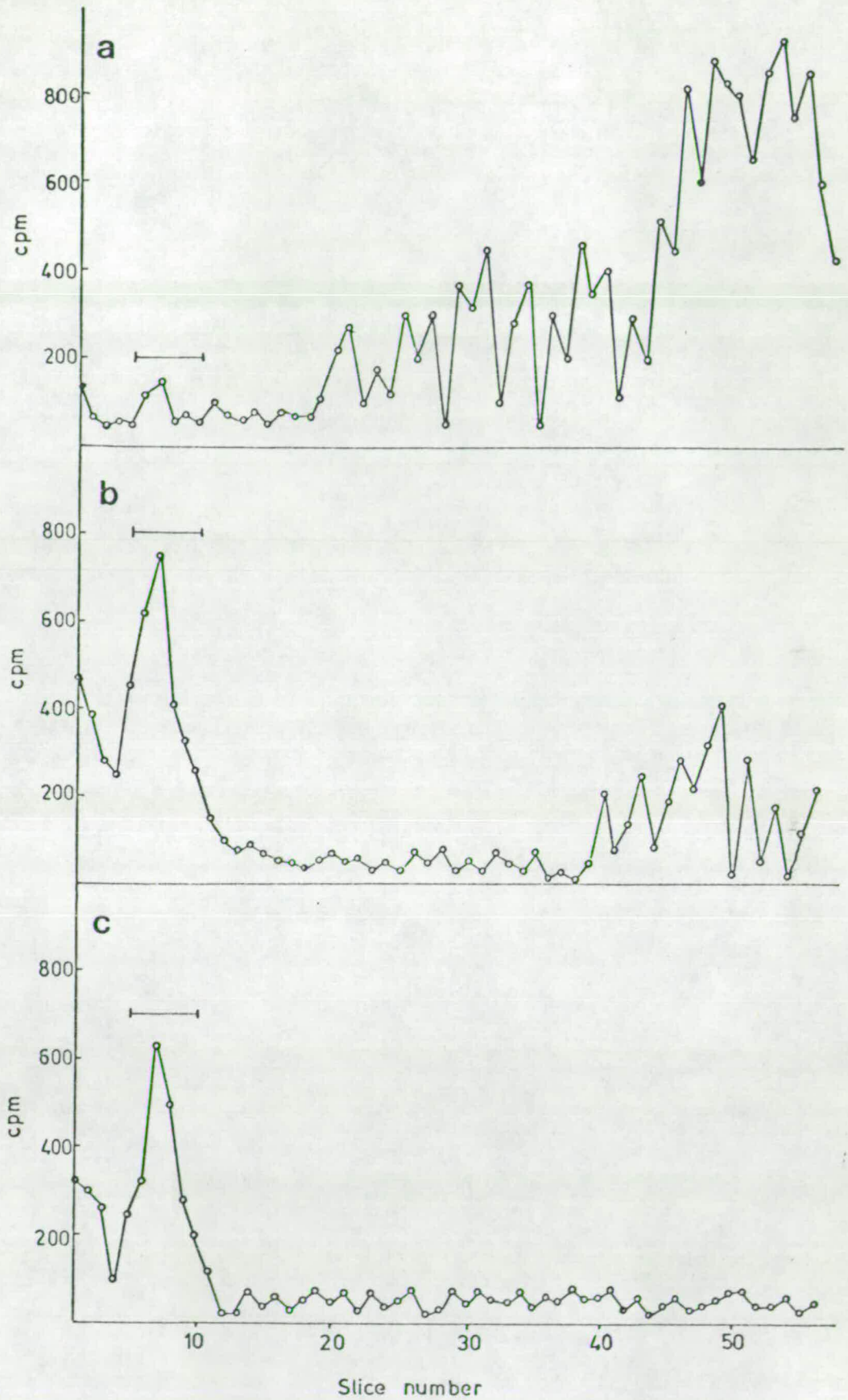

Figure 7.

could direct/ in a heterologous cell-free system, the synthesis of a protein which co-migrated with myosin heavy chain. Separation on the presence of SDS is a crude one based on the molecular size and since there might exist another protein that can co-migrate with MHC, due to identical M.W., a number of other criteria were used for identifying the synthesized protein (for a detailed account see Appendix). In summary, evidence for the synthesis of myosin heavy chain originated from (a) co-purification experiments with carrier myosin, (b) tryptic digest analysis and (c) iso-electric focussing which showed that the radioactivity formed two distinct but closely spaced peaks, which were in excellent correspondence with two visible bands of chick embryonic myosin heavy chain.

From the above mentioned results it was reasonable to conclude that the mRNA isolated from the polysomal subfraction of the large polysomes was MHC mRNA, since it could direct in a heterologous cell-free system the synthesis of a polypeptide, characterized by a number of criteria as myosin heavy chain. In addition, no contamination of the MHC mRNA with other active mRNAs could be detected.

(d) In vitro synthesis of MHC cDNA

The isolated MHC mRNA was used as an efficient template for the production of ^{3}H-cDNA molecule, under the conditions described in the Materials and Methods Section. As has been reported before (Verma et al, 1972; Kacian et al., 1972), the incorporation of 5'-deoxyribonucleotides into the DNA product was completely dependent upon the inclusion of template mRNA and oligo(dt) primer in the reaction mixture. Actinomycin D was added in order to prevent the

synthesis of a double-stranded DNA product. 18S or 28S rRNA were not effective templates. The synthesized cDNA was substantially free of base-paired regions since it showed 93% sensitivity to the single-strand specific nuclease S_1. Since the amount of cDNA synthesized *in vitro* was never sufficient for optical measurements of the mass of DNA present, only the theoretical specific activity was estimated which was found 1.5×10^7 cpm/µg (assuming that all deoxyribonucleotides were equally represented in the DNA product).

An estimate of the size of the cDNA transcript was obtained on alkaline sucrose gradient. Unlabelled sonicated mouse DNA of known size (6.5S) was used as an internal marker. As can be seen in Fig. 8, the cDNA had an average size similar to the one of the marker, that is about 530 nucleotides. The size distribution appeared not very broad, but indicated the presence of some transcripts shorter and longer than average. On the total, it appeared that the cDNA molecules were much shorter than the template mRNA, for which a size of 5.830 nucleotides has been estimated (Sarkar *et al.*, 1973; Mondal *et al.*, 1974). Under the same conditions cDNA synthesized on a rabbit globin mRNA template was also found to have a size of 530 nucleotides.

The synthesis of cDNA products with an average size smaller than that of the template has been a common observation, for example with ovalbumin mRNA or immunoglobin mRNA (Sullivan *et al.*, 1973; Schechter, 1975). It has been reported that the average size of the cDNA product can be increased by the presence of relatively high concentrations of dNTP's in the reaction mixture (Imaizumi *et al.*, 1973).

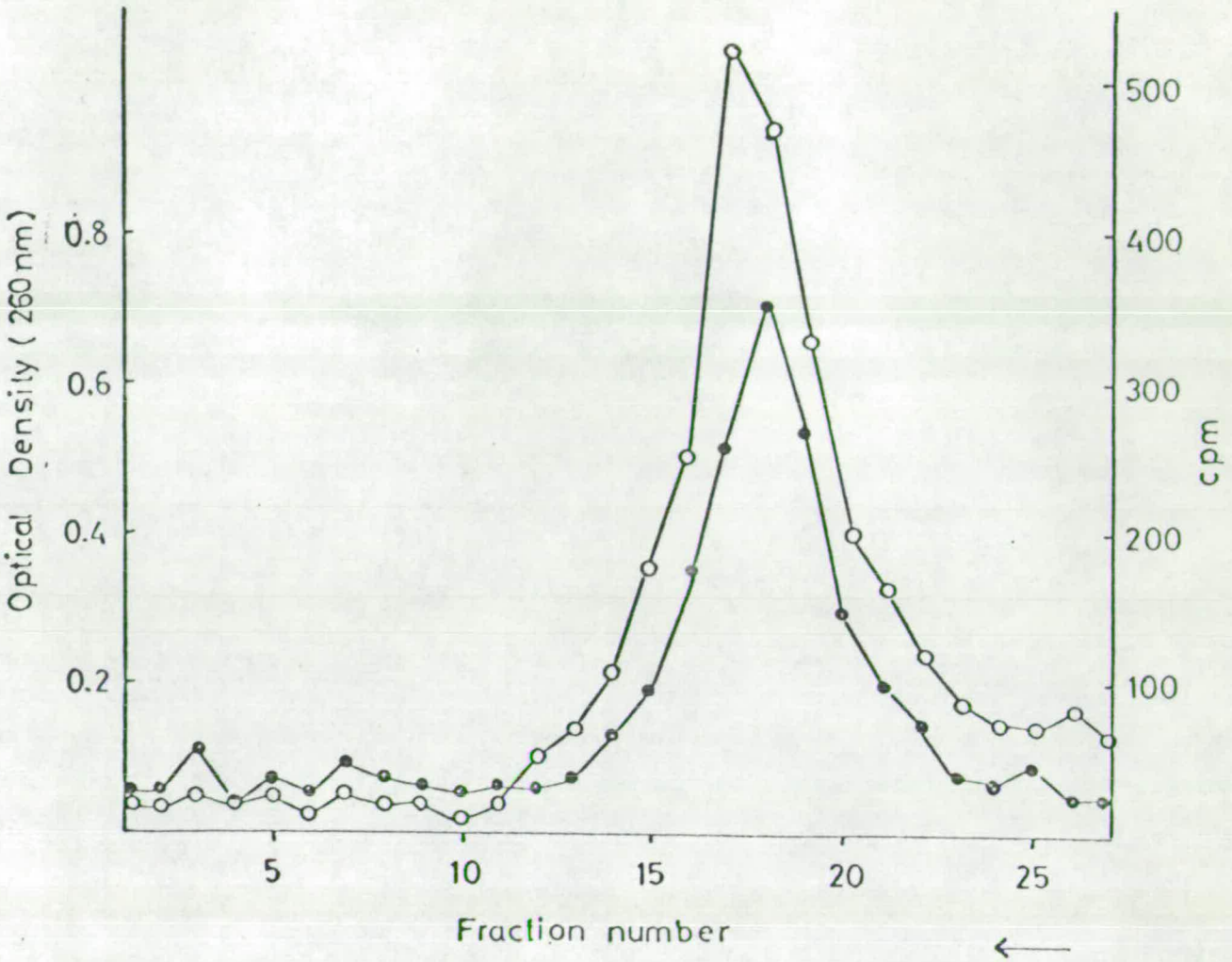

Figure 8.

Alkaline sucrose gradient centrifugation of MHC cDNA. The cDNA was centrifuged in a 5-20% sucrose gradient for 41 hrs at 24,000 rpm at 24°C in a Beckman L-5 SW 27 rotor. Unlabelled sonicated mouse DNA was run on the same gradient as size marker. Sedimentation was from right to left.

o—o—o cpm of MHC cDNA

•—•—• optical density of marker DNA.

Efstratiadis et al. (1975) have reported the production of full copies of different mRNA templates by using up to 600 μM dNTPs in the reaction. Although in the present work a fairly high concentration of dNTPs (550 μM) was used, the transcript was far from complete. Moreover, increase in the dNTP concentration did not increase the size of MHC cDNA. If secondary structure of the RNA plays an important role in the mode of action of the enzyme (Monahan et al., 1976), then incubation conditions for complete cDNA synthesis may differ for different mRNAs depending upon the particular mRNA's template secondary structure.

It is well established that synthesis of cDNA starts at the 3' end of the mRNA (Ross et al., 1972). Thus the cDNA that was prepared should represent a fraction of the 3' end of the MHC mRNA. Non-translated region present at each side of the coding region have been reported for many eucaryotic mRNAs (Dinna et al., 1973; Firtel and Lodish, 1973). There had been no evidence for the presence of non-translated segment at the 3' end of MHC mRNA. But even if the synthesized cDNA contained transcripts of this region, the results that followed showed that the cDNA included at least part of the mRNA coding sequences, enough to provide it with specificity.

(e) Estimation of the complexity of embryonic MHC mRNA

The kinetic standard which was used in the hybridization studies was the hybridization of rabbit globin mRNA to its cDNA, in large RNA excess. The Rot curve for this reaction (Fig. 9) was a close approximation to a homogeneous reaction of this type. The globin cDNA and

globin mRNA hybridized in a sharp transition with a mid point (Rot ½) of 5.3×10^{-4} (log Rot = -3.27). This Rot ½ value corresponded to an mRNA molecule that had a total base sequence complexity of approximately 4×10^{5}, since rabbit globin mRNA is composed of 1a and 1b globin mRNAs (Ross et al., 1972).

MHC mRNA hybridized to its cDNA with a similar sharp transition, but with a Rot ½ value of about 6.309×10^{-3} (log Rot ½ = -2.2) (Fig. 9). The nearly complete annealing indicated that the cDNA product was a faithful copy of the MHC mRNA. The low Rot ½ value and the sharpness of the transition indicated that the MHC mRNA was not contaminated to any significant extent with other mRNAs.

The theoretical Rot ½ of an RNA with a M.W. of 2.05×10^{6} (which is the M.W. of MHC mRNA according to Sarkar et al., 1973) was calculated from the relation:

$$\frac{\text{Rot ½ MHC mRNA}}{\text{Complexity MHC mRNA}} = \frac{\text{Rot ½ RNA Standard}}{\text{Complexity RNA Standard}}$$

to be 2.7×10^{-3}. By comparison of the experimentally determined Rot ½ of the MHC mRNA with the theoretically expected Rot ½ it was estimated that 2.3 different RNA sequences existed in the MHC mRNA preparation. The sharpness of the Rot curve indicated that both of these mRNA sequences were at nearly identical concentrations.

