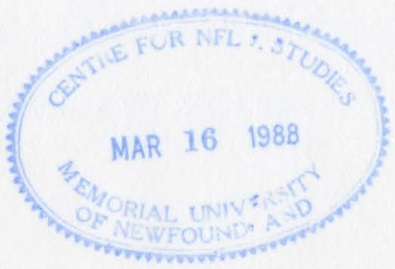
REGULATION OF TROPONIN C SYNTHESIS IN CHICKEN CARDIAC MUSCLE CELL CULTURES

SUMAN BALA MALHOTRA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REGULATION OF TROPONIN C SYNTHESIS IN CHICKEN CARDIAC MUSCLE CELL CULTURES

By


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ABSTRACT

Most of the contractile muscle protein genes have been extensively examined to understand the mechanisms controlling tissue-specific gene expression in skeletal muscle. However, very little is known at the molecular level about another major contractile muscle protein, troponin C. Furthermore, very little is known about the regulation of gene expression in cardiac muscle. In this study, slow troponin C has been examined to understand its differentiation and regulation in chicken cardiac myocytes.

Dot blot hybridisation and restriction endonuclease analysis indicated that the chicken slow troponin C gene was present as a single copy. Although, troponin C is a very conserved protein, the conservation at the DNA level is not known. When DNA from various species was compared by Southern blot hybridisation with a quail troponin C cDNA probe, I found that quail troponin C DNA was non-homologous to DNA from other classes.

The regulation of troponin C protein synthesis in cardiac myocyte cell cultures was examined in this study. Cultured myocyte cells were pulse-labelled with ^{35}S-methionine, at different days after plating, and the protein synthesis levels were compared by two-dimensional gel electrophoresis. At the same time, total cellular RNA was extracted and troponin C mRNA levels were examined by Northern blot analysis, using a quail troponin C cDNA probe. The results showed that mRNA accumulation closely paralleled the synthesis

of troponin C, suggesting its principal mode of regulation to be transcriptional. However, the decrease in the level of troponin C polypeptide synthesis was somewhat greater than the observed decrease in the mRNA level, suggesting a possible translational control of gene expression. Furthermore, when troponin C protein synthesis levels, at different days of plating, were compared with those of actin and tropomyosin, the results indicated that unlike skeletal contractile muscle proteins, these cardiac contractile muscle proteins were not co-regulated.

The possible association of the transcriptionally active troponin C gene of cardiac myocytes with a nuclear sub-structure called nuclear matrix was also examined. Results indicated that the transcriptionally active troponin C gene was not preferrentially enriched in the nuclear matrix fraction.

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CHAPTER 1

INTRODUCTION

The fundamental problem in the study of development and differentiation is the way in which a multitude of specialised cells and tissues are formed from the fertilised egg cell. At the molecular level, this becomes a question of the way in which different genes are expressed in different cells. Muscle cells have been examined in a variety of ways to understand the mechanisms controlling the tissue-specific gene expression. Developmentally distinct stages are readily recognised by monitoring morphological and biochemical properties of muscle cells and the conversion of one stage to another stage can be mimicked under the controlled conditions of tissue culture (Yaffe, 1968).

Determined, but non-differentiated, muscle cells are known as myoblasts and during differentiation, myoblasts fuse to form elongated, multinucleated myotubes. Other morphological changes following differentiation include the appearance of electrically excitable membranes containing nicotinic acetylcholine receptors (Kidokoro, 1973), and the appearance of myofibrils and specialised membrane systems (Klier, Schubert & Heinemann, 1977). During differentiation, the rate of synthesis of most of the contractile muscle proteins increases (Garrels, 1979). Also the key metabolic enzymes such as creatine phosphokinase and myokinase are induced (Shainberg, Yagil & Yaffe, 1971).

On the other hand, some proteins, eg. the C1 series of collagen-like pro-

teins, are made in myoblasts and not in myotubes (Garrels, 1979), suggesting the existence of complex patterns of gene expression.

This study was carried out to gain further insight into the mechanisms of tissue-specific expression of genes. Specifically, the regulation of synthesis the of the muscle protein troponin C in cardiac muscle cells is studied here.

1.1. Structure and function of cardiac troponin C

Vertebrate cardiac muscle fibres contain striated filaments similar in appearance to those of skeletal muscle fibres. The major contractile proteins in striated muscle are localised in regular arrays of thick and thin filaments. The thick filaments are composed of myosin molecules and the thin filaments contain actin, tropomyosin and the troponin complex. The sliding of thick and thin filaments relative to each other generates force. The energy for this force is generated by the hydrolysis of ATP by actomyosin ATPase. Calcium ions regulate this process through the troponin complex in the following way. When a muscle is stimulated, calcium ions released from the sarcoplasmic reticulum bind to troponin inducing a conformational change which causes the heads of the myosin in the thick filaments to attach and detach cyclically from the actin-containing thin filaments. This cyclic attachment and detachment causes the filaments to move relative to each other resulting in contraction of of the muscle fibre (Mannherz & Goody, 1976). Troponin is composed of three subunits. Troponin C is the Ca^{2+}-binding subunit, troponin T is the

tropomyosin binding subunit and troponin I inhibits actomyosin ATPase.

The amino acid sequence of troponin C from the fast skeletal muscle of mammals, birds, and reptiles is known and as is that from bovine cardiac muscle (Wilkinson, 1980). It is a highly acidic protein (isoelectric point 3.7, Murakami & Uchida, 1984) with a molecular weight of 18,000. The three-dimensional structure for fast skeletal troponin C contains four Ca^{2+} binding loops, each loop being bound by two α-helices, and the whole structure is stabilised by interactions between specific hydrophobic side chains in the helices (Romero-Herrera, Castillo & Lehmann, 1976). Sites 1 and 2 are the low affinity Ca^{2+} binding sites while sites 3 and 4 are the high affinity Ca^{2+} and Mg^{2+} binding sites. However, the cardiac troponin C contains only three Ca^{2+}-binding sites - two high affinity Ca^{2+} and Mg^{2+} sites and only one low affinity Ca^{2+} specific site. The site 1 in this protein has lost the ability to bind Ca^{2+}. The two high affinity Ca^{2+} / Mg^{2+} binding sites are in the C-terminal region of cardiac muscle troponin C (Leavis & Kraft, 1978).

It is believed that the C-terminal part of cardiac troponin C is responsible for the interaction with troponin I. Binding of Ca^{2+} to troponin C breaks the links between troponin I and tropomyosin, and tropomyosin moves to the active position with the troponin complex remaining attached to tropomyosin via the troponin T subunit (Barskaya & Gusev, 1981).

1.2. Muscle protein isoforms and their genes

The contractile proteins of muscle are members of large families of related isoproteins. In some cases the different members of an isoprotein family are so closely related that amino acid sequences differ in only small regions. For example, within the actin family found in birds and mammals (of which there are six members) there are only 27 amino acid differences, from the total of 375, between the most widely diverged members, and the difference between muscle isoforms of actins can be due to as few as three amino acid changes (Obinata, Reinach, Bader, Masaki, Kitani & Fischman, 1984). Despite this similarity in primary structure, each member of a contractile protein family is synthesized in a tissue-specific and developmental stage-specific manner. Proteins synthesized in embryonic skeletal and cardiac muscles are qualitatively different from the adult forms (Dhoot & Perry, 1980; Toyota & Shimada, 1981). During skeletal muscle differentiation, both the light and heavy chains of myosin undergo complex, stage-specific programmes of isoform switching (Lowey, Benfield, LeBlanc & Waller, 1983).

Similarly, the components of troponin complex exist in multiple molecular forms. Troponin I has three isoforms, which are expressed in fast, slow and cardiac muscle fibres (Dhoot, Gell & Perry, 1979). Troponin T also has isoforms that are specific in most animals for for slow, fast and cardiac muscle, and chicken fast skeletal muscle itself has three isoforms, whereas troponin C has only two isoforms, one of which is expressed in fast muscle and the other

in slow and cardiac muscle (Hastings & Emerson, 1982a, Wilkinson, Moir & Waterfield, 1984).

In rat muscle there are three different mRNAs which code for different α isoforms of tropomyosin. The synthesis of these three different mRNAs is regulated in a tissue and developmental stage-specific manner. The α_1- and α_2-tropomyosin mRNAs are produced only in striated muscle, whereas a third α-form seems to be expressed predominantly in smooth muscle (Ruiz-Opazo, Weinberger, & Nadal-Ginard, 1985). The α_1-tropomyosin mRNA is induced early in muscle development and accumulates to high levels in the adult, whereas the α_2-tropomyosin mRNA is present only in adult muscle. All three α-isoforms have been shown to be the product of a single gene.