There is evidence that the kinetics of hybridization between RNA and cDNA are affected by a variation in size of cDNA molecules, in the sense that larger cDNA molecules hybridize faster than small ones. Thus, the short MHC cDNA would give higher Rot ½ values than expected, although it must be pointed out that the cDNA used for the

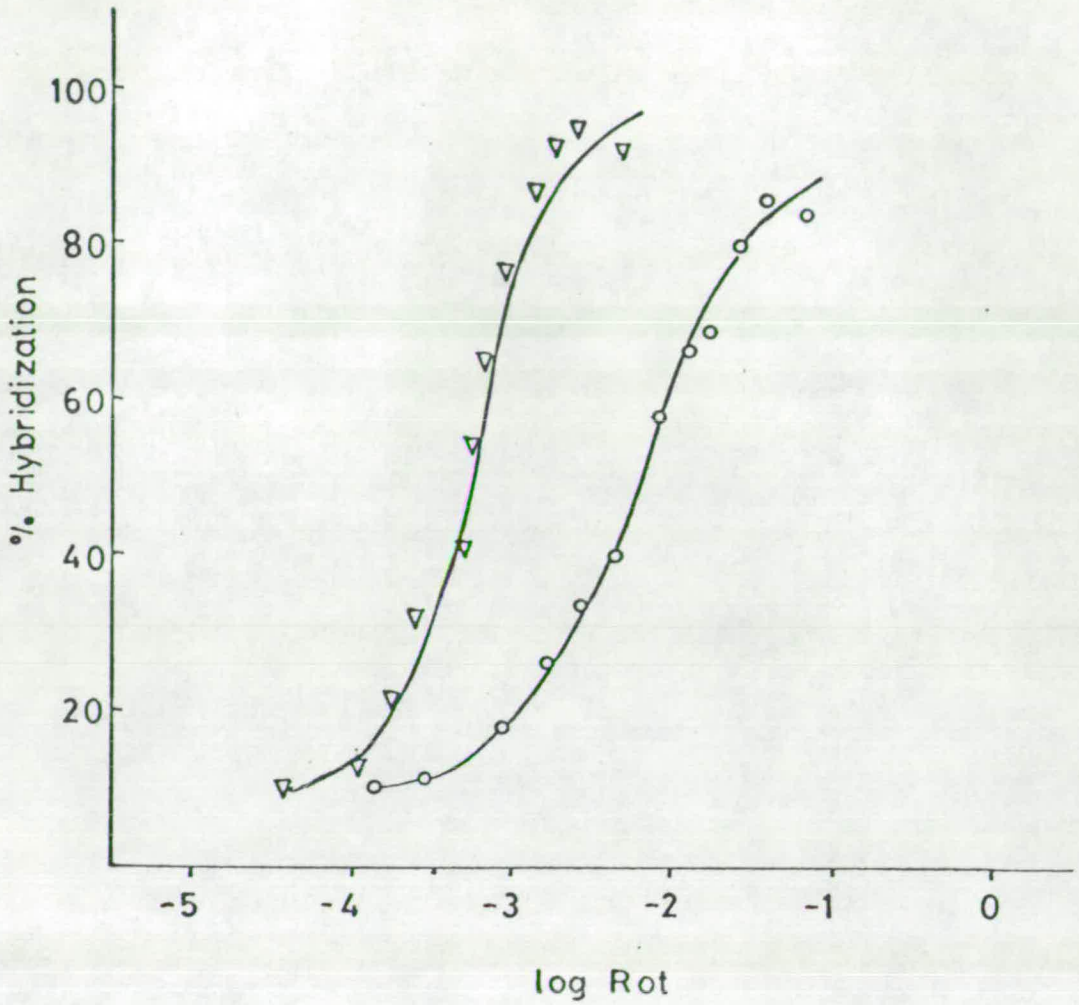

Figure 9.

Hybridization reaction in RNA excess between MHC mRNA-cDNA (o——o——o) and globin mRNA-cDNA (Δ——Δ——Δ). Hybridization was at 70°C in 0.24 M PEB. Percentage of hybrid was estimated by resistance to S_1 nuclease digestion. Rot values for MHC mRNA-cDNA reaction were based on the assumption that Poly(A) forms 3% of MHC mRNA.

kinetic standard had a similar size to MHC cDNA. However it is likely that the high Rot ½ value for the MHC cDNA-mRNA reaction cannot be attributed (at least entirely) to the incomplete MHC cDNA copy, since the iso-electric focussing of the in vitro synthesized protein also indicated the presence of two different mRNA species. There was no indication that one of these mRNA species coded for a protein different than MHC.

In order to check the specificity of the MHC cDNA probe, it was hybridized with 28S rRNA, 18S rRNA and globin mRNA. The hybridizations were carried out in RNA excess and taken to high Rot to ensure completion of the reaction. The results are shown in Table 1. The higher cross-hybridization of the MHC cDNA with 28S rRNA could be explained on the assumption that the 28S rRNA used in the hybridization contained some MHC mRNA sequences. An alternative explanation was that the MHC mRNA was contaminated with 28S rRNA which for some unknown reason was transcribed into cDNA.

(f) Reiteration frequency of embryonic MHC genes in the chicken genome

Highly-labelled DNA was prepared from chick fibroblast cultures labelled with ^{3}H-Thymidine. The ^{3}H-DNA was sonicated to fragments of 490 nucleotides and was allowed to reanneal. The dublex formation was monitored by S_1 digestion. As illustrated in Fig. 10, approximately 28% of the chick DNA renatured prior to a Cot value of 200 and could be considered as repeated sequences. The remaining 72% of the chick DNA was designated as non repetitive or unique sequences. The Cot ½ of total chick DNA had a value of 390 (log Cot ½ = 2.6). These results were in close agreement with previous reports (Rosen et al., 1973).

TABLE 1 Extent of cross hybridization of MHC cDNA

RNA added	Rot (mol s l^{-1})	Hybridization (%)
globin mRNA	3.1×10^{1}	6
18S rRNA	4.8×10^{3}	7
28S rRNA	4.8×10^{3}	15
MHC mRNA	8.5×10^{-2}	85

In order to achieve a separation of repeated and unique sequences, the sonicated ^{3}H-DNA was allowed to reanneal to a Cot value of 200. The reaction mixture was fractionated through a HAP column into double stranded and single stranded DNA. The single stranded DNA represented 73% of the total and was considered as the non repetitive or unique fraction of the ^{3}H-chick DNA. As can be seen from Fig. 11, most of the ^{3}H-DNA that reassociated by a Cot value of 200 was eliminated. To determine the Cot ½ for unique sequences, the isolated non-repetitive ^{3}H-DNA fraction was reannealed to an excess of unlabelled sonicated (550 nucleotides) chicken DNA. The reaction shown in Fig. 11 had a Cot ½ of 8×10^{2} (log Cot = 2.9).

When MHC cDNA was hybridized with an excess of unlabelled sonicated chick DNA a similar curve was obtained, which went over 90% in completion with a mid point of 4.8×10^{2} (log Cot = 2.68) (Fig. 11). This value was quite close to the value estimated for unique chicken DNA indicating that the MHC genes were not reiterated. Since the MHC cDNA and the ^{3}H-unique chick DNA fragments were of similar size, the difference in their rates of hybridization to chicken DNA should reflect the number of the embryonic MHC genes in the chicken genome. By comparing the Cot ½ values for unique DNA and MHC cDNA an approximate value of 2 could be estimated. This value represented the number of each embryonic MHC genes from which the MHC mRNA had been transcribed.

It must be stressed that the cDNA-DNA hybridization technique used results in only an approximate estimation of the number of genes.

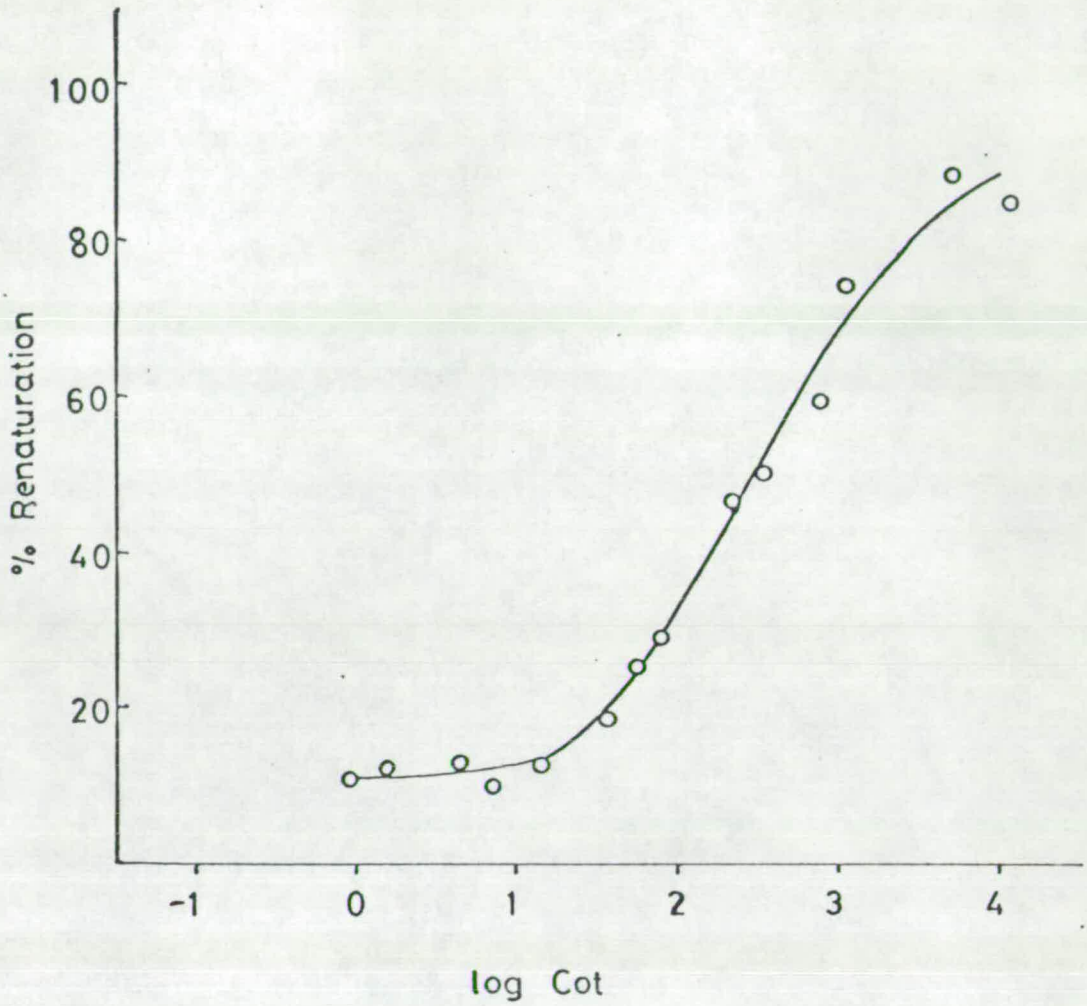

Figure 10.

Renaturation of ^{3}H-labelled chick fibroblast DNA in 0.24M PEB at 70°C. The ^{3}H-DNA was sheared to fragments of 490 nucleotides. Percentage of renaturation was estimated with S_1 nuclease digestion.

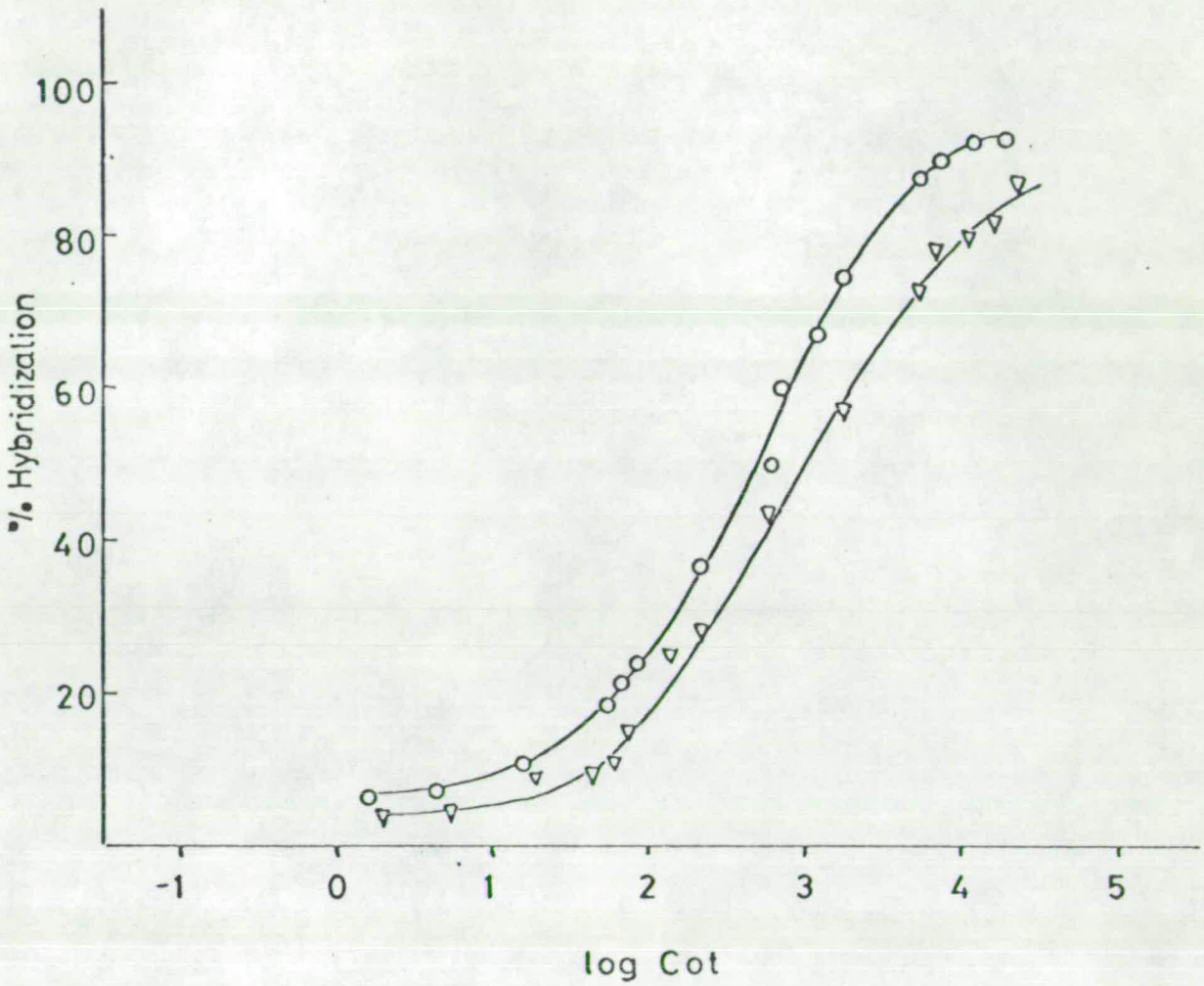

Figure 11.

Hybridization of MHC cDNA (o——o——o) and unique sequence chick ^{3}H-DNA (▽——▽——▽) to a vast excess of unlabelled chicken DNA. The reaction was carried out at 70°C in 0.24 M PEB. Percentage of hybridization was estimated by resistance to S_1 nuclease digestion.

For a more accurate calculation, Bishop et al.(1975) have introduced a further refinement of the same technique, which involves the titration of DNA sequences with a moderate excess of cDNA. However, the present results suggested the presence of limited copies of the embryonic MHC genes in the chicken genome.

MHC mRNA synthesis during myogenesis in vitro

(a) Growth and differentiation of embryonic chick myoblasts in cell culture

In the first 46 hours of primary culture of chick embryonic leg muscle, cells underwent intense replication. From about 46 to 60 hours approximately half the cells fused into multinucleated syncytia, which in the course of the next days enlarged and became cross-striated muscle fibers. After day 7 the cells started detaching from the dish. Cells collected 24 - 30 hours and 96 - 140 hours after plating were considered to represent "replicating myoblasts" and "standard myotubes"* respectively.

As the cells fused to form syncytia, the remaining mononucleate cells continued to divide. Some of these mononucleate cells were myogenic, while others were fibroblasts which had not been removed by the "preplating" step. The mononucleated cells increased in number filling in all the spaces between the myotubes so that eventually a network of cells, several layers thick was formed. Thus, the standard myotube cultures were of a "mixed" nature. In order to obtain almost pure myotubes, cultures were treated with cytosine-arabinoside from day 3 to 5 or 6. Cytosine-arabinoside (Ara-C) selectively kills most

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* as opposed to Ara-C treated myotubes.

of the replicating cells (Fischback, 1972) and there is no indication that it interferes with the normal differentiation of the myotubes (Chi et al., 1975a). Ara-C treated cultures collected on day 5 had a high proportion of myotubes and relatively few mononucleate cells.