Some cardiac-specific contractile isoproteins are also expressed as embryo-specific isoforms in skeletal muscle, eg. mouse actin (Minty, Alonso, Caravatti, & Buckingham, 1982), rat myosin heavy chain (Whalen, Sell, Eriksson & Thornell, 1982), chicken myosin heavy chain (Sweeney, Clark, Umeda, Zak & Manasek, 1984), chicken troponin T and troponin C (Toyota & Shimada, 1981). For each example above, immunological criteria and peptide mapping criteria show that the isoprotein present in adult heart is indistinguishable from that present transiently in embryonic skeletal muscle. Also, Cooper and Ordhal (1984), using Northern blot analysis to quantitate mRNA, have demonstrated that the gene encoding one of the chicken troponin T isoforms is also expressed transiently during the early stages of *in vivo* muscle development.

Chicken embryonic and adult cardiac muscle both synthesize the slow troponin T isoform, whereas the embryonic skeletal muscle synthesizes both slow and fast troponin T. During development the slow troponin T synthesis is repressed and only the fast troponin T is produced in the adult skeletal muscle (Cooper & Ordhal, 1984). Thus, in the development of cardiac and skeletal muscles, a single gene appears to be governed by two different regulatory programmes.

Isoforms of many contractile proteins are encoded by multigene families and the diversity of these proteins is further augmented by alternative RNA splicing of individual gene transcripts. This has been documented for myosin heavy chain (Rozek & Davidson, 1983), myosin light chains 1 and 3 (Nabeshima, Fuji-Kuriyama, Muramatsu & Ogata, 1984; Robert, Daubas, Akimenko, Cohen, Garner, Guenet & Buckingham, 1984), α-tropomyosin (Ruiz-Opazo *et al.* 1985), and troponin T (Breitbart, Nguyen, Medford, Destree, Madhavi & Nadal-Ginard, 1985; Cooper and Ordhal, 1985).

The *Drosophila* genome contains a single copy of the myosin heavy chain gene, which however produces three different transcripts whose levels vary during development. These RNAs differ primarily in their patterns of splicing at the 3' end (Rozek & Davidson, 1983). Similarly, in chicken and mouse, skeletal muscle myosin light chains (MLC), MLC_{1F} and MLC_{3F} are encoded by a single gene. This gene has two transcription initiation sites from which two precursor RNAs are transcribed. These RNAs are processed by different

modes of splicing to form mRNAs encoding distinct light chain proteins. In rat a single gene which codes for three different α-tropomyosin mRNAs has an unusual organization with common and exchangeable exons that can be spliced in several different combinations (Ruiz-Opazo *et al.* 1985). Similarly, the rat fast skeletal muscle troponin T gene encodes 10 different isoforms by differential splicing of its mRNA (Breitbart *et al.* 1985). Also, in chicken a single cardiac troponin T gene produces adult and embryonic isoforms by alternative splicing (Cooper and Ordhal, 1985).

1.3. Regulation of muscle gene expression

Regulation of gene expression during muscle cell differentiation has been extensively examined in skeletal muscle cells. Myoblasts isolated from developing skeletal muscles initially proliferate in culture. Later, they stop dividing and enter a phase of cell fusion which culminates in the formation of multinucleated myotubes. Early studies reported that fusion of myoblasts and the expression of muscle proteins can take place when mRNA synthesis was inhibited with actinomycin D prior to fusion (Yaffe & Dym, 1972). This observation was further supported by the presence of large amounts of myosin heavy chain and actin mRNAs in muscle cells in the form of untranslated mRNA-protein complexes. The RNA isolated from these complexes was translatable in cell-free translation systems (Heywood, Kennedy & Bester, 1975; Bag & Sarkar, 1975; Bag & Sarkar, 1976). This was interpreted to suggest that the

regulation of myosin synthesis during myoblast differentiation is controlled at the level of translation of mRNAs. However, by measuring the accumulation of eight major contractile mRNAs using cloned cDNAs from quail skeletal muscle myoblasts, Devlin and Emerson (1979) demonstrated that all of these mRNAs accumulate in a closely coordinated manner during quail skeletal myoblast differentiation. In addition, changes in the levels of these translatable contractile protein mRNAs occur at the same time as the changes in the relative rate of synthesis of the proteins occur *in vivo*. Thus the major control of contractile protein synthesis during quail skeletal muscle myoblast differentiation appears to be at the transcriptional level. Caravatti, Minty, Robert, Montaraas, Weydert, Cohen, Daubas and Buckingham (1982) studied expression of mRNA for contractile proteins during differentiation in a mouse skeletal muscle cell line using cDNA probes. Cloned cDNA probes for actins, myosin heavy chain, and myosin light chains were employed in Northern blotting experiments with total cellular poly(A)$^+$ RNA extracted from cultures at different times after plating. At the same time, the myoblast cultures were pulse-labelled and newly synthesized proteins were analysed by two dimensional gel electrophoresis. Their results also show that mRNA accumulation closely parallels the synthesis of the corresponding muscle protein, and that there is no major accumulation of non-translatable muscle RNA prior to differentiation. It is now clear that differentiation of skeletal muscle cells is regulated at the level of transcription, that is, by selectively turning on the

genes for contractile proteins during differentiation. It is not known however, whether translational control plays a role during isogene switching.

On the other hand, Ouellette, Croall, Van Ness & Ingwell (1982), detected evidence for post-transcriptional control of some muscle mRNAs in rat cardiac muscle. Based on the analysis of the products of cell-free translation directed by mRNAs isolated from the cardiac muscle of fetal, neonatal and adult mice, and by the kinetics of mRNA - cDNA hybridisation, they found that the diversity and complexity of total cellular poly(A)$^+$ RNA is unchanged in development. However, polypeptides synthesized from fetal heart muscle mRNA were abundant in cell-free translation products even though the corresponding proteins were not synthesized in the intact heart muscle of similar age. Therefore, even though the control of gene expression during myogenesis of skeletal muscle is primarily transcriptional, post-transcriptional control may play a large role during the myogenesis of cardiac muscle.

1.4. Tissue culture system for cardiac muscle differentiation

As mentioned above, the availabilty of immortal skeletal muscle cell lines of rat and mice (Yaffe & Dym, 1972), and the primary cultures of chicken (Holtzer, Rubenstein, Fellini, Yeoh, Chi, Burnbaum & Okayama, 1975),and quail (Hastings & Emerson, 1982a) have facilitated the study of the regulation of gene expression during muscle differentiation. However, there are no

immortal cell lines available to study differentiation during cardiac myogenesis. Chick cardiac myoblasts differentiate very early in embryonic development and start beating spontaneously and rhythmically at about the 10-somite stage (Johnstone, 1925, sited in Chacko, 1973). The myocardium at that stage consists of a pure population of muscle cells, which increases in size considerably thereafter by addition of new cardiac muscle cells, vascular smooth muscle cells, endothelial cells and interstitial fibroblasts (DeHaan, 1967; Manasek, 1968). Hence, in contrast to skeletal muscle cells, embryonic cardiac cells synthesize DNA and undergo mitosis even after the formation of cross-striated myofibrils. Using single muscle cells isolated from 5-day chick embryonic heart, Chacko (1973) has shown that these cells incorporate labelled thymidine into their nuclei and divide frequently. This suggests the regulation of DNA and contractile muscle protein synthesis in cardiac muscle cells is different from that observed in skeletal myoblasts. Functional cardiac muscle cells withdraw from the mitotic cycle after a few divisions. It has been suggested that cardiac muscle cells might employ some regulatory mechanism controlling the number of divisions after the first appearance of contractile proteins (Chacko, 1973).

It is rather difficult to study the mechanisms involved in the regulation of protein synthesis during cardiac myogenesis, since cardiac cells differentiate very early in embryonic development. Also, problems are often encountered in obtaining a pure population of myocytes using primary culture since they

comprise only about 40% by number and 65-70% by mass of the total cell population in chick embryonic hearts from 10-18 days of age (DeHaan, 1967). However, it is possible to obtain an enriched population of myocytes by selectively inhibiting fibroblast cell division using 5-bromodeoxyuridine (Chacko & Joseph, 1974).

Some work has been done on the differentiation of precardiac mesodermal cells using 5-bromodeoxyuridine (Chacko and Joseph, 1974). Although the myofibrils do not appear in the precardiac cells until stage 9, the precardiac mesodermal cells of stage 7 embryos treated with bromodeoxyuridine are capable of forming a beating heart. It is possible that this synthesis of myofibrilar proteins is taking place by translation of stored preformed mRNAs. This has been further supported by the observation that precardiac mesodermal cells from stage 7, 8 and 9 embryos treated with actinomycin D, to inhibit RNA synthesis, are capable of differentiating into beating hearts (Chacko & Joseph, 1974).