It has been shown that EGTA prevents fusion of myogenic cells by lowering the levels of extracellular Ca^{2+} (Paterson and Strohman, 1972). After a series of divisions in the presence of EGTA, mononucleated cells cease dividing and become post-mitotic having many of the characteristics of fully differentiated myogenic cells (Dienstman and Holtzer, 1975). Therefore, in order to obtain a cell population enriched in mononucleated post-mitotic myoblasts, cultures were maintained in EGTA (1.67 mM) from 24 to 72 hr after plating. (Higher EGTA concentrations caused detachment of the cells from the plate). EGTA-treated cultures, collected at 48 - 72 hr after plating, were composed almost entirely of mononucleated cells while control cultures of the same age showed widespread myotube formation.

(b) Myosin heavy chain (MHC) estimation

The amount of MHC present in a single dish at different stages of the myogenic tissue cultures and in the fibroblasts, was estimated. From the results shown in Table 2, it was apparent that MHC was present in all stages of the myogenic cultures as well as in fibroblasts. The percent weight MHC increased from 2.6% of the total protein in dividing myoblasts to 7.5% in Ara-C treated myotubes. As has been reported before (Vertel and Fishman, 1976), the amount of MHC in EGTA treated myoblasts was higher than in replicating myoblasts but lower than in standard myotubes. Standard myotubes contained less MHC

TABLE 2 Myosin HC content of *in vitro* differentiating myogenic cells and fibroblasts

Cell Types	DNA µg/dish	Total Protein µg/dish	Myosin HC µg/dish	Myosin HC/ Total Protein %
Replicating myoblasts	19	420	11	2.62
EGTA-treated myoblasts	46	980	56	5.71
Standard myotubes	119	2840	181	6.37
Ara-C treated myotubes	45	1080	81	7.50
Fibroblasts	49	1100	32	2.90

than Ara-C treated myotubes, presumably due to the presence in the former of mononucleated cells, having low amounts of MHC.

(c) Detection of MHC mRNA by *in situ* hybridization

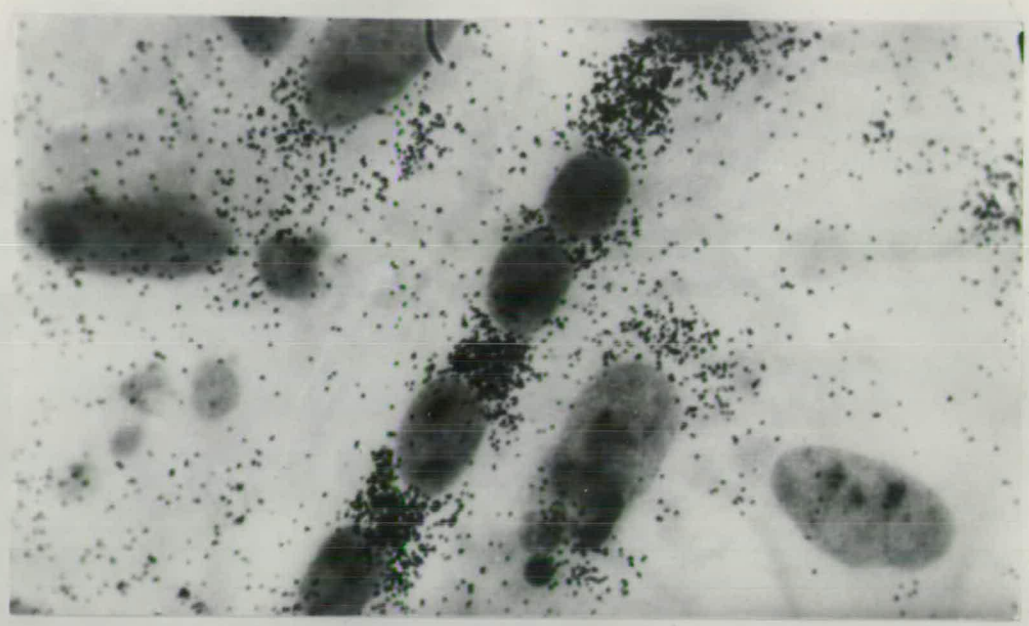
A controversy exists on whether myogenic cell fusion by triggering gene activation is an essential prerequisite for further differentiation (See Introduction, page 6). In the present investigation this issue was approached by studying the appearance of the MHC mRNA and the corresponding protein (MHC) in relation to fusion, during *in vitro* myogenesis. The technique of *in situ* hybridization was used since it would allow detection of MHC mRNA in individual specific cells, at different stages of differentiation.

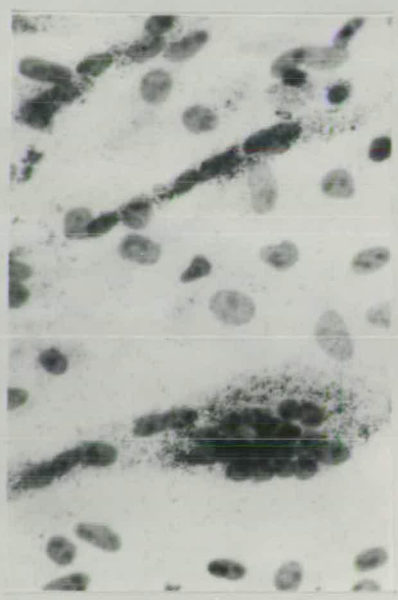
The cDNA transcribed from the MHC mRNA of chick embryonic leg muscle was hybridized to cytological preparations of cells at different stages of differentiation (Plates 1-5). Myotubes from both standard and Ara-C treated cultures were heavily labelled in the cytoplasm with cDNA (Plates 1,3) indicating the presence of large amounts of MHC mRNA. Occasionally grains were clustered in the nuclei, sometimes localized in the nucleolar region (Plate 2). Labelled cDNA was also found in mononucleated cells, particularly in the Ara-C treated cultures. The mononucleated cells of EGTA-treated cultures hybridized with MHC cDNA but to a lesser extent than myotubes (Plate 4). The label was almost uniformly distributed among EGTA-treated cells and it was mainly cytoplasmic. Slides from cultures of replicating myoblasts showed that the majority of these cells did not undergo molecular hybridization with MHC cDNA. However, some patches of small cells, distributed in clusters among the myoblasts, formed extensive hybrids with MHC cDNA (Plate 5). The

label was localized around and inside their nuclei. The identity of these small cells has not been established but they were also heavily labelled in the EGTA-treated cultures after hybridization with MHC cDNA. Such small cells could not be seen in the myotube preparations. Fibroblasts did not show any grains in either the nucleus or the cytoplasm (Plate 6) and such cultures contained no small cells.

To test whether the label represented cDNA-RNA hybrid formation, MHC cDNA was hybridized to slides of standard myotubes which had been previously treated with RNase or DNase. The amount of label was reduced to background in the pre-RNased slides (Plate 7) but there was no reduction in the labelling of the pre-DNased ones (Plate 7), indicating that the cDNA probe was reacting with endogenous RNA. Furthermore, the DNase treatment did not eliminate the *in situ* hybrid of the myotube nuclei.

When MHC cDNA was hybridized *in situ* to slides of mouse LS cells or HeLa cells, no labelling could be detected after 8 weeks exposure (Plate 8). Furthermore no hybridization was observed when globin cDNA was hybridized to standard myotubes (Plate 9). These observations suggested that the conditions of *in situ* hybridization did not allow non specific absorbtion of cDNA to fixed cells and that the subsequent washing of slides at elevated temperature ($55^{o}C$) removed the non-hybridized cDNA molecules.





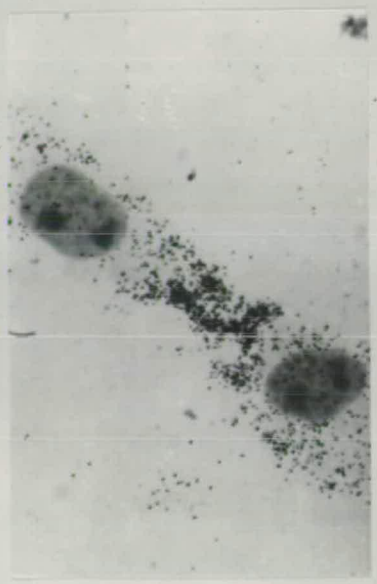


Plate 1

In situ hybridization of MHC cDNA to standard myotubes.

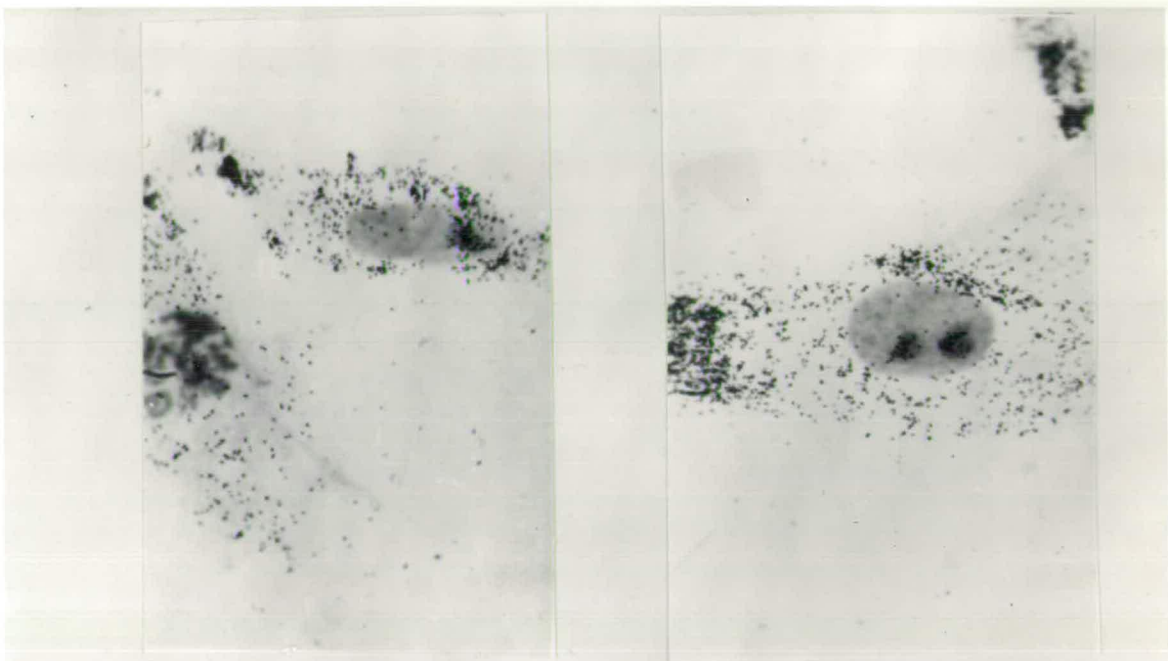


Plate 2

In situ hybridization of MHC cDNA to standard myotubes (nuclear labelling).

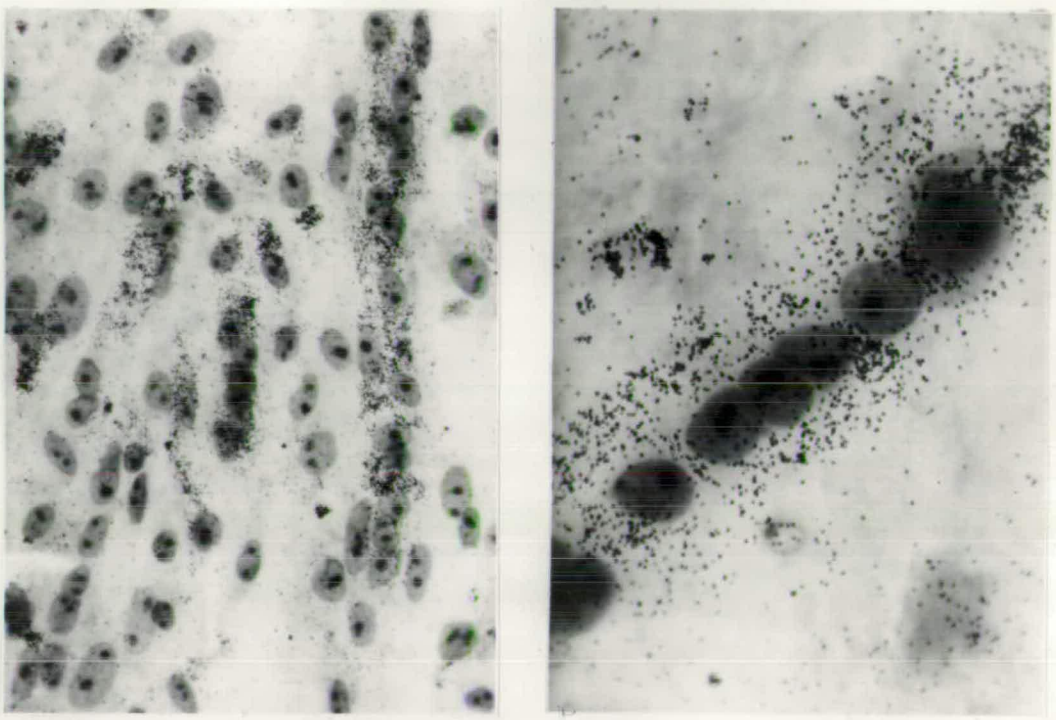
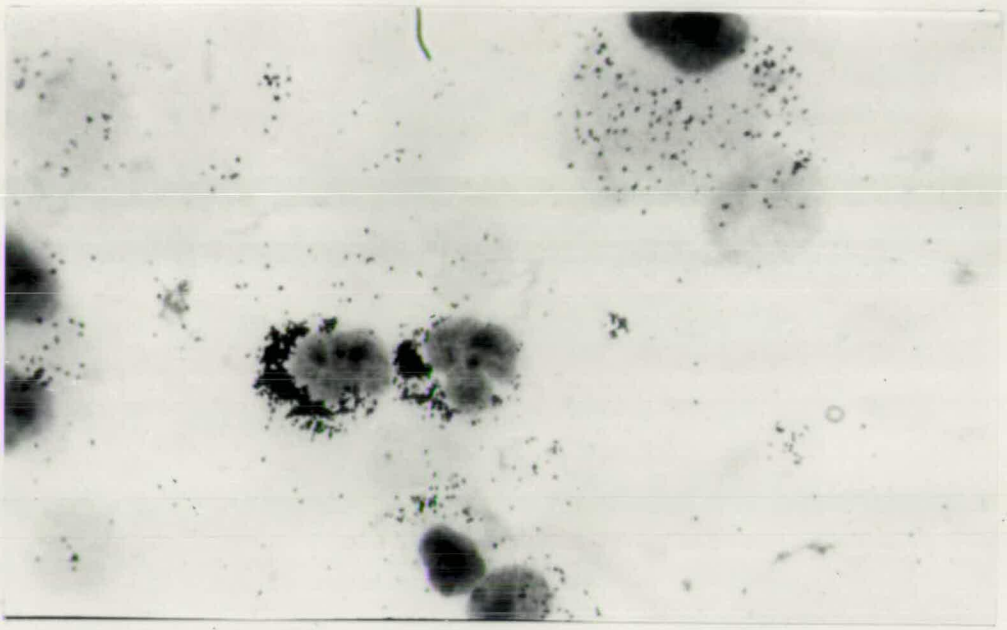
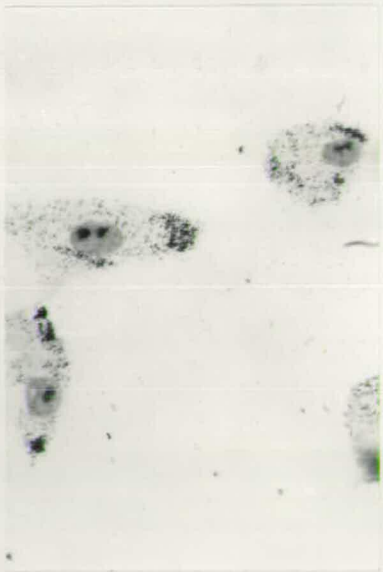


Plate 3

In situ hybridization of MHC cDNA to Ara-C treated myotubes.





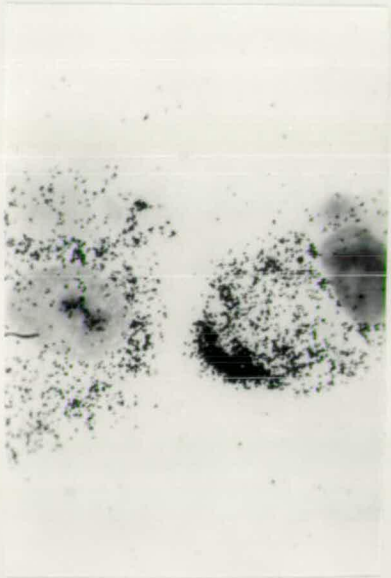


Plate 4

In situ hybridization of MHC cDNA to EGTA-treated myoblasts.

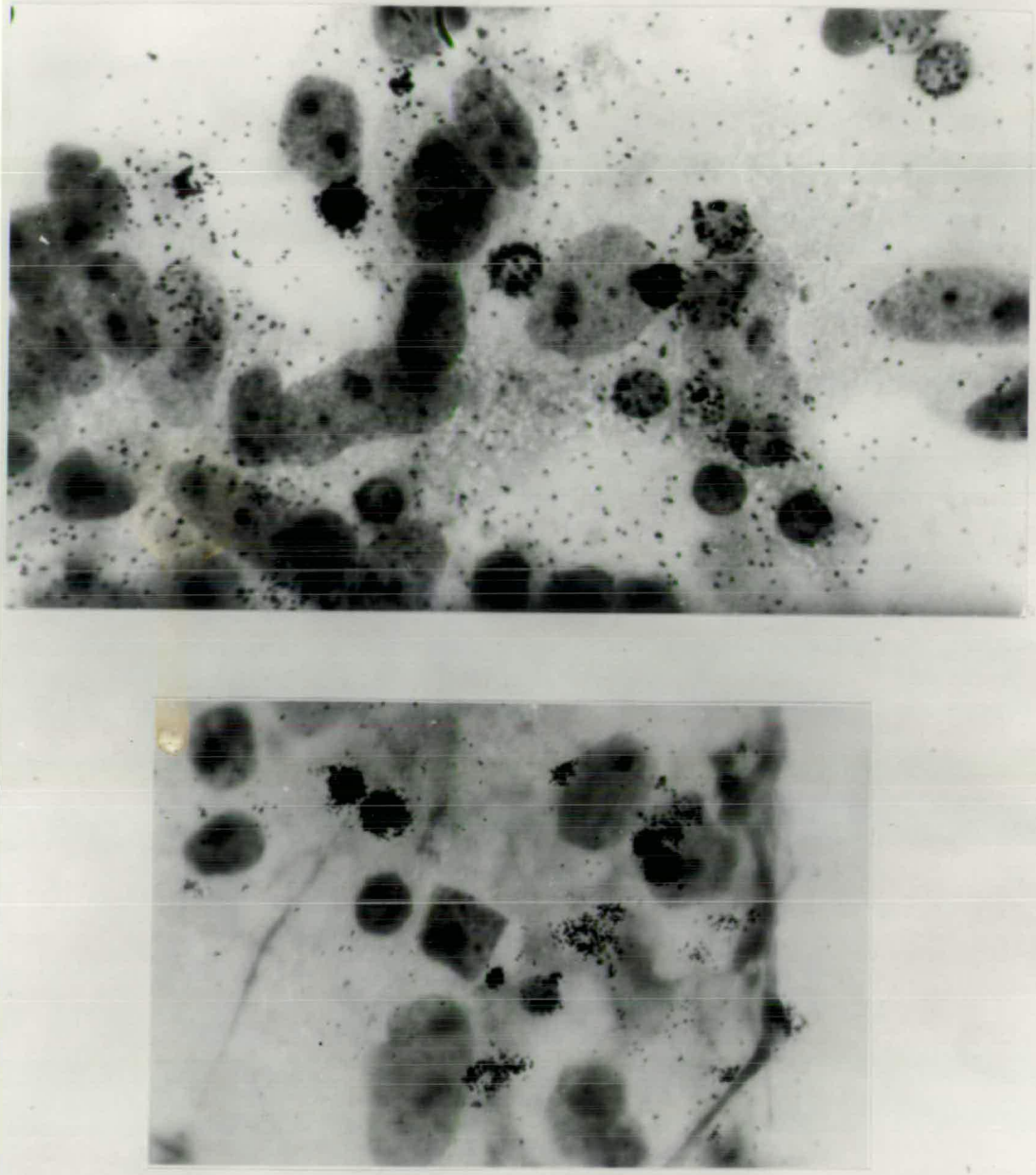


Plate 5

In situ hybridization of MHC cDNA to replicating myoblasts.

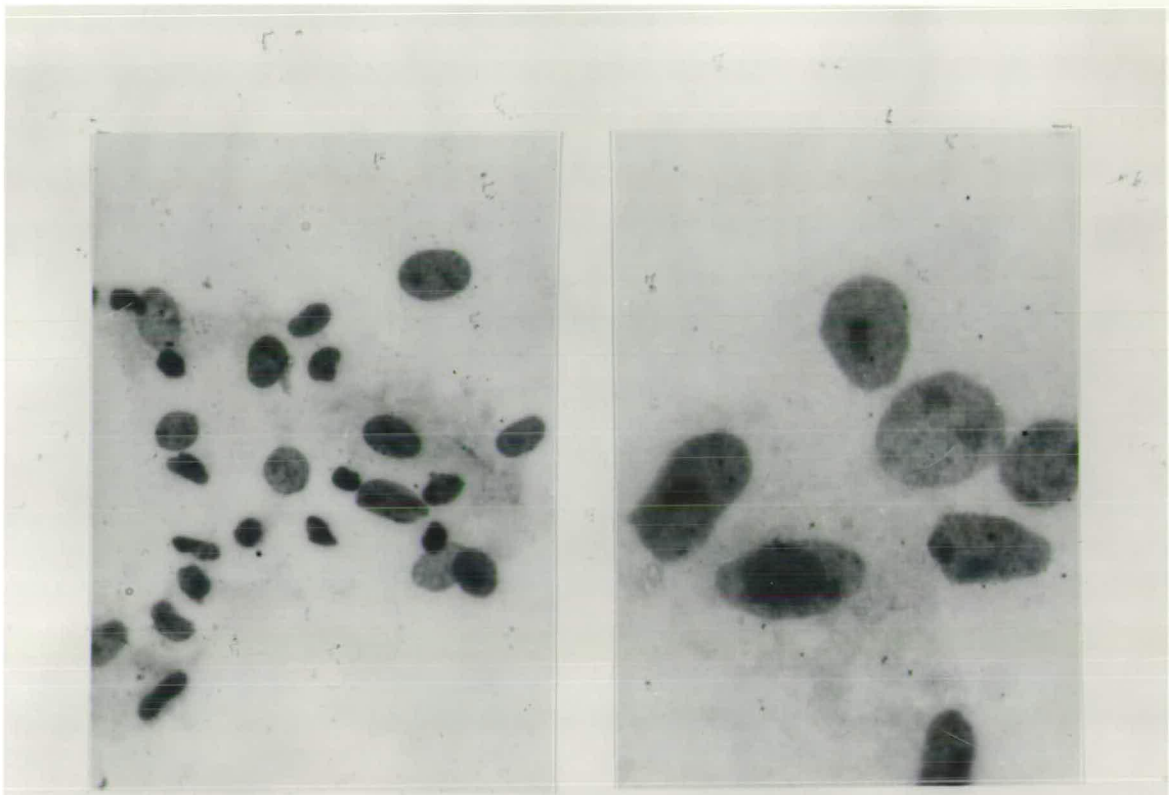


Plate 6

In situ hybridization of MHC cDNA to fibroblasts.

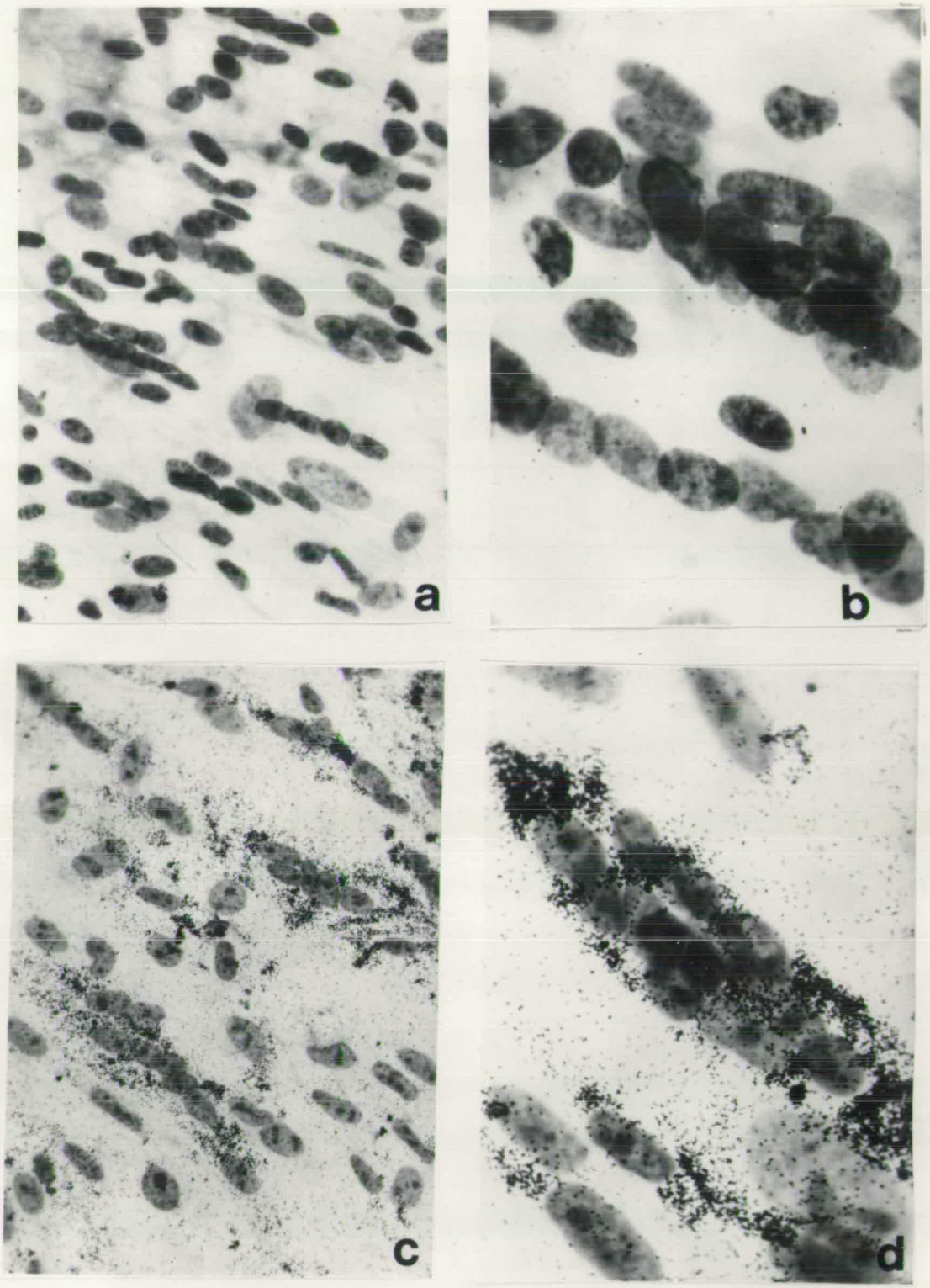


Plate 7

In situ hybridization of MHC cDNA to myotubes pretreated with (a-b) RNase (c-d) DNase.

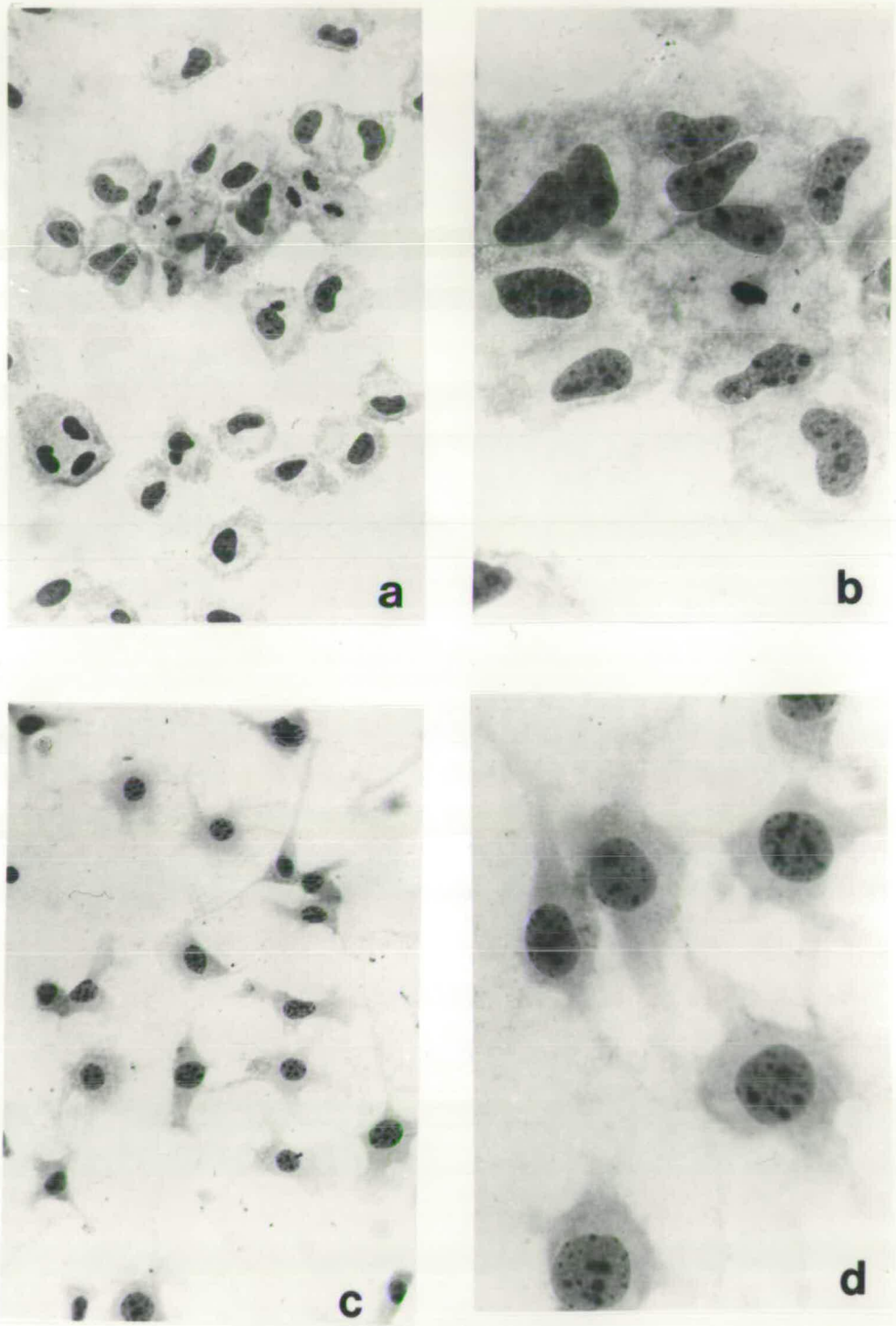


Plate 8

In situ hybridization of MHC cDNA to (a-b) HeLa cells and (c-d) L cells.