1.5. Chromatin structure and gene expression

The role of specific conformations of DNA sequences in the intact nucleus during gene expression has been extensively studied (Weintraub, 1985). Genes which are transcriptionally active, or which have the potential for rapid expression in response to the appropriate stimuli, have been shown to exhibit a preferential susceptibility to cleavage by nucleases. Micrococcal nuclease and

DNase 1 preferentially attack newly replicated DNA and nucleosome linker DNA in transcriptionally active chromatin (Weintraub & Groudine, 1976). Also, the 5' regions of active genes are hypersensitive to DNase 1 and S1 nuclease (Mathis, Oudet & Chambon, 1980).

Another difference between active and inactive genes is that active genes are often hypomethylated. Experiments with mouse retroviruses have provided strong evidence that methylation can suppress transcription (Jaenisch & Jahner, 1984). Methylation or hypomethylation is probably a means of stabilising the structure of a gene in an inactive or active state respectively. Thus, the hypomethylated state might be a prerequisite for transcriptional competence. It has been suggested that prior to gene activation, trans-acting molecules may trigger a gene to switch from an inactive to a competent state. This switch may involve alterations of chromatin structure as well as demethylation.

Another difference between active and inactive genes is the association of active genes with a nuclear substructure known as the nuclear matrix. Linear DNA is organized into a series of loops, of 50-200 kb, by attachment to a subnuclear structure called the nuclear-matrix, scaffold, or cage (Berezney & Coffey, 1974). The structure remaining after extraction of nuclei with salt to remove histones and other proteins is the nuclear matrix. Morphologically, this structure is composed of three elements - a peripheral nuclear lamina, an internal protein network, and a residual nucleolar structure. The peripheral

lamina is the best studied component and is composed of three major proteins of molecular weight 60,000 to 70,000 daltons (Gerace, Blum & Blobel, 1978).

The nuclear matrix is believed to be the key site in the regulation of gene activity. The attachment sites of the supercoiled loops to the nuclear matrix are the sites at or near which DNA replication is believed to take place (Pardoll, Vogelstein & Coffey, 1980). DNA polymerase has also been shown to be associated with the nuclear matrix (Smith & Berezney, 1980). The functional replisomes are associated with the matrix and the DNA is replicated as it passes through these matrix-associated complexes (Pardoll *et al.* 1980).

The nuclear matrix also selectively binds steroid receptor complexes. The high affinity steroid-binding sites are tightly associated with the nuclear matrix of hormone responsive tissues and these binding sites are diminished following hormonal depletion of the animal (Colvard & Wilson, 1984). Transcription complexes are also bound to the nuclear matrix, and nascent RNA is closely associated with the matrix (Jackson, McCready & Cook, 1981). The processing of precursor mRNAs has also been shown to be associated with the nuclear matrix. RNA splicing occurs within hnRNP particles and involves a series of low molecular weight RNAs (lmw RNAs). These lmw RNAs remain associated with the nuclear matrix (Maundrell, Maxwell, Puvion & Scherrer, 1981), suggesting that splicing might take place at or near the nuclear matrix. The ribosomal RNA precursors are also associated with the nuclear matrix and this association progressively decreases as rRNA processing proceeds (Rennie,

Bruchovsky & Cheng, 1983). Actively transcribing genes are also selectively associated with the nuclear matrix (Robinson, Small, Idzerda, McKnight & Vogelstein, 1983). Ovalbumin and conalbumin genes are associated with the nuclear matrix of the oviduct, which is actively synthesizing egg white proteins, but not with the nuclear matrix of other organs where egg white proteins are not being synthesized (Ciejek, Tsai & O'Malley, 1983). Similarly, Cook, Lang, Hayday, Lania, Fried, Chiswell & Wyke (1982), have shown that polyoma and avian sarcoma viral genes in transformed cells are closely associated with the nuclear matrix. Also, globin genes from chicken erythrocytes (Hentzen, Rho, & Bekhor, 1984), ribosomal rRNA genes of the rat liver cells (Pardoll & Vogelstein, 1980), vitellogenin II genes of the chicken liver cells (Jost & Seldran, 1984), and the SV40 sequences in several SV40-transformed 3T3 cell lines (Nelkin, Pardoll & Vogelstein, 1980) have been shown to be nuclear-matrix associated.

A detailed analysis of the chicken ovalbumin gene in oviduct tissue has shown that only the transcribed regions of a defined DNase 1 sensitive domain are associated with the matrix. However, the non-transcribed regions at both ends of the DNase 1 sensitive domain are not associated with the matrix (Ciejek *et al.* 1983). Therefore, there is no general structural correlation between the DNase 1 sensitive chromosomal domain and the nuclear matrix, even though a good correlation between the transcribed region of the gene and the attachment to the nuclear matrix exists.

Recently, however, it has been suggested that the preferential association with the matrix in the cell types in which the gene is actively transcribed is an artifact of isolation (Mirkovitch, Mirault & Laemmli, 1984). It is suggested that the exposure of nuclei to high salt would induce precipitation of proteins or transcriptional complexes onto the matrix as well as allow sliding of DNA attachment sites. Mirkovitch *et al.* (1984) have developed a low-salt procedure of nuclear lysis to investigate nuclear matrix association of histone and heat shock genes in *Drosophila.* They do not find any preferential attachment of these genes in the matrix fraction obtained by this method. However, it could also be argued that the low ionic strength extraction destroys the attachment of transcriptionally active DNA to the nuclear matrix. Low ionic strength induces the depolymerisation of intermediate filaments of the vimentin type, and also, F-actin, a structural component of nuclear matrix, is unstable in hypotonic solutions (Razin, Yarovaya & Georgiev, 1985). Using isotonic conditions, Jackson and Cook (1985) also demonstrated the transcriptionally active gene to be associated with the nuclear substructure and this association was partly disrupted by hypotonic treatments. Recently, Keppel (1986) showed that nuclear matrices prepared from HeLa nuclei in both high or low-salt buffers are enriched in actively transcribing ribosomal RNA genes. Therefore, the argument for the use of high-salt methods for the study of nuclear matrix is still strong. The high-salt method of nuclear matrix extraction is still very useful even if the enrichment of active genes in the matrix fraction is an

artifact, since it will be useful in examining the proteins of transcription complexes.

1.6. Objectives

The objective of this research project was to examine how the expression of genes for contractile proteins is regulated in cardiac muscle. In the studies reported here, an enriched primary culture of chick cardiac muscle cells was used to examine the regulation of troponin C synthesis. The activation of the cardiac troponin C gene was examined following plating of myocyte cell cultures. The correlation between troponin C synthesis and its cytoplasmic mRNA levels was studied to examine whether cardiac troponin C synthesis is regulated only at the level of transcription. Furthermore, an attempt was made to examine the difference in conformation between the expressible troponin C genes of cardiac muscle and the repressed troponin C genes of hepatocyte cells. In this context the nuclear matrix association of the transcriptionally active troponin C gene of cardiac myocytes was studied.

Troponin C has been studied very little at the molecular level. Although troponin C is a conserved protein, it is not known to what extent the conservation extends to the gene level. By Southern blot hybridisation we have found that this conservation did not extend to the gene level, especially between avian and mammalian genomes.

One of the first steps in elucidation of troponin C regulation was to compare the levels of the troponin C protein and its mRNA. At different days after plating, the protein synthesis levels of cultured myocyte cells were compared by two-dimensional gel electrophoresis and troponin C mRNA levels were compared by Northern blot analysis. The results showed that mRNA accumulation closely paralleled the synthesis of troponin C, indicating the principal mode of regulation was transcriptional. The possible attachment of the transcriptionally active troponin C gene of cardiac myocytes to the nuclear matrix was also studied. The results showed that this gene was not preferentially enriched in the nuclear matrix.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell isolation and culture

2.1.1. Cardiac myocyte culture

Primary cultures of cardiac myocytes were obtained by modification of a procedure for the isolation of rat heart cells (Claycomb, 1979). Hearts were removed from 14-day old chick embryos, cleaned of attached tissues, minced and washed in chilled Hank's balanced salt solution (GIBCO). The minced tissue pieces were dissociated by multiple 15 min enzyme treatments with 0.1% collagenase (Worthington class II) and 0.1% hyaluronidase (Sigma type IV) in Hank's balanced salt solution at 37 °C. The supernatants from the first two enzymatic treatments were discarded (they contained mainly epithelial cells). The enzymatic treatments were repeated 8-10 times for 20 hearts and the supernatants were decanted into centrifuge tubes containing an equal volume of chilled Hank's balanced salt solution. The cells were collected by centrifugation for 5 min at 200 rpm, washed in cold nutrient medium, and re-spun. This low speed centrifugation helped to eliminate fibroblasts and red blood cell contamination (Coetzee, Van der Westhuyzen & Gevers, 1977; Claycomb, 1979). Myocytes sediment more quickly and the supernatant contains mainly

fibroblasts and red blood cells. The final pellet was resuspended in 0.5 ml of nutrient medium and dispensed into gelatin-coated petri dishes containing nutrient medium supplemented with 0.1 mM 5-bromo-2'-deoxyuridine. Cultures were incubated in a humidified incubator at 37 ° C in an atmosphere of 95% air and 5% CO_2. After 24 h of culture, the culture medium was replaced with fresh medium without 5-bromo-2'-deoxyuridine. When the culture was to be maintained for long periods, the medium was replaced every third day.