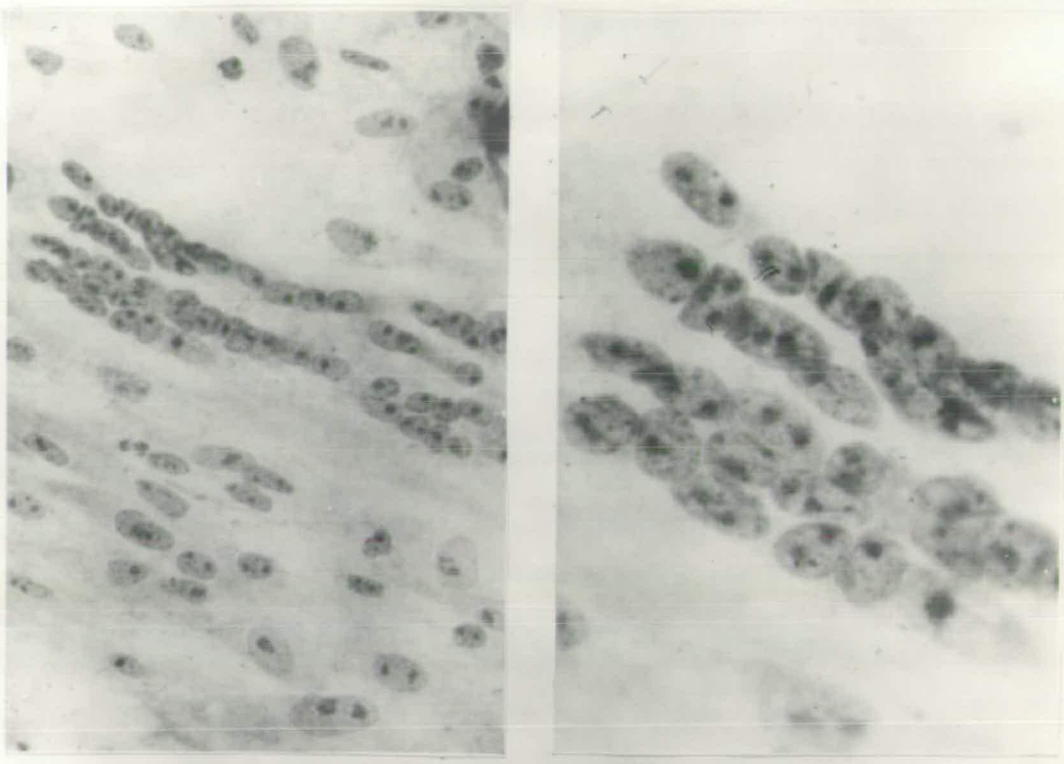


Plate 9

In situ hybridization of globin cDNA to myotubes.

(d) Quantitation of MHC mRNA, during myogenesis in tissue culture, by hybridization in RNA excess

The amount of MHC mRNA present in muscle cells at different stages of differentiation in tissue culture was also determined by conventional hybridization. Total RNA was extracted from the different cultures and quantitative estimates of MHC mRNA sequences in the RNA samples were obtained by hybridization to MHC cDNA under conditions of RNA excess. This approach also served to confirm the results of in situ hybridization.

The kinetics of hybridization of MHC cDNA with RNA from dividing myoblasts, EGTA-treated myoblasts, standard and Ara-C treated myotubes and fibroblasts are shown in Fig. 12. 70-75% hybridization was usually achieved and most curves were parallel within a given experiment. However, in the case of RNA preparation with extremely low concentration of RNA sequences complementary to MHC cDNA (i.e. RNA from replicating myoblasts), the hybridization reaction continued but did not go to completion and only an approximate estimate could be made for the Rot ½. The even slighter reaction observed with fibroblast RNA could be attributed to hybridization of contaminants present in the cDNA preparation and not of MHC cDNA, since at high Rot values the percentage of hybridization did not increase and it was similar to the one observed with 28S rRNA (Table 1).

By estimating the Rot ½ values of these reactions and comparing them with the Rot ½ value of the purified MHC mRNA-cDNA reaction, the fraction of MHC mRNA represented in the total RNA sample could be calculated (see legend Table 3). These calculated values could be used

Legend to Figure 12.

MHC cDNA hybridization to an excess of ^{total}RNA from:

Standard myotubes(▽——▽)

Ara-C treated myotubes (●——●)

EGTA-treated myoblasts (■——■)

Replicating myoblasts (□——□)

Fibroblasts (▼——▼)

MHC mRNA (Fig. 9). (o——o)

Reactions were carried out at 70°C in 0.24 M PEB and the percentage of hybridization was estimated by resistance to S_1 nuclease digestion.

Figure 12.

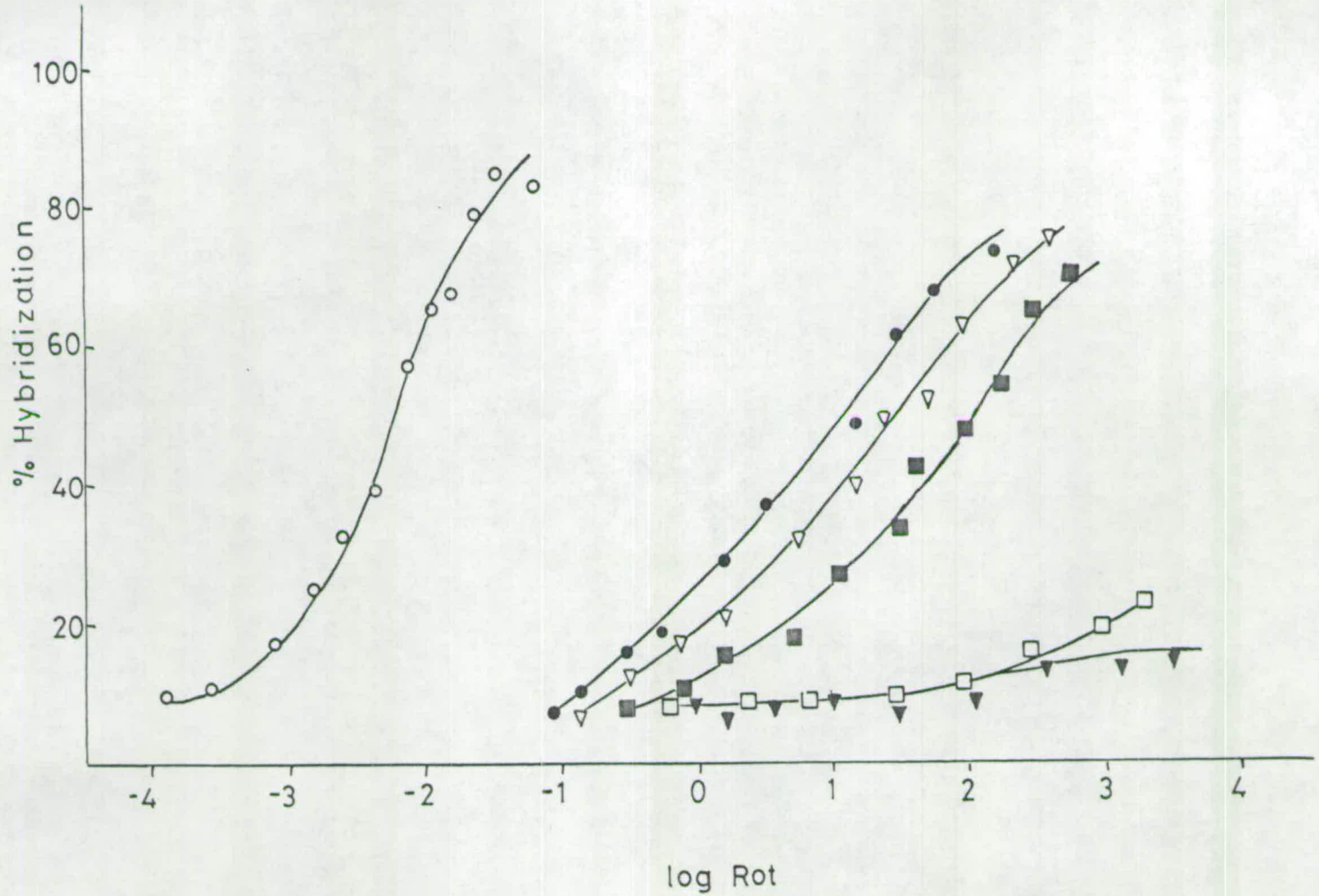


TABLE 3 Quantitation of MHC mRNA during myogenesis in vitro

Cell Types	log Rot½	Rot½ (mol s l^{-1})	Fraction of MHC mRNA[a] (MHC mRNA / Total RNA %)	µg total RNA / dish	µg MHC mRNA / dish	No.cells / dish	µg MHC mRNA / cell	Molecules of MHC mRNA[b] / cell
Replicating myoblasts	2.65	446.7	1.41×10^{-5} (0.0014)	48	6.77×10^{-4}	1×10^{6}	6.77×10^{-10}	0.02
EGTA-treated myoblasts	1.15	14.12	0.45×10^{-3} (0.045)	92.8	4.14×10^{-2}	2.1×10^{6}	1.97×10^{-8}	5,700
Standard myotubes	0.9	7.94	0.8×10^{-3} (0.08)	204.4	16.35×10^{-2}	3.8×10^{6}	4.22×10^{-8}	12,400
Ara-C treated myotubes	0.6	3.98	1.5×10^{-3} (0.15)	94.4	14.16×10^{-2}	2.2×10^{6}	6.43×10^{-8}	18,800

a: Under the assay conditions used the Rot ½ of MHC mRNA was found 6.3×10^{-3} (see page 44). The fraction of MHC mRNA = Rot ½ MHC mRNA/Rot ½ given RNA.

b: The M.W. of MHC mRNA was taken as 2.05×10^{6}, thus 1 molecule of MHC mRNA = 3.42×10^{-12} µg and molecules of MHC mRNA/cell = µg MHC mRNA per cell/3.42×10^{-12}.

along with the values for the RNA content and the number of cells in the different cultures, to determine the MHC mRNA content of each individual myogenic cell. The results are shown in Table 3.

EGTA-treated myoblasts, standard and Ara-C treated myotubes accumulated increasing amounts of MHC mRNA. The amount of MHC mRNA in dividing myoblasts represented less than one molecule per cell and was attributed to the simultaneous presence in these cultures of the small cells, shown by in situ hybridization to contain detectable amounts of MHC mRNA. Ara-C treated myotubes were comparatively enriched in MHC mRNA, which represented ≈ 0.15% of the total RNA. The lower value obtained for standard myotubes (0.08%) was presumably due to the dilution of the myotube population with dividing myoblasts and fibroblasts.

The estimation for the MHC mRNA molecules content of the myotubes was in good agreement with a previous report of 2,000 to 6,000 MHC mRNA molecules per nucleus (Emerson and Beckner, 1975), based on the rate of MHC synthesis in quail myotubes.

Comparison of MHC mRNA and 26S mRNA

Polysomal and cytoplasmic RNA were extracted from 14-day old chick leg muscle, passed two times through the oligo(dt) cellulose column and the Poly(A) minus and Poly(A)+ RNA fractions were run on parallel sucrose gradients (Fig. 13). The size distribution and the amount recovered of the Poly(A)+ RNA fraction from both polysomal and cytoplasmic RNA preparations was almost identical, indicating that most of the mRNA found in the cytoplasm appeared to be associated with polyribosomes. The size of the mRNA present in the Poly(A)+ fraction was measured by hybridizing

Legend to Figure 13.

Sucrose gradient analysis of (a) cytoplasmic and (b) polysomal Poly(A) + RNA from chick leg muscle. Sedimentation was from left to right in a 15-30% gradient. Centrifugation was for 16 hrs at 25,000 rpm at 22°C in the Beckman L-5 SW 27 rotor. 500 µl fractions were collected and 50 µl aliquots of each gradient fraction were used for ^{3}H-poly(U) hybridization.

———— optical density of Poly(A) + RNA

●—●—● hybridization of ^{3}H-Poly(U) (cpm)

– – – – – optical density of Poly(A) minus RNA run on parallel gradient.

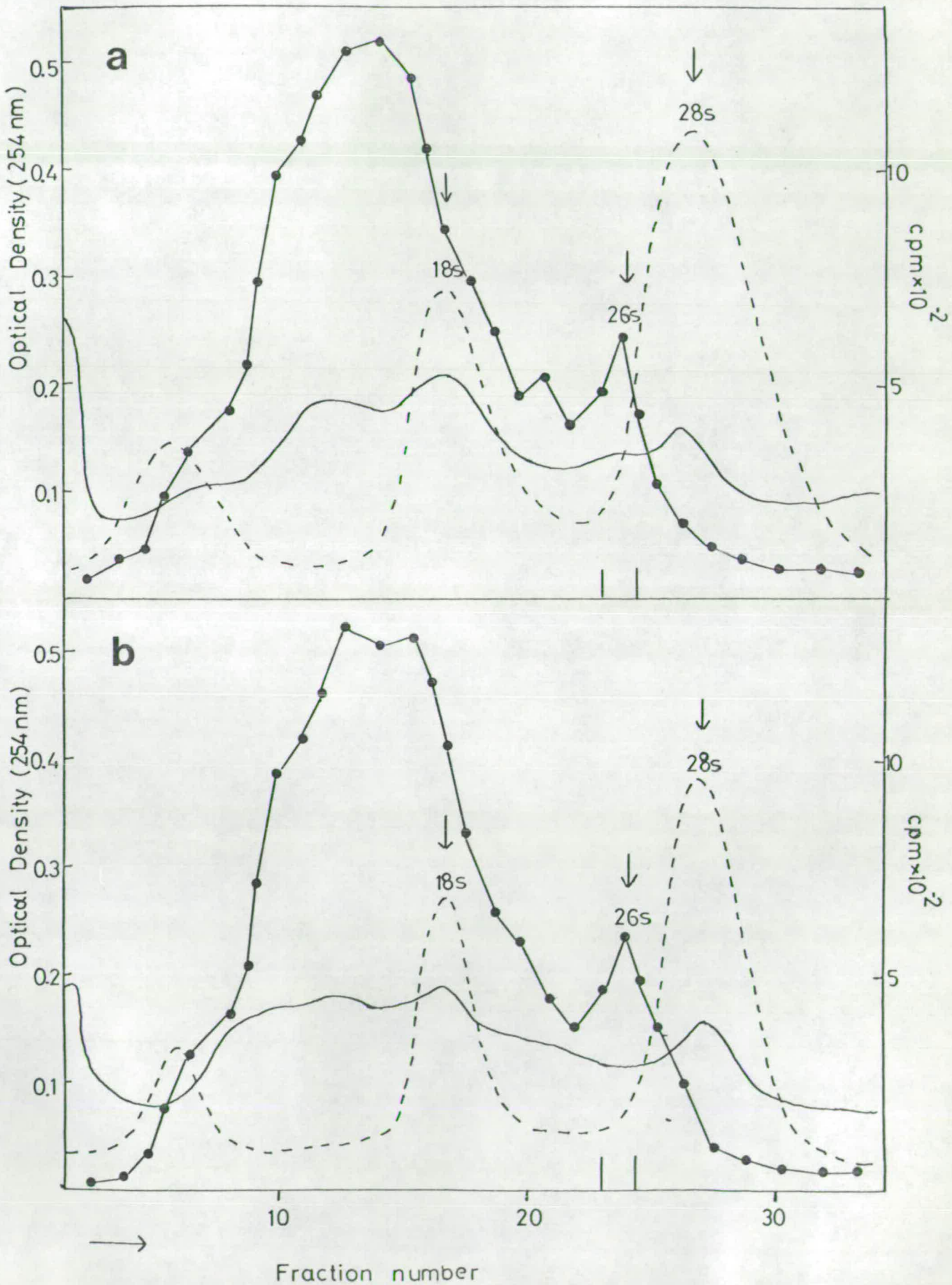

a
b
Optical Density (254 nm)
cpm×10⁻²
28s
18s
26s
0.5
0.4
0.3
0.2
0.1
10
5
10
20
30
Fraction number

Figure 13.