2.1.2. Chicken hepatocyte culture

Livers were removed from 14-day old chick embryos, cleaned of attached tissues, minced and washed in chilled Hank's balanced salt solution to wash out blood. Cells were dissociated from the minced tissue by multiple 10 min trypsinizations, at 37 ° C in 0.05% trypsin (GIBCO) in calcium-and magnesium-free Hank's balanced salt solution. The cells were collected in cold minimum essential medium (MEM) containing 50% horse serum. The diluted suspension was centrifuged at 750 rpm for 10 min in a clinical centrifuge. The cells were resuspended in MEM containing 50 U ml^{-1} penicillin (5,000 U ml^{-1}), 50 μg ml^{-1} streptomycin (5,000 μg ml^{-1}), and 5% fetal bovine serum and plated into gelatin-coated petri dishes containing the same medium. The cultures were maintained at 37 ° C in a humidified incubator under in an atmosphere of 95% air and 5% CO_2. Initially the medium was changed 24 h after plating and thereafter every third day.

2.1.3. Nutrient medium

The culture medium employed to maintain the cardiac myocytes was CMRL 1066 liquid medium with glutamine (GIBCO) containing 10% horse serum (GIBCO), 3% fetal bovine serum (GIBCO), 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, and 40 μg ml^{-1} bovine pancreatic insulin (26.2 I.U. per mg, Sigma) (Nath, Shay & Bollon, 1978). The hepatocytes were cultured in MEM containing 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 5% fetal bovine serum.

2.1.4. Gelatin coating of petri dishes

The tissue culture petri dishes were coated with 100 μg ml^{-1} of gelatin (Hauschka, 1972). 10 mg ml^{-1} stock solution of gelatin was prepared by gentle heating in distilled water. The solution was filter-sterilised and stored at 4 °C until used. The petri dishes were flooded with a large volume of dilute gelatin and the protein was allowed to adsorb to the surface for about 1.5 h at 37 °C. After aspirating the excess, plates were rinsed with sterile distilled water and used immediately.

2.2. 2-Dimensional electrophoretic analysis of proteins

2.2.1. Labelling and extraction of proteins in cell culture

Cardiac myocytes were cultured in 25-mm petri dishes for the determina-

tion of the relative rates of synthesis of proteins. After various intervals, cells were washed with Hank's balanced salt solution and incubated for 1 h in MEM without methionine, supplemented with 2.5% dialysed horse serum. Cultures were then labelled for 4 h with 400 μCi of [^{35}S] methionine (1000 Ci/mmol; Amersham) in 0.75 ml of methionine-free MEM containing 2.5% dialysed horse serum. After labelling, cultures were washed with Hank's balanced salt solution and incubated for 30 min in non-radioactive medium. Cells were washed twice with Hank's balanced salt solution and twice with 10 mM Tris-HCl, pH 7.5. Cells in 150 μl of 9.5 M urea, 2% NonidetP-40 (NP-40, Sigma) (w/v), 5% 2-mercaptoethanol and 2% pH 3 to 10 ampholines (v/v, Bio Rad) were scraped from the petri dishes and disrupted using a Dounce homogeniser. The cellular homogenates were incubated with 10 μg each of DNase 1 and RNase A for 30 min and stored at -70 ° C until used.

2.2.2. Electrophoretic analysis of proteins

Two-dimensional gel analysis of labelled proteins was performed using the technique described by O'Farrell (1975), with a few modifications. Total cell homogenates each containing the same amount of acid-precipitable counts (about 500,000) were run on 110 mm long and 1.5 mm thick isoelectric focusing gels containing 2% (v/v) ampholines pH 3 to 10. To make 5 ml of gel mixture (about 0.5 ml of gel mixture per gel tube), 2.75 g of urea was dissolved in 0.75 ml of 30% acrylamide stock (28.38% acrylamide and 1.62 % bisacrylam-

ide), 1 ml of 10% (w/v) NP-40 in water, 0.90 ml of water and 0.25 ml of ampholines pH range 3 to 10 (stock concentration 40%). When the urea was completely dissolved, the solution was degassed under vacuum for about 30 min. Then 6 μl of 10% ammonium persulphate and 3.5 μl of TEMED was added and the solution was loaded into the glass tubes using a syringe with a long narrow-gauge hypodermic needle. The gels were overlayed with n-butanol and allowed to set overnight. They were then placed in a standard tube gel electrophoresis chamber, n-butanol removed from their surfaces, and rinsed twice with the sample overlay solution containing 9 M urea and 1% ampholines pH 3 to 10. The surfaces were covered with 10 μl of sample overlay solution and the tubes were filled with 0.02 M NaOH. The lower reservoir (anode) was filled with 0.01 M H_3PO_4 and the upper reservoir (cathode) filled with 0.02 M NaOH which was extensively degassed to remove CO_2. The gels were prerun according to the following schedule: (a) 200 volts for 15 min; (b) 300 volts for 30 min; (c) 400 volts for 30 min. The power was turned off and the samples were loaded onto the gels using micro syringes (Hamilton Co.). After the samples were loaded the gels were run at 1000 volts for 5.5 h. Cold water was circulated in the outer jacket of the electrophoresis chamber to avoid overheating.

After isoelectric focusing, the gels were extruded from the tubes and equilibrated in SDS sample buffer [10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% SDS and 0.0625 M Tris-HCl, pH 6.8] at room

temperature for 1 h, and stored at -70 ° C.

Electrophoresis in the second dimension was carried out on a 12.5% polyacrylamide (prepared from 33.8% stock solution containing 33.5% acrylamide and 0.3% bisacrylamide) slab gels (110 mm long and 1.5 mm thick) in the presence of 0.1% (w/v) SDS as described by Dreyfuss, Adam & Choi (1984). The isoelectric focusing gels were thawed at room temperature and were placed on the slab gels. Agarose solution (50 mg agarose dissolved in 2.5 ml of 0.5% Tris-HCl, pH 6.8, 0.4% SDS and 7.5 ml of water and 0.01% bromophenol blue, held at about 50 ° C) was used to keep the gels in place on the slab gel notches. The agarose was allowed to set for about 10 min and the gels were run according to Dreyfuss *et al.* (1984), at 120 volts until the dye front reached the bottom of the gel. The slab gels were treated for fluorography with PPO-DMSO (NEN, Laskey and Mills, 1975), dried and exposed to Kodak X-Omat films at -70 ° C. In order to obtain a relative quantitation of the radioactivity in proteins, specific spots were sliced out of the dried gel and the radioactivity was measured in Toluene-Omnifluor (NEN).

For the identification of newly synthesized troponin C, a preparation of troponin C from bovine cardiac muscle (a gift from Dr. C. Kay, University of Alberta, Edmonton) was added to the extract prior to electrophoresis. The gels were fixed in 10% TCA and stained for 15 min in 0.2% commassie blue dissolved in 10% acetic acid and 25% isopropanol. Destaining was carried out in 10% acetic acid and 25% isopropanol for 1 h.

2.3. Preparation of RNA

All glassware was baked at 180 ° C for 12-18 h to avoid RNase contamination. Polyallomer centrifuge tubes, magnetic stirrers and rubber policeman were treated with 0.2% diethyl pyrocarbonate (Fedorcsak & Ehrenberg, 1966, cited in Maniatis, Fritsch, & Sambrook, 1982) followed by autoclaving. All solutions were prepared using baked glassware and autoclaved distilled water, and were filtered through 0.22 μm Millipore filter membrane (Type GS) and reautoclaved. Gloves were worn at all stages during the preparation of materials and solutions used for the isolation of RNA and during all manipulations involving RNA.

Cells were washed twice with ice-cold phosphate-buffered saline (PBS, 1 x PBS = 145 mM NaCl, 80 mM $Na_2HPO_4.2H_2O$ and 1.5 mM KH_2PO_4, pH 7.4). Washed cells were lysed in a 150 mm petri dish for 5 min with 3 ml lysis buffer containing 25 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 5 mM $MgCl_2$, 0.5% (w/v) NP-40, 200 μg ml^{-1} heparin, 50 μg ml^{-1} cyclohexamide, 5 mM dithiothreitol. The lysed cells were scraped from the petri dishes using a rubber policeman and homogenised in a loose-fitting homogeniser to recover the cytoplasmic remnants from the nuclei which were then quickly removed by centrifugation at 20,000 x g for 15 min at 0-4 ° C. The pellet containing intact nuclei and other cellular debris was discarded. SDS was added to the supernatant to a concentration of 1% (w/v) and the samples were heated at 65 ° C for 5 min. This cytoplasmic extract was used either for total cytoplasmic RNA or

poly(A)$^+$ RNA extraction.

2.3.1. Isolation of total cytoplasmic RNA

Total RNA was isolated from the cytoplasmic extract using phenol and chloroform. An equal volume of phenol (equilibrated in 10 mM Tris-HCl, pH 7.5) was added to the cytoplasmic extract, mixed and heated for 5 min at 65 °C in a 50 ml polypropylene tube. This was followed by addition of an equal volume of chloroform. After mixing well, the organic and aqueous phases were separated by centrifugation at 2,000 rpm at room temperature for 10 min. The upper aqueous phase was re-extracted with phenol and chloroform, followed by an extraction with an equal volume of chloroform. Sodium acetate, pH 5.0, was added to the aqueous phase to a final concentration of 0.2 M, and the nucleic acid was precipitated with 2.5 volumes of pre-chilled Aristar ethanol (BDH) and stored at -20 °C for at least 12 h. The precipitated RNA was collected by centrifugation in a Beckman SW 27 rotor at 20,000 rpm for 3 h at 4 °C. The precipitates were washed twice in 66% ethanol, dried under vacuum, and dissolved in water. The composition of the RNA solution was adjusted to 20 mM Tris-HCl, pH 7.8 and 10 mM $MgCl_2$, and the RNA treated with 10 μg ml^{-1} of RNase-free DNase (Worthigton) for 30 min at 37 °C. The RNA was phenol and chloroform extracted to remove the DNAse and precipitated with 2.5 volumes of ethanol in the presence of 0.2 M sodium acetate as above. The RNA precipitates were dissolved in water, and stored at

-70 ° C.

2.3.2. Isolation of poly(A)$^+$ RNA

The poly(A)$^+$ containing mRNA was isolated by affinity chromatography using oligo-(dT)-cellulose (Aviv & Leder, 1972). The original procedure was modified to obtain quick purification of mRNA (Bag & Pramanik, unpublished observations).

Briefly, 0.3 g of oligo-(dT)-cellulose, type III (Collaborative Research Inc.) was packed in a column (100 mm long and 7 mm internal diameter), washed with 10 volumes of 0.5 M NaOH, then with water and finally with the binding buffer, containing 25 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.5% SDS. The concentration of NaCl in the cytoplasmic extract was adjusted to 0.5 M which was then heated at 65 ° C for 2 min to inactivate any residual nuclease activity and then chilled in ice. The cytoplasmic extract (10-15 ml/column) was warmed to dissolve the SDS and passed through the column three times. The column was then washed with binding buffer until the optical density of the effluent at 260 nm became zero. The poly(A)$^+$ RNA was then eluted with 3 ml of water. The eluted RNA was adjusted to the composition of binding buffer, heated at 65 ° C, chilled on ice, warmed to dissolve SDS and again passed through the oligo-(dT)-cellulose column. The bound RNA was then eluted with water, adjusted to 0.5% SDS, 0.2 M sodium acetate and precipitated with 2.5 volumes of pre-chilled ethanol, and stored at -20 ° C for at least

12 h. The precipitated RNA was collected by centrifugation in a Beckman ultra-centrifuge (Beckman SW 27 Rotor, 20,000 rpm, 3 h, 0-4 °C), washed twice in 66% ethanol, dried under vacuum, and stored at -70 °C. This high speed centrifugation was necessary to obtain 90-100% recovery of the small amount of RNA precipitate.

This procedure for the purification of poly(A)$^+$ RNA was preferred to the more conventional phenol extraction procedure (Feramisco, Smart, Burridge, Helfman & Thomas, 1982) since total recovery of poly(A)$^+$ mRNA was achieved. Bag and Pramanik (unpublished results) have shown that the above method gives absolute recovery of the poly(A)$^+$-containing RNA. They further extracted the unbound fraction from the oligo-(dT)-cellulose column with a mixture of phenol and chloroform (1:1). The RNA was precipitated and the poly(A)$^+$ mRNA was selected again by oligo-(dT)-cellulose chromatography, but no further binding of mRNA to oligo-(dT)-cellulose was observed. This indicates that the non-phenolic affinity chromatography in the presence of SDS is sufficient for isolating polyA - containing translatable RNAs from tissue culture cells.

2.4. Preparation of nuclear matrix

Nuclear matrices were prepared by the method described by Cook *et al.* (1982) with a few modifications. To monitor the extent of digestion of nuclear DNA with restriction enzymes, the cells were labelled for 24 - 48 h with 3 μCi

ml^{-1} of [^{3}H–methyl] thymidine. The labelled cells were washed twice with phosphate-buffered saline (PBS). The cells in PBS were scraped from the petri-dishes, spun down and resuspended in PBS (about 3 x 10^6 cells ml^{-1}) and were lysed on ice for 15 min in 3 volumes of 1.33 x lysis buffer (1 x lysis buffer = 1.95 M NaCl, 10 mM Tris-HCl, pH 8.0, 100 mM EDTA, and 0.5% Triton X-100). The lysed cells (about 1 x 10^6 cells ml^{-1}) were spun (Beckman SW 27 Rotor, 4,500 rpm, 1 h, 0-4 ° C) through 4 ml of 7.5% sucrose in lysis buffer onto a 3 ml shelf of 30% sucrose in the lysis buffer. The white aggregates of nucleoids were removed from the interface between the 7.5 and 30% sucrose, diluted with 20 volumes of Eco RI (BRL) restriction enzyme buffer without NaCl (100 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$) to adjust the NaCl concentration to 100 mM and incubated with 5 U ml^{-1} of Eco RI at 37 ° C for various times to obtain partial digestion. The nuclear matrices so prepared were centrifuged at 8,000 rpm for 40 min, and the pellets dissolved in 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% Sarkosyl and 100 μg ml^{-1} proteinase K (Sigma) and incubated at 56 ° C for about 2 h. Samples of dissolved pellet and supernatants were counted to determine the percentage of total DNA remaining associated with the nuclear matrix. The pellet DNA was purified by phenol-chloroform (1:1) extraction (Maniatis *et al.* 1982), 100mM NaCl added and precipitated with pre-chilled ethanol. The phenol-chloroform step was omitted for the supernatant DNA purification due to the large volumes of supernatant, the NaCl concentration was adjusted to 100 mM and was ethanol

precipitated directly. The precipitates were centrifuged and dissolved in TE, pH 7.5 (TE = 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA). The DNA preparations were treated with 100 μg ml^{-1} of RNase A at 37 °C for 30 min, followed by 100 μg ml^{-1} of proteinase K at 37 °C for 30 min before further extraction with phenol-chloroform (1:1) and ethanol precipitated. The deproteinised DNAs were dissolved in TE buffer, pH 7.5 and were digested to completion with Eco RI restriction enzyme for Southern analysis..

2.5. Isolation of genomic DNA

Quail, herring gull, duck, pigeon and yeast DNAs were generously provided by Dr. W. Davidson. Human and hamster DNAs were provided by Dr. H.B. Younghusband, and the mycoplasma DNA was provided by Dr. P. Barnsley.

Chicken and rat genomic DNAs were prepared from embryonic heart, liver or skeletal muscle tissue. Tissue was washed in cold PBS and homogenised using a Teflon-coated homogeniser. The homogenate was suspended in four volumes of PBS, and pronase and SDS to the final concentrations of 1 mg ml^{-1} and 1% (w/v) were added respectively. This was incubated at 55 °C till it became clear (about 2-3 h). It was subjected to several rounds of phenol:chloroform (1:1) extraction until no precipitated material was found at the interface. The aqueous layer was either dialysed overnight with 3 changes of TE, pH 7.5, or was ethanol precipitated. The precipitate was spun down

and dissolved in TE, pH 7.5. The DNA was treated with 100 μg ml^{-1} of RNase A at 37 °C for 30 min followed by incubation with 0.5% SDS and 250 μg ml^{-1} of pronase for a further 30 min. It was once again phenol-chloroform extracted and was either dialysed or ethanol precipitated as above.

2.6. Electrophoresis, blotting and hybridisation

2.6.1. Gel electrophoresis of DNA and Southern transfer

The restriction endonuclease digested DNA samples were subjected to horizontal gel electrophoresis in 0.8% agarose in Tris-borate buffer (0.089 M Tris, 0.089 M boric acid and 0.002 M EDTA) containing 0.5 μg ml^{-1} of ethidium bromide to stain DNA fragments. Samples were loaded into sample wells, beneath a covering layer of electrode buffer and electrophoresed to the desired extent as indicated by migration of tracking dye. The gels were then removed and the DNA was visualised and photographed using a Polaroid MP-3 land camera over a UV-transilluminator (Chromato-vue transilluminator, mode C-61).