^{3}H-Poly(U) to each fraction of the gradient. The results indicated that the mRNA had an heterodisperse size distribution, similar to that from most kinds of cells, ranging from 10S to 28S. Among the species of Poly(A)+ RNA, 26S mRNA could be clearly distinguished after the ^{3}H-Poly(U) hybridization.

The fractions of gradients containing the 26S mRNA were pooled, as indicated in Fig. 13, and the RNA was ethanol precipitated several times until no traces of sucrose or SDS could be detected. The 26S mRNA was dissolved in distilled H_2O and kept at -70°C until it was used for *in vitro* protein synthesis, cDNA preparation and hybridization.

The template properties of the 26S mRNA was examined by cell-free translation in the wheat germ protein synthesizing system. 26S mRNA stimulated ^{3}H-leucine incorporation into TCA-precipitable material to the same extent as an equal amount of MHC mRNA. Translation was in the presence of 150 mM KCl, since elevated KCl concentration favours the complete translation of high M.W. mRNAs (Harwood *et al.*, 1975; Benveniste *et al.*, 1976). The *in vitro* synthesized products under the direction of 26S mRNA were analyzed by SDS-Polyacrylamide gel electrophoresis. From the results shown in Fig. 14 it was clear that although myosin heavy chain represented a major product it was not the only protein synthesized under the direction of 26S mRNA, which suggested that 26S mRNA contained more than one species of mRNA. The presence of radioactivity in the lower part of the gel had been previously discussed (page 40).

The 26S mRNA was used as an efficient template for the synthesis of highly radioactive cDNA using reverse transcriptase from avian myoloblastosis virus. This cDNA was hybridized with a vast excess

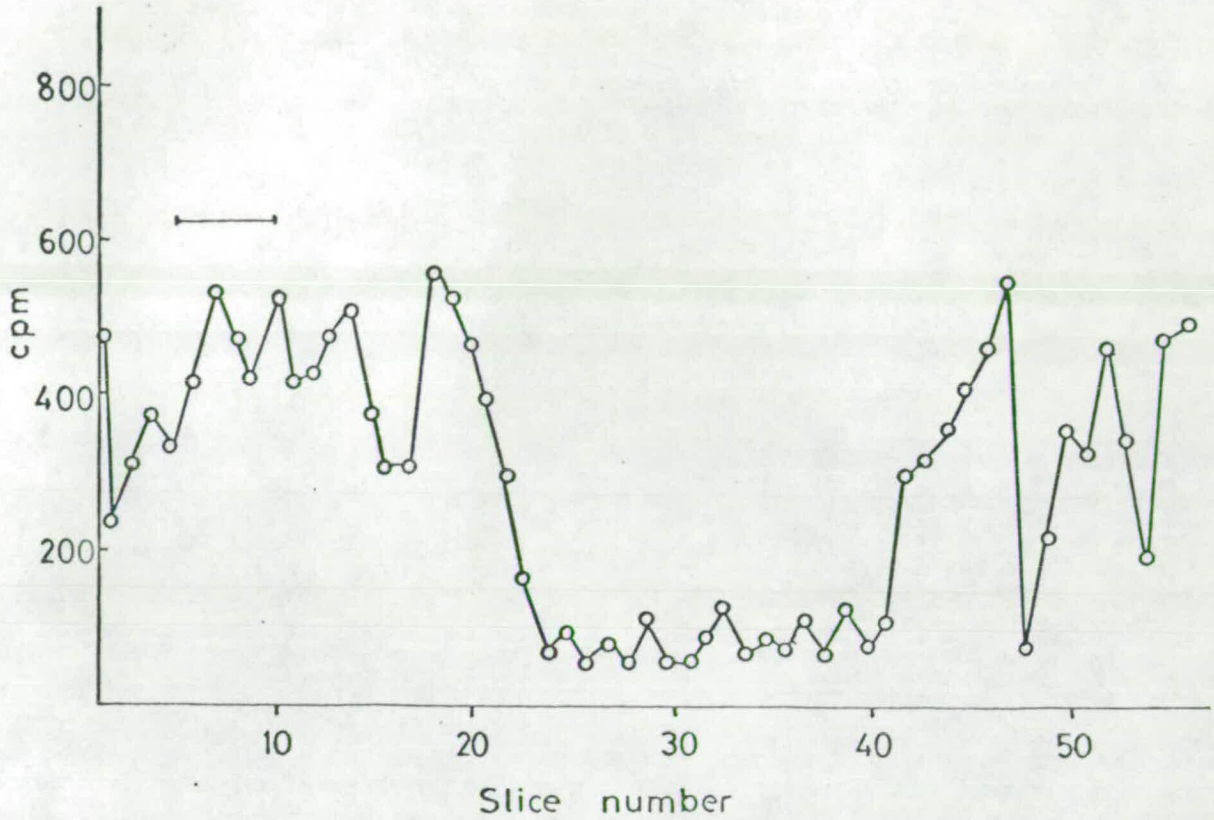


Figure 14.

Direct Analysis on SDS-polyacrylamide gel of the translation products of 0.9 µg 26S mRNA in the wheat germ cell-free system. Incubation was in presence of 160 mM KCl. Gel contained 10% acrylamide and 0.134% bisacrylamide. Migration is to the right. The position and width of the myosin heavy chain marker band, run on the same gel, is indicated by horizontal bar.

of the template 26S mRNA,in order to determine the complexity of the 26S mRNA preparation. The kinetics of hybridization of 26S cDNA with 26S mRNA were complex, with the reaction proceeding to completion only after about 5 orders of magnitude in Rot (Fig. 15). This profile indicated the presence of heterogeneous mRNA populations, which were present in different frequencies. The reaction could be easily resolved into two transitions which reflected the frequency classes of mRNA (Table 4). The first transition included 27% of the cDNA and had an observed Rot ½ of 2.4×10^{-2} (log Rot = -1.6). The second transition included 44% of the cDNA and had an observed Rot ½ of 4.47 (log Rot = 0.65).

To calculate the number of different sequences present in these transitions, the Rot ½ values were corrected to the values which would be obtained if the RNA responsible for any transition was present on its own. It had been calculated (page 44) that an mRNA molecule with M.W. 2×10^{6} and which is known to have a sedimentation coefficient of 26S (MHC mRNA) has a theoretical Rot ½ of 2.7×10^{-3}. If the corrected values of Rot ½ for each transition were divided by 2.7×10^{-3} (used in this case as a kinetic standard), the number of sequences which were represented in each transition could be calculated (Bishop et al., 1974; Axel et al., 1976). Table 4 shows that the RNA represented in the first transition consisted of 2 sequences highly reiterated in the 26S mRNA population, while the RNA represented in the second transition consisted of 729 sequences present in much fewer copies.

The first transition had a corrected Rot ½ of 6.5×10^{-3}, very close to the Rot ½ observed when pure MHC mRNA was annealed to the

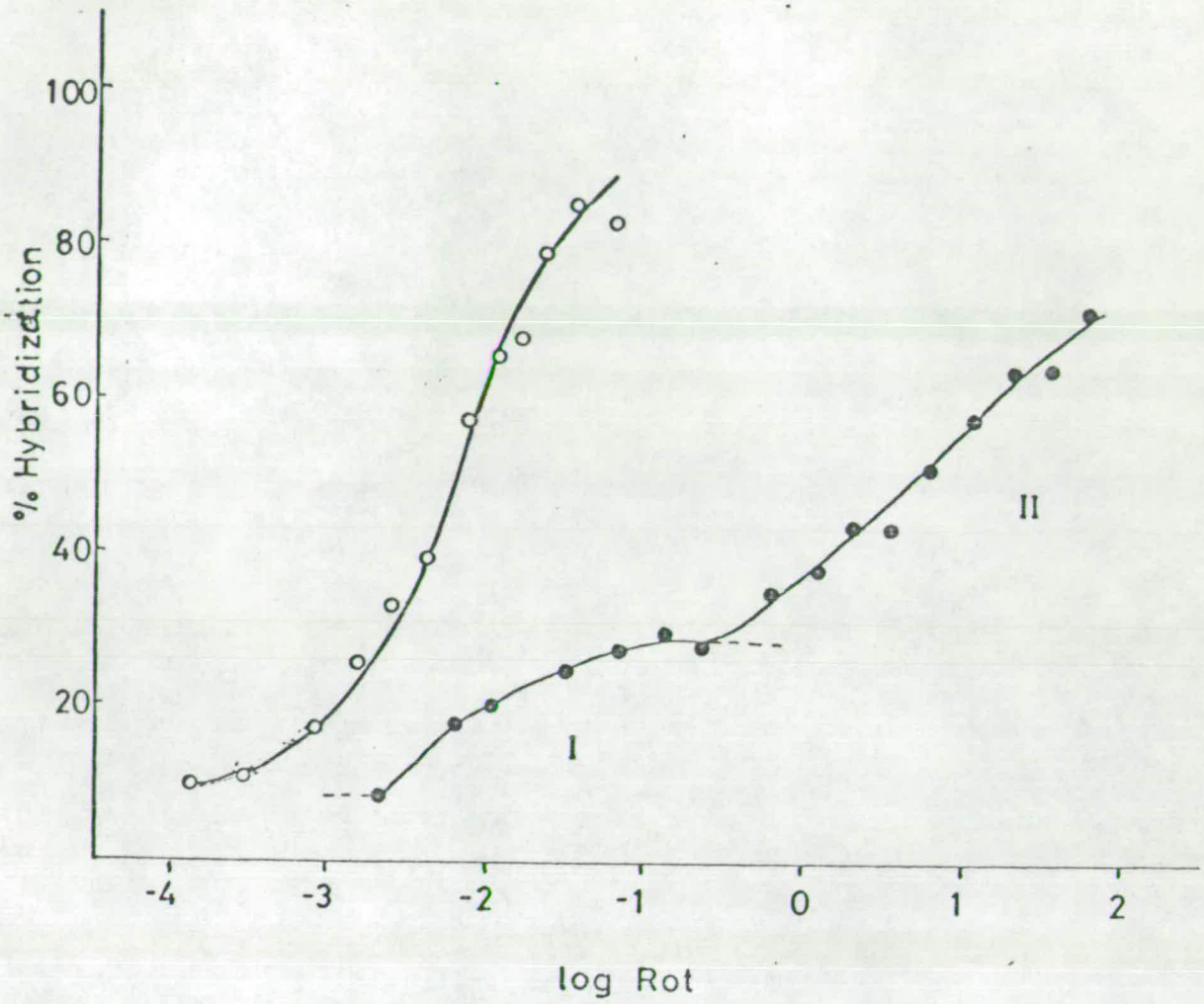


Figure 15.

Hybridization of 26S mRNA and its cDNA, in RNA excess. The reaction was carried out at 70°C in 0.24 M PEB. Percentage of hybridization was estimated by resistance to S_1 nuclease digestion. Rot values were based on the assumption that Poly(A) forms 3% of 26S mRNA. The hybridization reaction between MHC mRNA-cDNA (taken from Fig. 9) is also shown.

●—●—● 26S mRNA-cDNA

○—○—○ MHC mRNA-cDNA

TABLE 4 Analysis of 26S mRNA-cDNA Hybridization Reaction

Transition	Fraction of Hybridizable cDNA	Observed Rot $\frac{1}{2}$[a]	Corrected Rot $\frac{1}{2}$[b]	Number of Sequences
I	0.27	2.4×10^{-2}	6.5×10^{-3}	2.4
II	0.44	4.47	1.97	729

a: Rot is expressed in terms of moles nucleotides per liter x time of annealing in seconds.

b: Corrected Rot $\frac{1}{2}$ = Observed Rot $\frac{1}{2}$ x fraction of hybridizable cDNA.

template MHC mRNA (Fig. 15). Thus, it is believed that the first transition represented the hybridization of MHC mRNA sequences, which would indicate that less than 30% of the 26S mRNA was pure MHC mRNA.

DISCUSSION

Different mRNA sequences in embryonic MHC mRNA

Limited number of embryonic MHC genes

Homogeneous chick embryonic MHC mRNA was isolated from a fraction of large polysomes and its identity was determined by translation in a heterologous system. A cDNA copy was used for determining the number of different MHC mRNAs for the embryonic MHC and the reiteration frequency of the corresponding genes. The results strongly indicated the presence, in the 14 day chick embryo leg skeletal muscle, of at least two different MHC mRNA sequences, which transcribed from a limited number of structural genes (most probably two) belonging to the non-reiterated fraction of the genome.

The sole product of *in vitro* translation of the mRNA was identified as myosin heavy chain by a number of criteria. The synthesis of myosin heavy chain in a heterologous system prepared from plant tissue (wheat germ) suggests that muscle specific factors are not essential for the translation of MHC mRNA as has been suggested by Heywood (Rourke and Heywood, 1972; Heywood *et al.*, 1974). The translation of muscle specific actin mRNA in a heterologous system has also been reported (Paterson *et al.*, 1974; Bag and Sarkar, 1975; Sorti and Rich, 1976). Nevertheless, the possibility that the addition of initiation factors from muscle tissue may enhance the translation of MHC mRNA cannot be ruled out.

The validity of many of the conclusions in this study depends on the purity of the template RNA used to synthesize complementary cDNA.