The DNA fragments were transferred to a nylon membrane *Zetabind* (AMF-CUNO Co.) by the transfer technique described by Southern (1975), with a few modifications. The DNA was partially hydrolysed by acid depurination (by soaking the gel twice in 0.25 M HCl for 15 min at room temperature). The gel was rinsed with water to remove excess acid and the DNA was dena-

tured by soaking the gel in a solution of 1.5 M NaCl and 0.5 M NaOH twice for 15 min each time, with constant shaking. It was then neutralised by soaking in a solution of 1.0 M Tris-HCl, pH 7.5, and 1.5 M NaCl twice for 15 min each time, with constant shaking.

The *Zetabind* membrane was pre-wet by first boiling it twice in distilled water, then soaking it for 30 min in 20 x SSC (1 x SSC = 0.15 M sodium chloride, and 0.015 M sodium citrate, pH 7.0). The DNA was transferred onto the membrane using 20 x SSC as the transfer buffer (Southern, 1975; Maniatis,*et al.* 1982). For transfer, the gel was placed on a sponge platform covered with 2 sheets of Whatman 3MM paper, in a reservoir containing 20 x SSC. The *Zetabind* membrane was carefully positioned on the gel. Two additional sheets of Whatman paper were then placed in an uninterrupted contact over the top of *Zetabind* membrane and a thick layer of paper towels supporting a light weight was added to complete the apparatus. The transfer was carried out overnight at 4 °C. At this point restaining the gel with ethidium bromide in Tris-borate buffer followed by examination under UV light confirmed the complete transfer of DNA from the gel.

After transferring the DNA, the membrane was washed twice, for 15 min each, with 2 x SSC at room temperature to remove any agarose. It was then sandwiched between 3MM sheets and baked in a vacuum oven at 70 °C for 2 h. The membrane was then washed in 0.1 x SSC and 0.5% SDS at 60 °C for 1 h to minimize the background on subsequent hybridisations.

2.6.2. Gel electrophoresis of RNA and Northern transfer

Before electrophoresis, RNA was denatured by heating at 65 °C for 5 min in 10 mM sodium phosphate, pH 7.4 containing 50% formamide, 2.2 M formaldehyde and 0.5 mM EDTA. After denaturation the RNA sample was cooled to room temperature and 1/5 volume of 5 x sample buffer (5 x sample buffer = 0.5% SDS, 0.025% bromophenol blue, 25% glycerol, 25 mM EDTA) was added. RNA was fractionated by electrophoresis in 1.5% agarose gels containing 10 mM sodium phosphate, pH 7.4 and 1.1 M formaldehyde at 30V (Meinkoth & Wahl, 1984).

Following electrophoresis the gels were stained with 33 μg ml^{-1} acridine orange in 10 mM sodium phosphate, pH 6.7, for 10 min. These were then destained for 60 min in sodium phosphate, pH 6.7 with 3 changes of buffer, and photographed over the UV-transilluminator.

The RNA was transferred onto nitrocellulose filters (0.45μm, Schleicher & Schuell). Prior to transfer, the gels were soaked for 5 min in several changes of water, and the nitrocellulose filters were pre-wet by soaking them first in distilled water and then in 20 x SSC. The RNA was transferred directly onto the nitrocellulose filters without further manipulations using the procedure similar to that described above for Southern transfers. Following transfer, the filters were washed in 2 x SSC for 5 min to remove residual agarose, and baked at 80 °C under vacuum for 2 h in a vacuum oven.

2.6.3. Spot blotting of DNA and RNA

Spot blotting of nucleic acids was carried out essentially by the method of Wahl (1983), using an Schleicher & Schuell Minifold II. Briefly, for DNA slot blotting and dot blotting, DNA was denatured with 0.2 M NaOH for 15 min at room temperature. The denatured DNA was neutralised by the addition of 0.4 M Tris-HCl, pH 7.5, and 5 x SSC. Loading the DNA onto nitrocellulose was essentially the same as described Wahl (1983). To slot blot the RNA, it was denatured by the addition of 3 volumes of 6.15 M formaldehyde and 10 x SSC at 65 ° C for 15 min, and applied directly to the nitrocellulose filter through the minifold. The filter was placed between sheets of 3MM paper and baked in a vacuum oven for 2 h.

2.6.4. Densitometer scanning of gel negatives and autoradiographs

In some nuclear matrix preparations, the concentration of DNA was too low to be measured by optical density. This was overcome by densitometer scanning the negatives of the photographs from agarose gel electrophoresis. Briefly, a small volume of DNA solution and 3 serially-diluted samples of a DNA solution of known concentration were electrophoresed in an agarose gel. After the electrophoresis the gels were photographed and the DNA lanes in the negatives were scanned and traced onto Bond paper, using a Corning 750 Scanning Densitometer. The area under each scan was cut out and weighed. Using the standard DNAs a graph of weight vs DNA concentration was plot-

ted, and the concentration of DNAs in question was read from the graph. The lanes in some autoradiographs were also scanned to compare band intensities, and the area under each peak was calculated.

2.6.5. Nick-translation, prehybridisation and hybridisation

The plasmids cC111 and cC118 were generously provided by Dr. Charles P. Emerson (University of Virginia). Both plasmids were isolated from a cDNA library of quail myofiber mRNA sequences and the cDNAs were inserted at the Pst1 site of the vector pBR322. The cC111 plasmid contains a 420-bp insert complementary to the mRNA for the slow form of troponin C and the cC118 contains a 590-bp insert complementary to the skeletal muscle α-actin mRNA (Hastings & Emerson, 1978).

[^{32}P]-labelling of plasmid DNA was performed using a nick-translation kit and 3000 μCi/mol [α- ^{32}P]dCTP (Amersham) according to the procedure of Rigby, Dieckmann, Rhodes and Berg (1977). The reaction mixture contained 5 μl nucleotide buffer, (containing 1.5 μM each of dATP, dGTP and dTTP in buffer solution); 5 μl of enzyme solution (containing 2.5 units of DNA polymerase 1 and 50 pg DNase 1 in a buffer solution); 100 μCi of [^{32}P]dCTP; and 0.5 μg of plasmid DNA, in a total volume of 50 μl. The reaction was carried out at 15 ° C for 70 min and then stopped by addition of EDTA to a concentration of 10 mM and SDS to the final concentration of 1.5%. The products of the reaction mixture were diluted to 100 μl with STE (STE = 10mM Tris-

HCl, pH 8.0, 1 mM EDTA and 0.1 M NaCl) and applied to a 1 ml spun down column of Sephadex G-50 (medium) (Sigma) previously equilibrated with the same buffer (Maniatis *et al.* 1982). The effluent from the spun down column was collected and the specific activity of the probe was calculated, based on the Cerenkov counting of 1 μl aliquots of probe. The probe was denatured by heating at 100 ° C for 10 min and cooling it quickly.

Prehybridisation and hybridisation conditions for the Zetabind and nitrocellulose membranes were different and each of them are described below:

Zetabind membrane:

Membranes were sealed in polythene bags and prehybridised overnight (8 to 24 h) at 42 ° C in a prehybridisation mix (20 ml) containing 5 x SSC, 5 x Denhardt's reagent (1 x Denhardt's = 0.02% each of ficoll, M.Wt. 400,000, polyvinylpyrollidine and BSA), 5% dextran sulphate, 0.01 M sodium phosphate, pH 6.7, 100 μg ml^{-1} sonicated denatured salmon testes DNA, and 50% deionised formamide (deionised by shaking with a mixed bed resin- Bio Rad AG 501-X8 - until the pH was neutral). The hybridisations were usually carried out for 2 to 3 days at 42 ° C in a hybridisation mix (7 - 10 ml) containing 5 x SSC, 0.05 x Denhardt's reagent, 10% dextran sulphate, 0.01 M sodium phosphate, pH 6.7, 100 μg ml^{-1} of denatured, sonicated salmon testes DNA, 50% formamide and 2 x 10^6 cpm/ml of [^{32}P]-labelled nick-translated, denatured probe DNA.

After hybridisation, the hybridised blots were removed and the filters were washed under the following conditions: (a) Twice for 20 min each in 2 x SSC at room temperature; (b) twice for 20 min each in 2 x SSC at 42 ° C; and (c) twice for 20 min each in 0.1 x SSC and 0.1% SDS at 60 ° C. Slightly moist filters were wrapped in Saran Wrap and exposed to Kodak X-Omat RP films for various times.

To reuse the blots, the probe was removed by washing the membrane in 0.4 M NaOH for 30 min at 42 ° C with constant shaking. The membrane was then washed 4 times, 15 min each with 0.1 x SSC, 0.5% SDS, 0.2 M Tris-HCl, pH 7.5 at 42 ° C with constant shaking.

Nitrocellulose filters:

These filters were prehybridised overnight at 42 ° C in a prehybridisation mix (20 ml) containing 5 x SSPE (1 x SSPE = 0.18 M sodium chloride, 10 mM sodium phosphate, pH 7.7 and 1 mM EDTA), 5 x Denhardt's reagent, 0.1% SDS, 200 μg ml^{-1} of denatured, sonicated salmon testes DNA and 50% deionised formamide.

Hybridisation was carried out at 42 ° C in 7 - 10 ml of 5 x SSPE, 1 x Denhardt's reagent, 10% dextran sulphate, 0.1% SDS, 100 μg ml^{-1} of denatured salmon testes DNA, 2 x 10^6 cpm/ml of denatured [^{32}P]dCTP-labelled nick-translated probe DNA and 50% formamide for 2-3 days.

After hybridisation, the filters were washed according to the following conditions: (a) 3 times, 15 min each with 2 x SSC and 0.1% SDS at room temperature and (b) 2 times, 15 min each with 0.1 x SSC and 0.1% SDS at 50 ° C. After washing, the slightly moist filters were wrapped in Saran Wrap and exposed to Kodak- X-Omat RP films for various times.

The probe was removed by washing in 0.005 M Tris-HCl, pH 8.0, 0.002 M EDTA, and 0.05% sodium pyrophospate at 65 ° C for 2 h.

CHAPTER 3

RESULTS

3.1. Behaviour of cardiac muscle cells in culture

It was important to isolate cardiac myocytes free from fibroblasts and in high yield, and to maintain them for long periods for the nuclear matrix and *in vivo* protein synthesis experiments. During initial trials, hearts from chick embryos of various ages - 10, 12, 14 and 17 days were used to isolate and culture the myocyte cells. Hearts from 10 and 12 day old embryos were quicker to process due to less connective tissue and the cultures were least contaminated with fibroblasts, but the yield of myocyte cells was very low when plated. Hearts from 17 day old embryos gave a very high yield of cells, but the majority of them were of fibroblast origin. Hearts from 14 day old embryos gave the best yield of myocyte cells with the minimum fibroblast contamination.

Within 10 hours of plating, spontaneous contraction could be observed in some of the myocyte cells, and after 24 hours of plating, most of the myocyte cells were beating spontaneously. Chicken cardiac myocyte cells adopted heterogeneous shapes once they became attached to the petri dishes. During the first 3 days in culture, most of the cells became flattened, often with branches. These branches joined to the neighbouring branches and formed

clumps (Fig. 3.1). Cells in a clump contracted synchronously. No significant difference was noticed in the frequency of contraction amongst cells of different morphology. During observation most of the cardiac muscle cells cease contraction temporarily. Cells quiescent at one examination may later beat spontaneously and rythmically.

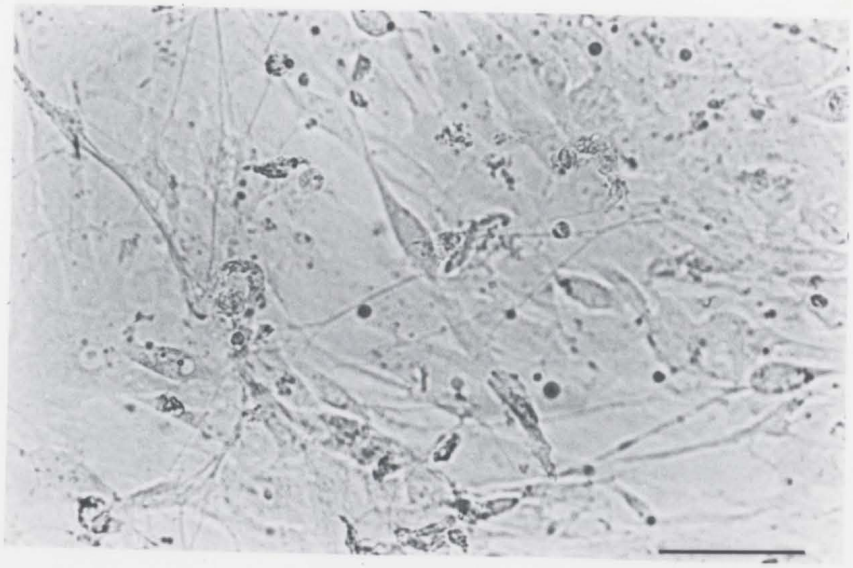
The chicken cardiac muscle cells synthesize DNA as deduced by their ability to incorporate [^{3}H]-thymidine into their DNA. This property was important later for the nuclear matrix experiments for measuring the percentage of DNA associated with nuclear matrix. However, it was not clear whether these cells underwent mitosis. Counting of cells before plating would have given erroneous results due to the presence of fibroblast cells and counting after plating was made difficult due to the myocytes forming clumps. However, indirect evidence from the nuclear matrix experiments, where the yield of nuclear matrix DNA was very low from cells 2 days post-culture compared to cells 4 or 7 days post-culture, suggests that at least some of these cells undergo cell divisions before withdrawing from the mitotic cycle.

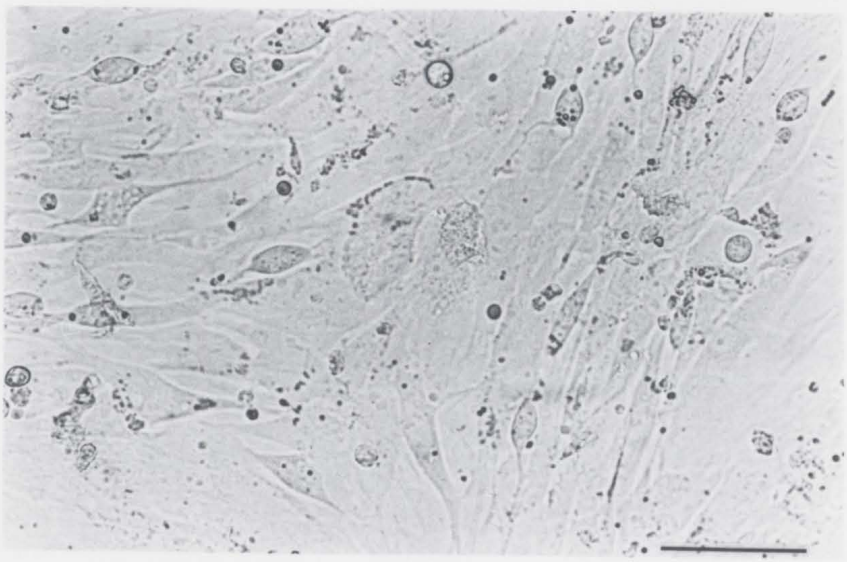
3.2 Gene copy number and restriction endonuclease analysis of chicken troponin C gene

To examine the difference between transcriptionally active and inactive genes, it is important to know whether the gene is present in a single or multiple copies. There is no information available regarding the copy number of the

Fig. 3.1. Phase-contrast micrographs of contractile aggregates of cells.

The photographs illustrate the appearance of beating cells when attached to substratum, 3 days after plating. The cells adopt heterogenous shapes and branch. Branches adjoin the neighbouring branches and form clumps. Bar indicates 15 μm.





Phase-contrast photomicrographs of contractile aggregates of myocyte cells.

slow troponin C gene. The number of slow troponin C genes in chicken DNA was assayed by dot blot hybridisation and restriction endonuclease analysis. The dot blot analyses were chosen to obtain at least an approximate estimate of gene number. However, when complemented with the restriction analysis data, it was possible to estimate the gene copy number more accurately.