It is necessary to ensure that cDNA complementary to minor contaminating RNA does not make a substantial contribution. The MHC mRNA used in the present investigation was prepared from a size-class of polysomes that synthesized myosin heavy chain and not other protein, as judged by M.W., suggesting that only this mRNA was present. Further purification of the RNA by oligo(dt) cellulose chromatography served to increase the relative proportion of MHC mRNA by removing most of the ribosomal RNA. The ribosomal RNA which remained acts as a poor template for reverse transcriptase (Daubert and Dahmus, 1976). Although there was an indication that a fraction of the MHC cDNA cross-reacted with ribosomal RNA (Table 1), the contribution to the total hybridization reaction has been small because the MHC cDNA did not hybridize to the intermediate repetitive DNA sequences to which the ribosomal genes belong (Fig. 11).

Hybridization of cDNA back to its MHC mRNA template revealed the presence of two different mRNA sequences. In order to establish that they represent different MHC mRNA species it was necessary to establish that both sequences coded for different heavy chains with identical M.W. (200,000 daltons). The possibility that one of the mRNA sequences coded for a different high M.W. protein could not be completely excluded. The MHC mRNA was isolated from a fraction of the large polysomes and therefore the most likely of its contaminants could be the mRNA for a large protein close to the size of myosin heavy chain, such as B-protein with a M.W. of 180,000 (Starr and Offer, 1971) or filamin with a M.W. of 250,000 (Wang *et al.*, 1975b). The electrophoretic analysis of the *in vitro* synthesized product in the homologous or heterologous cell-free system under the direction of the isolated mRNA, showed that the bulk of the radioactivity formed

a single peak, co-migrating with MHC marker (M.W. 200,000). In the SDS-polyacrylamide gels used, B-protein or filamin were clearly separated from myosin heavy chain and therefore it cannot be argued that two proteins were included in the radioactive peak. Furthermore the isoelectric focussing of the radioactive polypeptide product showed that it contained two radioactive peaks of almost equal size which co-focussed with two prominent bands of equal intensity present in the embryonic myosin heavy chain marker.

The conclusion that embryonic MHC mRNA contained at least two different sequences was based on the estimation of the Rot ½ value for the cDNA-mRNA hybridization reaction. The amount of MHC mRNA used was determined by hybridization of radioactive poly(U) of known specific activity to the poly(A) tail of the mRNA. The calculation of Rot figures was made on the assumption that poly(A) segment comprises 3% of the MHC mRNA molecule (Sarkar et al., 1973; Mondal et al., 1974). If the Poly(A) value of 3% was inaccurate, then the Rot values would have been different, obviously affecting the calculation of the number of different mRNA sequences in the MHC mRNA preparation. However the independent evidence from the isoelectric focussing of the in vitro synthesized protein and from the estimation of the number of embryonic MHC genes argued for the presence of two MHC mRNAs in the RNA preparation. Furthermore, it is unlikely that the higher than expected Rot ½ value of the MHC cDNA-mRNA hybridization was due to a higher molecular weight of the MHC mRNA molecule. The estimated value of 2.05×10^6 Daltons for the molecular weight of MHC mRNA (Sarkar et al., 1973) is in good agreement with the number of amino-acid residues in the MHC

after making allowances for untranslated sequences (e.g. poly(A)).

Analysis of the kinetics of the annealing of MHC cDNA to total chicken DNA indicated that each of the two MHC mRNAs from 14 day embryonic chick leg skeletal muscle was coded by a gene, which was present in two copies. The limitations to this interpretation are (1) the sensitivity of the technique used (see page 46), (2) the purity of the MHC mRNA fractions used as a template for the synthesis of the cDNA, as discussed above, and (3) the observation that the cDNA is a partial copy of the MHC mRNA. The cDNA is complementary to the 3'-end of its template mRNA which codes for the C-terminal fraction of the corresponding protein. Therefore, the *in vitro* synthesized MHC cDNA would be complementary to that part of the MHC mRNA or MHC gene that carried the information for the LMM fraction of the myosin heavy chain, and most probably excluded that part coding for HMM, which would obviously affect the specificity of the probe (Diagram 1).

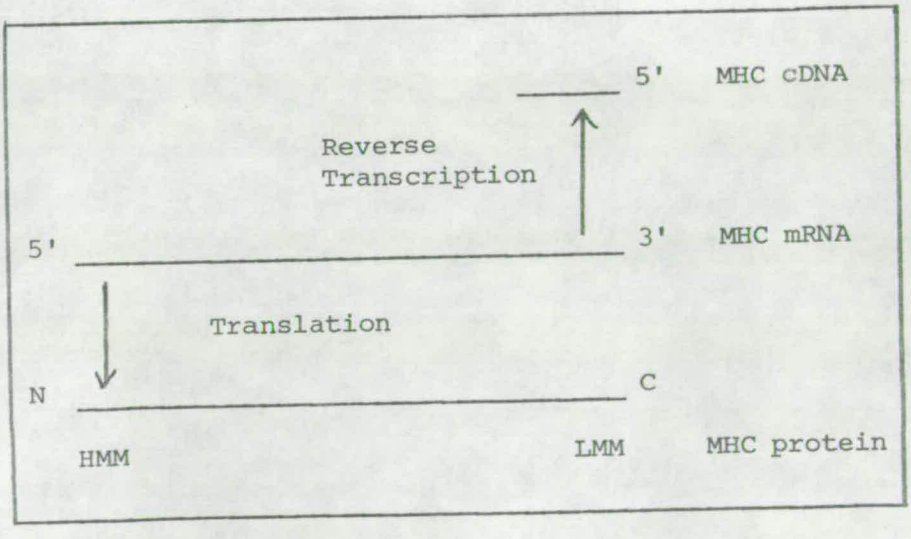


Diagram 1.

The limited size of the cDNA would also be expected to influence the rate of cDNA-DNA hybridization. However, in the present experiments this should not have affected the results since the "unique" DNA fragments,used as a standard in the calculation of the MHC gene's reiteration frequency,had a size similar to the MHC cDNA.

Masaki and Yoshizaki(1974) using fluorescent antibody technique have shown that myotubes of early chick embryo contain three kinds of heavy chain (namely cardiac, fast and slow),but that only one of these persists in the respective muscle fiber after hatching while the others are gradually lost during development. Furthermore,myosin heavy chain synthesized in vitro by polysomes prepared from skeletal muscles of chick embryos at various stages, contained these three immunochemically distinguishable heavy chains (Masaki and Kinoshita, 1974). These results together with the observation that it was the primary structure of MHC which determined its antigenicity (Masaki, 1974),suggested the existence of three kinds of MHC mRNA in the chick embryonic muscle (8 - 18 day old). It is possible that some MHC mRNA present in chick embryonic muscle was not detected in the present investigation. As was shown in Fig. 6, myosin heavy chain was in vitro synthesized by the bottom six polysomal fractions. The heavy chain synthesized by the fractions 3, 4, 5 and 6 has a M.W. of 200,000, the same as that synthesized by fraction 2 (from which the MHC mRNA was isolated), but it is possible that there may be sequence difference. The possibility that different myosin heavy chains coded by different mRNAs occur in the different polysomal fractions cannot be ruled out. However the results of isoelectric focussing of embryonic myosin showed the presence

of only two heavy chain bands (John, 1976b).

In view of all the evidence cited above it is proposed that 14 day chick embryonic skeletal myosin contains at least two different forms of myosin heavy chain,which are coded by distinct mRNA species. Although previous reports had shown differences in the primary structure of heavy chains (Starr & Offer, 1974; Burridge & Bray, 1975) this is the first demonstration that myosin heavy chain forms are the products of different structural genes.

The finding that embryonic MHC mRNA existsin two different forms is not surprising since John (1976b) has shown that chick embryonic myosin contains 2 heavy chains in almost equal amounts. The present results on the isoelectric focussing of the *in vitro* synthesized myosin heavy chain polypeptides indicated that the two MHC mRNAsexisted in equal proportions.

The conclusion that there are two copies of each MHC gene expressed in the chick embryonic leg skeletal muscles, is consistent with the accumulating evidence on a very low reiteration frequency (if any) for most structural genes (Harrison *et al.*, 1974a; Harris *et al.*, 1973; Bishop and Rosbash, 1973).

Chemical and immunochemical evidence indicates substantial heterogeneity of the myosin heavy chains, suggesting the presence of different structural genes for fast, slow and foetal skeletal heavy chains and in addition cardiac, smooth and non-myogenic heavy chains. The present experiments indicate that two structural genes are expressed in embryonic chick leg skeletal muscle, coding for two myosin heavy chains the identity of which is uncertain. Two possible explanations

exist on the source of the two heavy chains of the embryonic myosin: (1) that they are found in foetal and fast adult skeletal myosin (supported by Sreter et al., 1972) and (2) that one heavy chain is fast and one is slow, because of evidence from the light chain pattern of embryonic myosin presented by John (1976b). However, the same work indicated that there is a distinguishable slow myosin heavy chain which is not present in embryonic myosin.

Further experiments are required in order to establish the relationship between the embryonic MHC genes and the ones for adult fast and slow myosin.

MHC mRNA synthesis during myogenesis in vitro

A cDNA copy of purified MHC mRNA, isolated from chick embryonic leg muscles, was employed as a specific probe in in situ and in vitro (RNA excess) hybridization experiments designed to detect and measure the amount of muscle-specific MHC mRNA at different stages of myogenesis in tissue culture. The amount of a myosin heavy chain protein was also estimated. The results of the present study indicated that myosin heavy chain was accumulated during all stages of myogenesis in vitro (i.e. in replicating myoblasts, postmitotic myoblasts and myotubes) and in fibroblasts as well. The lack of hybridization of muscle MHC cDNA to the majority of replicating myoblasts and fibroblasts suggested that the MHC protein synthesized in these cells was different enough from that present in myotubes for the mRNAs not to cross hybridize. EGTA-fusion blocked myoblasts showed accumulation of muscle MHC mRNA but to a lesser extent than myotubes.

There was excellent correspondence between the detectability of MHC mRNA by *in situ* hybridization and the amount of mRNA estimated by *in vitro* hybridization. However, it must be stressed that the figures for the number of MHC mRNA molecules/cell, presented in Table 3, may not represent the real situation because at each myogenic stage different cells were present having a variable content for MHC mRNA, as was revealed by *in situ* hybridization.

The observed *in situ* hybridization of MHC cDNA represented a specific reaction, since no hybridization was observed when HeLa cells, L cells or fibroblasts were used, or when globin cDNA was hybridized to myotubes. Furthermore the lack of hybridization reaction in RNase pretreated cells and the lack of effect of DNase pretreatment indicated that the hybrid was formed between cDNA and RNA. When cells, whose RNA was pulse-labelled with ^{3}H-uridine, were processed through the *in situ* hybridization reaction but without cDNA ("dummy *in situ* hybridization"), there was loss of labelled RNA. A similar observation had been previously made for other types of cells (Dr C. Godard, unpublished data). However, no indication was found for an unequal loss of RNA from the different myogenic stages, which may have accounted for the different levels of hybridization. Furthermore, the results of the conventional hybridization (RNA excess), strongly support the specificity of the *in situ* results.

The kinetic curves, representing the hybridization between MHC cDNA and RNA from EGTA-myoblasts, Ara-C treated and standard myotubes (Fig. 12) were broad, extending over approximately 3 log units, i.e. their range was greater than expected for a homogeneous reaction between cDNA-RNA (Getz *et al.*, 1975). One possible explanation is that

the MHC cDNA contained minor contaminants, reacting with other RNAs apart from the MHC mRNA (e.g. 28S rRNA). An alternative view is that the hybridization curve was composed of two or more components (transitions), each one representing the hybridization of MHC cDNA with different MHC mRNAs. In this case the low hybridization observed with RNA from replicating myoblasts or fibroblasts would have to be explained as indicating that these cells contained only one of the MHC mRNAs. However the fact that in the in situ hybridization experiments no reaction was observed between fibroblasts and MHC cDNA would seem to argue against this second possibility.

The morphologically distinct small round cells, sporadically seen in the cultures of dividing myoblasts, have not been identified. Since their number decreased with the age of the culture it is proposed that they do not represent myoblasts altered as a consequence of the tissue culture conditions. Most likely they derive from the chick embryonic tissue. Given that they contained significant amounts of muscle MHC mRNA (as detected by in situ hybridization), it can be postulated that they were myoblasts which at the time of the isolation of the tissue were becoming postmitotic and for some reason were slow in recovering from the trypsinization procedure. Their heavy nuclear labelling with MHC cDNA indicated intense transcription of MHC mRNA, possibly due to their withdrawal from the cell cycle. The presence of post-mitotic myoblasts accumulating muscle MHC mRNA in the leg muscle of 12 day old chick embryos is consistent with Heywood's report on the presence of stored MHC mRNA in the muscle cells of this developmental stage (Heywood et al., 1975a).

The small amount of muscle MHC mRNA detected in the cultures of

replicating myoblasts by *in vitro* hybridization was attributed to the small cells, since the *in situ* hybridization revealed that MHC cDNA labelling was associated only with those. However, if all the myosin heavy chain present in the replicating myoblasts culture was ascribed to the small cells, then there would have to be substantially more myosin heavy chain per mononucleated (small) cell than in fully differentiated myotubes. Since this contention is unlikely, it is suggested that replicating myoblasts accumulate myosin heavy chain, an observation that has been reported before (Rubinstein *et al.*, 1974; Chi *et al.*, 1975a). Chi *et al.* (1975b) examining the light chain pattern and the antigenicity of the LMM fragments of the myoblastic (replicating myoblasts) myosin, concluded that it was different from the one synthesized in differentiated myotubes or skeletal muscles, but indistinguishable from the "cytoplasmic" myosin synthesized in fibroblasts myoblasts. From the present results no conclusion can be drawn on such a relationship, although it is indicated that neither myoblastic (replicating myoblasts) nor fibroblastic myosin heavy chain are being coded by the muscle MHC mRNA, as it is the case in myotubes. Young *et al.* (1975) and Tepperman *et al.*,(1975) have reported that MHC mRNA was bound on the polysomes of replicating myoblasts. However, the observation that the MHC synthesizing polysomes of the replicating myoblasts had a slightly smaller size than the corresponding ones in myotubes (Young *et al.*, 1975) may indicate that the MHC mRNAs present in those two cell populations were not identical.

The detection of muscle MHC mRNA in the EGTA-fusion blocked mononucleated myoblasts indicate that fusion is not a necessary event

for the transcription of muscle MHC mRNA. A similar observation has been made by Yaffe and Dym (1972) and Buckingham et al. (1974), who also proposed that only after fusion was the MHC mRNA translated. Emerson and Beckner (1975) had proposed that it is the withdrawal of myoblasts from the cell division cycle and not the fusion that triggers the differential gene expression, which characterises fully differentiated myotubes. The data presented in the present report are compatible with this view, provided that muscle MHC mRNA synthesis is any indication of complete differentiation in the myogenic tissue-culture system. Nevertheless, the low number of muscle MHC mRNA molecules/cell in the EGTA treated cultures in comparison with the one in myotubes may suggest that fusion acts by enhancing mRNA transcription.

The estimated concentration of muscle MHC mRNA of 18.800 MHC mRNA molecules/cell in the myotubes (Table 3) compares quite well with that for ovalbumin mRNA, calculated in a similar way (Harris et al., 1975). In a developmental stage of the chicken oviduct where up to 60% of the total soluble protein is ovalbumin, there are 147,000 ovalbumin mRNA molecules/cell. The actual number of MHC mRNA molecules/myotube may be higher than the estimated one, since even in the Ara-C treated cultures mononucleated cells could be observed which presumably did not synthesize the high amounts of MHC mRNA. In addition, the possibility that further development of the myotubes may be accompanied by enhanced MHC mRNA transcription cannot be overlooked.

It is well established that fibroblasts along with a variety of non-myogenic cells contain a myosin like protein (see Pollard and Weihing, 1974). Although it is not proved whether all the myosins

from different types of non-myogenic cells comprise a single or more than one class of "cytoplasmic" myosin, several lines of evidence have indicated that they are different from the muscle-specific myosin (Ostlund et al., 1974; Willingham et al., 1974). However, it was the pattern of the MHC chemical cleavage at cysteine residues which suggested that cytoplasmic and muscle myosin heavy chains differ in their primary structure (Beveridge and Bray, 1975). The present observation that muscle MHC cDNA failed to react with fibroblasts RNA, further supported this view.

Recently it has been established that different forms of the myofibrillar proteins are present at the different stages of myogenesis in tissue culture. Apart from myosin, which according to Chi's report (Chi et al., 1975b), supported by the present results, seem to exist in polymorphic forms during in vitro myogenesis, actin also exhibits multiple forms. Whalen et al. (1976) found three forms of actin possessing similar biochemical properties and identical M.W.s but slightly different isoelectric points. Two of the actin forms were found in prefusion replicating myoblasts of foetal calf cultures and in non-myogenic cells, while the third one was predominent in cultures of fused muscle cells. A similar situation has also been reported for chick muscle cultures (Sorti et al., 1975). Suggestions have been made that the myosins and actins which are not characteristics of the fully differentiated myotubes may be found in microfilament type structures, rather than in thin and thick myofibrillar filaments (Weber and Groeschel-Steward, 1974). However, it is not clear whether fully differentiated myotubes contain predominantly or exclusively the muscle-specific types of myosin and actin.

Sorti and Rich (1976) have suggested that cytoplasmic and muscle actins have different structural genes. Further experiments with isolation of the cytoplasmic MHC mRNA are required in order to establish that this is the case also for the MHC genes. Nevertheless it is quite possible that eventually several closely related myosin genes will be discovered within one organism.

26S mRNA and MHC mRNA

A 26S mRNA from chick embryonic skeletal muscle (considered in the literature as MHC mRNA) was isolated and transcribed into a cDNA molecule. Hybridization of this cDNA to an excess of its template 26S mRNA, revealed the presence of a high number of different mRNA species, among which MHC mRNA was represented in a high proportion. The presence of many different mRNAs was also indicated by *in vitro* translation of 26S mRNA in the heterologous wheat germ cell-free system, which showed the presence of many polypeptides as well as myosin heavy chain.

High Rot $\frac{1}{2}$ values for the 26S mRNA-cDNA hybridizations have been observed in previous reports (Buckingham *et al.*, 1974; Robbins and Heywood, 1976), but they have not been considered as indicating the presence of multiple RNA species in the 26S mRNA preparation. Although the exact number of different myosin heavy chains is not known it is unlikely that all these different RNA sequences represent MHC mRNAs. In the present study it was shown that the RNA species present in the 26S mRNA comprised different frequency classes of which only the most abundant, representing 27% of the 26S mRNA preparation, was attributed to MHC mRNA.

Heywood and Nwagwu (1969) demonstrated that mRNA isolated from the MHC synthesizing polysomes and shown to direct MHC synthesis in a cell-free system, had a sedimentation coefficient of 26S. These large polysomes synthesized predominantly but not exclusively myosin heavy chain as was shown in Heywood et al., (1967) Fig. 3, and confirmed in the present results (Fig. 5). Electrophoretic analysis of total protein from embryonic chick leg muscle revealed the presence of multiple bands in the M.W. range of 200,000 - 160,000 (see Appendix) suggesting that a number of proteins exist, whose M.W. is high enough so that they could be synthesized on the same size of polysomes as myosin heavy chain. Thus it was not surprising that 26S mRNA contained several different mRNA sequences.

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APPENDIX

CHARACTERIZATION OF THE RADIOACTIVE MYOSIN HEAVY CHAIN SYNTHESIZED IN VITRO BY POLYSOMES AND mRNA

In collaboration with Dr Huw A. John

Translation of the large polysomes and the mRNA gave a radioactive protein which was shown by SDS-gel electrophoresis to have an identical M.W. (200,000) to the myosin heavy chain.

The radioactive protein had other properties characteristic of myosin. When mixed with carrier myosin it precipitated at low ionic strength and at 38 - 50% saturation ammonium sulphate, and co-chromatographed with myosin on DEAE-sephadex A 50. After these purification steps the radioactive protein still co-migrated with the myosin heavy chain when electrophoresed in SDS gels. If the radioactive protein had been other than myosin it would be expected that it would be removed by the purification procedure.

Analysis of the radioactive protein by iso-electric focussing showed that it consisted of two distinct peaks which co-focussed with the two closely spaced bands of chick embryonic myosin heavy chain (Fig. 16).

Preliminary evidence using limited tryptic digestion of the radioactive protein in the presence of carrier embryonic myosin further suggested that it was the myosin heavy chain. The crude preparations of LMM and HMM were analyzed by SDS-gel electrophoresis (Fig. 17). Both preparations contained some of the radioactive 200,000 M.W. chains undigested and some slightly smaller radioactive

fragments (120,000 - 150,000). In the crude LMM preparation all radioactive peaks coincided with visible fragements and the most prominent fragment had a M.W. of 70,000, which is the approximate size expected for the main fragment of LMM. In the crude HMM preparation the prominent radioactive fragments had M.W.s of 65,000 - 75,000 and 48,000, which coincided with visible fragments derived from the heavy chain. Smaller fragments, presumably derived from the light chains, were not labelled.

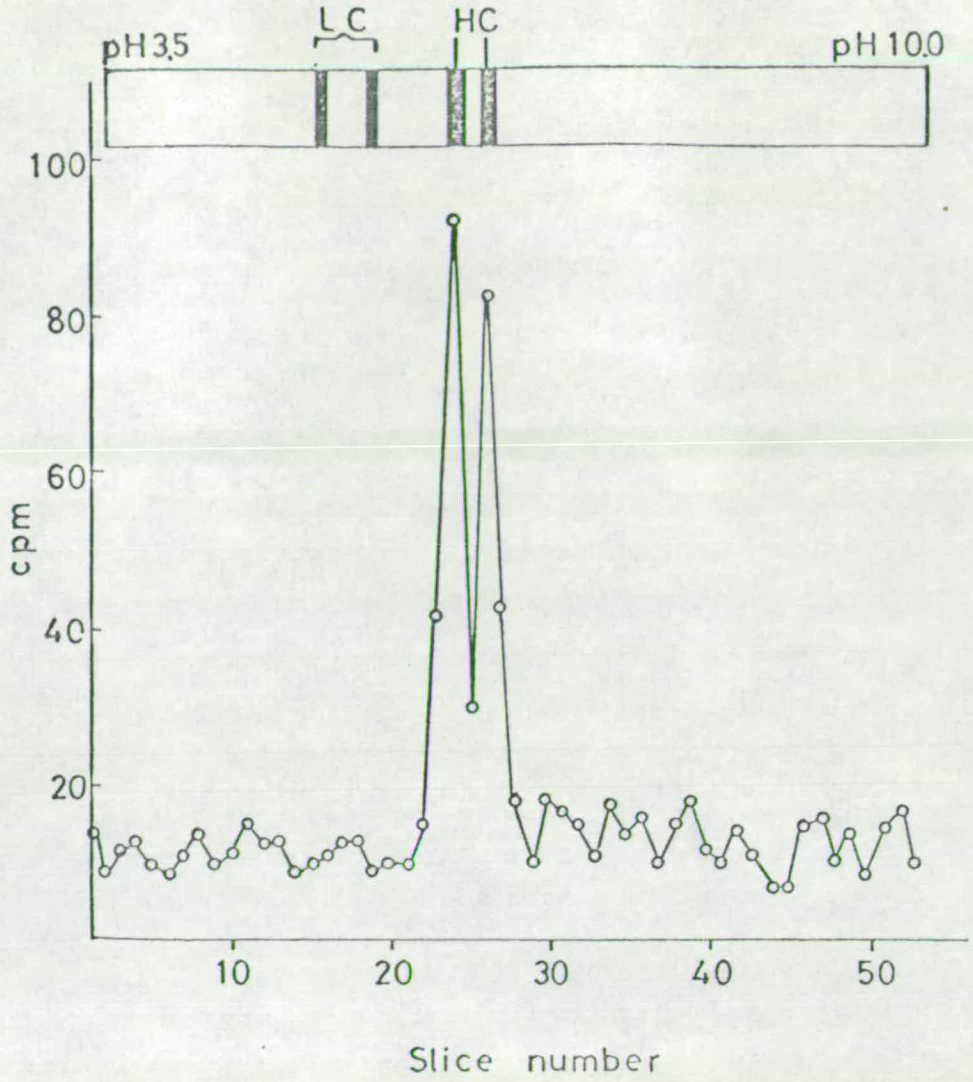

Figure 16.

Analysis by isoelectric focussing of the translation products of MHC mRNA *in vitro*. MHC mRNA (0.9 µg) was translated in the wheat germ cell-free system at a KCl concentration of 160 mM. 1 ml of 0.5 M KCl, 0.02 M Tris-HCl pH 7.2 containing 0.5 mg carrier embryonic myosin was added to 25 µl of the incubation mixture. The myosin was precipitated at low ionic strength by addition of 10 volumes of 0.5 mM dithiothreitol and pelleted by centrifugation at 20,000 g for 5 min. Myosin was redissolved in 0.5 ml of 9 M urea. Isoelectric focussing in 2.6% acrylamide gel containing Ampholine (pH 3.5 - 10.0) and 9 M urea was carried out for 2 hr as described by Florini *et al.* (1971), with the modification described by John (1976b).

Legend to Figure 17.

Analysis on SDS-polyacrylamide gels of tryptic fragments of translation products of MHC mRNA *in vitro*. MHC mRNA (0.9 µg) was translated in the wheat germ cell-free system at a KCl concentration of 160 mM. Radioactive protein was precipitated once at low ionic strength with 0.25 mg carrier chick embryonic myosin and redissolved in 0.5 ml 0.5 M KCl, 0.03M Tris-HCl pH 8.3. Digestion with 2 µg trypsin (trypsin : myosin ratio, *1 : 125 w/w) was carried out at 20°C for 5 min and was stopped by addition of 4 µg soyabean trypsin inhibitor (2 : 1 w/w ratio to trypsin). Following dialysis for 16 hours against 0.01 M phosphate pH 6.5 the samples were centrifuged at 20,000 g for 5 min. The supernatant of crude heavy meromyosin (HMM) was dialyzed against an excess of 8 M urea, 0.01M sodium phosphate pH 7.0, 1.0% SDS, 1.0% b-mercaptoethanol at 25°C overnight. The pellet of crude light meromyosin (LMM) was disolved directly in 0.5 ml of the dialysis medium. 100 µl samples were electrophoresed, stained and scanned, using a Joyce-Loebl densitometer as described previously (John, 1974, 1976a) before slicing and determination of radioactivity. Marker protein run on parallel gels were: myosin heavy chain (200,000), bovine serum albumin (68,000) and cytochrome C (13,000)

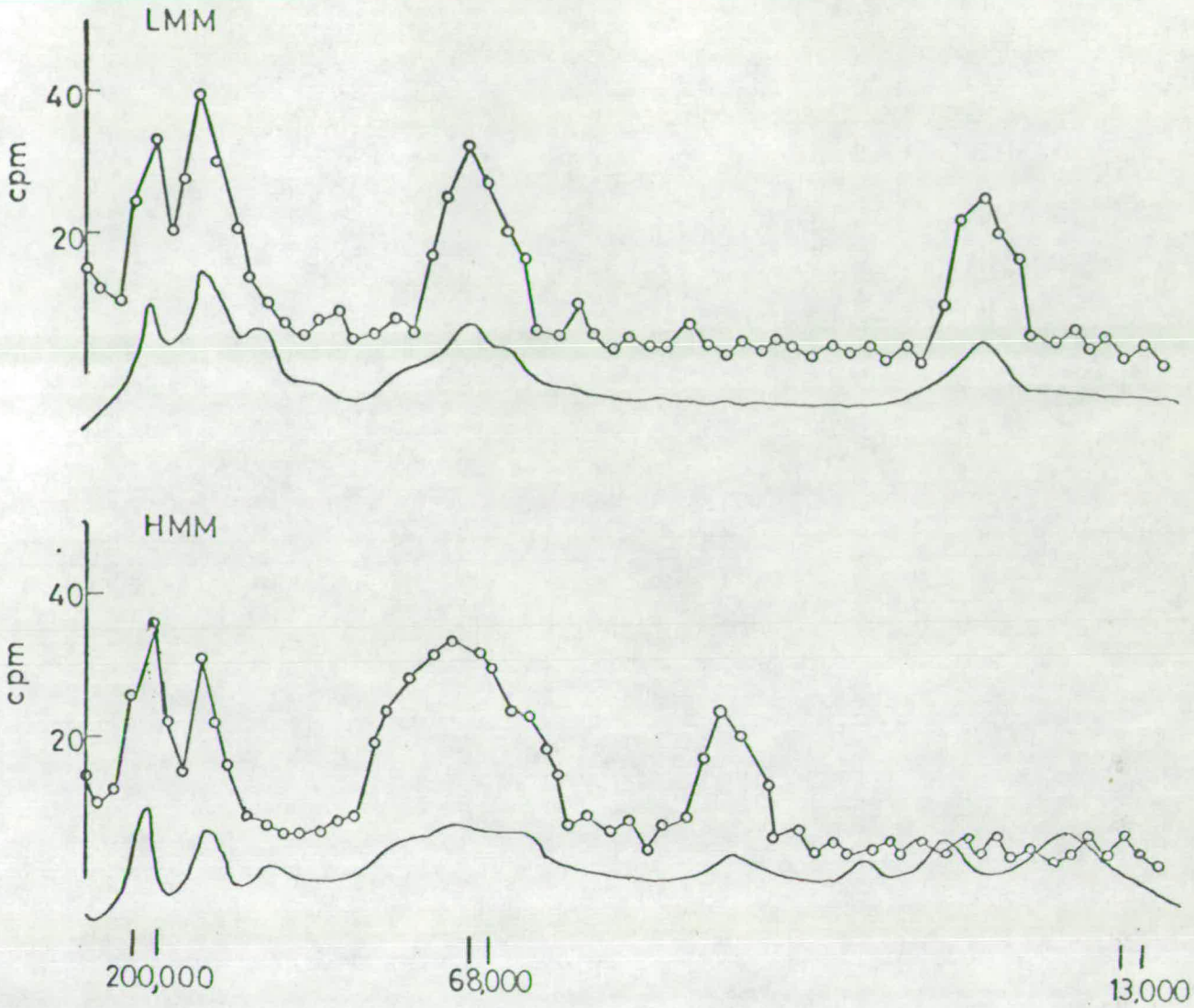

Figure 17.

Plate 10

Analysis on SDS-polyacrylamide gel of the total protein from 14 day old chick embryo leg muscle. Homogenization of the tissue and electrophoresis were carried out as described for the estimation of myosin heavy chain in tissue culture (page 34). The positions of myosin heavy chain and actin are indicated.

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