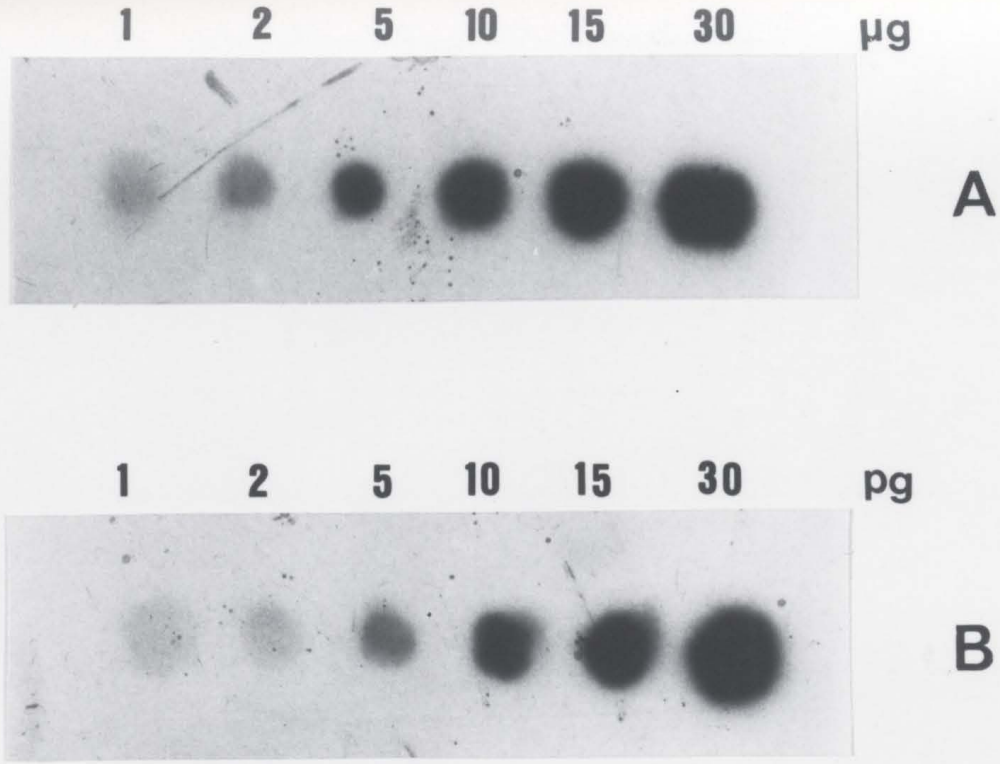
Denatured chicken genomic DNA was serially diluted and applied to a nitrocellulose filter. Denatured, unlabelled cCIII plasmid (containing quail troponin C cDNA insert) was also serially diluted and applied to the nitrocellulose filter in separate lanes. The filters were hybridised with [^{32}P]-labelled cCIII probe and exposed to X-ray film. The results of the experiment are shown in figure 3.2. Dots were excised from the filter and the amount of radioactivity hybridised to each dot was measured by scintillation counting in toluene-omniflour (Table 3.1). The troponin C gene copy number was calculated by assuming there is 2.5 pg of DNA/chicken genome (Fasman, 1978) and 1 pg of DNA is equivalent to 9.1 x 10^8 bp. The calculations are shown in the legend to figure 3.2. Results suggest that there are 2.6 copies of slow troponin C gene per haploid genome.

The dot blot analyses results were complemented with restriction endonuclease analysis data. Chicken genomic DNA was digested with a number of restriction enzymes and electrophoresed on agarose gels. Following transfer of DNA fragments (Southern, 1975) to a *Zetabind* membrane (AMF-CUNO), it was hybridised with [^{32}P]-labelled cCIII probe. The hybridisation patterns in

Fig. 3.2. Quantitation of chicken troponin C gene copy number by dot blot hybridisation.

1, 2, 5, 10, 15 and 30 μg of chicken genomic DNA (A) and 1, 2, 5, 10, 15 and 30 pg of cCIII plasmid DNA (B) were dot blotted directly onto nitrocellulose. DNA was hybridised to 1 $x10^7$ cpm of [^{32}P]-labelled cCIII probe (specific activity 2 x 10^8 cpm/μg) as described in 'Materials and Methods'. Filters were exposed to X-ray film for 20 h. Spots were excised and their radioactivity was measured (see table 3.1).

The radioactivity of 5, 10 and 15 μg of genomic DNA was equivalent to that of 5, 10 and 15 pg plasmid DNA. 1 μg of chicken DNA corresponds to 4 x 10^5 diploid genomes equivalents (Davidson & McIndoe, 1949, cited in Fasman, 1978) and from spot counts they are equivalent to 1 pg (9.1 $x10^8$ bp) of cDNA insert. Therefore, there is 2244 bp of DNA/genome homologous to the troponin C cDNA insert. Since the cDNA insert is 420 bp, there are 5.2 copies of troponin C gene per diploid genome.



Quantitation of chicken troponin C gene copy number by dot blot hybridisation.

Table 3.1

Dot blot hybridisation assay to determine troponin C gene copy number

Genomic DNA		cCIII DNA	
Amount μg	Counts Annealed	Amount pg	Counts Annealed
1	387.0	1	441.6
2	402.6	2	444.4
5	510.2	5	488.5
10	799.6	10	734.2
15	1142.6	15	1041.6
30	1579.6	30	2142.2

Blank 407.0

Blank represents background count (it is an average of four values). The above 'Counts Annealed' are the actual counts, these have not been substracted from the blank.

each case were quite simple (Fig. 3.3). Eco RI and Bgl II restriction endonuclease digests produced two cCIII hybridisable bands (molecular weight 4.8 and 2.3, and 6.2 and 1.8 kbp respectively, Fig. 3.3.II, lanes B and E), whereas, Pst I and Hind III generated only one band each (2.8 kbp and 8.0 kbp respectively, lanes lanes C and D). However, quail troponin C cDNA restriction analysis reveals that it has two Pst I sites (Hastings and Emerson, 1982). It is possible that the low molecular weight bands were lost in my restriction analysis or the two Pst I sites present in quail troponin C cDNA are absent in the chicken troponin C gene. Hae III, a restriction enzyme with only four nucleotide specificity, generated one relatively low molecular weight band of 300 bp (lane F). Kpn I produced three bands (6.5, 4.6 and 2.2 kbp), whereas Xho I and Bgl I, both partial digests, produced two bands each (9.5 and 8.6 kbp and 7.6 and 6.8 kbp respectively, lanes G, H and I). Sal I (lane J), also a partial digest, produced one band of 8.6 kbp. (All molecular weights were calculated by comparing the migration of bands with migration of linear plasmid bands and plotting the molecular weights on a log scale against the migration distance.)

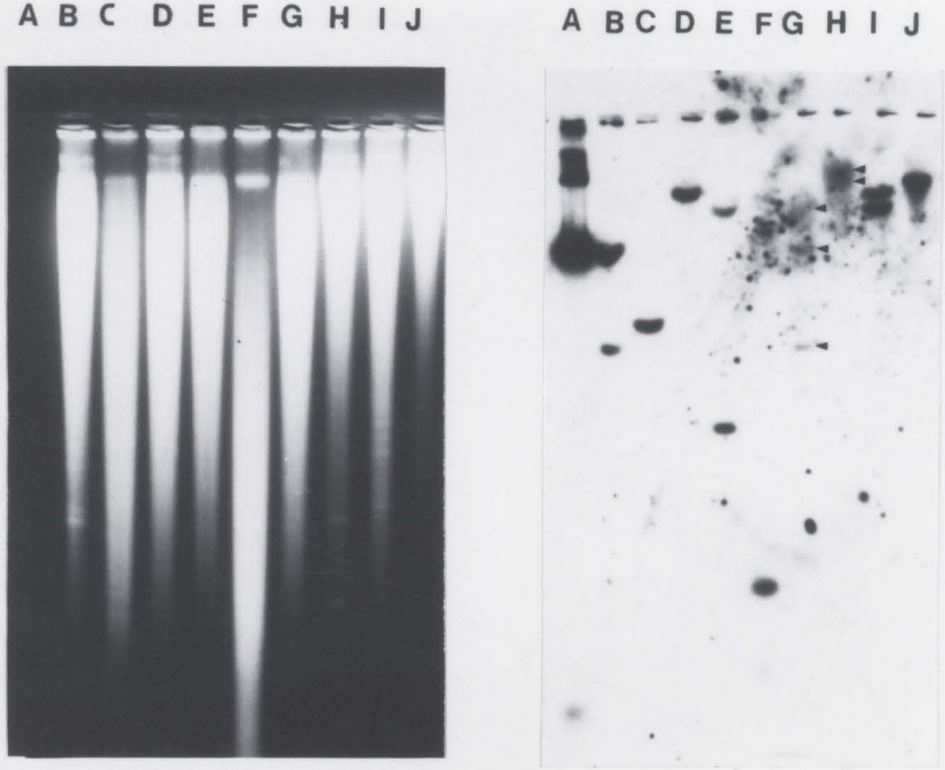
Both the restriction analysis and the dot blot analyses suggest that the troponin C gene is present in low copy number. Furthermore, the restriction analysis data showing a simple pattern of restriction fragments, hybridisable to the cCIII probe, suggests that it is likely there is a single copy of the slow troponin C gene per haploid genome.

Fig. 3.3. Analysis of genome complexity for the troponin C gene.

15 μg of chicken genomic DNA was digested with restriction endonuclease (B) Eco RI, (C) Pst 1, (D) Hind III, (E) Bgl II, (F) Hae III, (G) Kpn I, (H) Xho I, (I) Bgl I, and (J) Sst I, and resolved on a 0.8% agarose gel. The DNA was transferred to a *Zetabind* membrane and hybridised to 5 x 10^6 cpm of [^{32}P]-labelled cCIII probe (specific activity 2 x 10^8 cpm/μg) as described under 'Materials and Methods'. In lane A, 100 pg of cCIII plasmid was run as a control. (H),(I) and (J) are partial digestions. I. Photograph of the ethidium bromide stained agarose gel. II. The corresponding autoradiograph following 18 days' exposure.

I

II



Analysis of genome complexity for the troponin C gene.

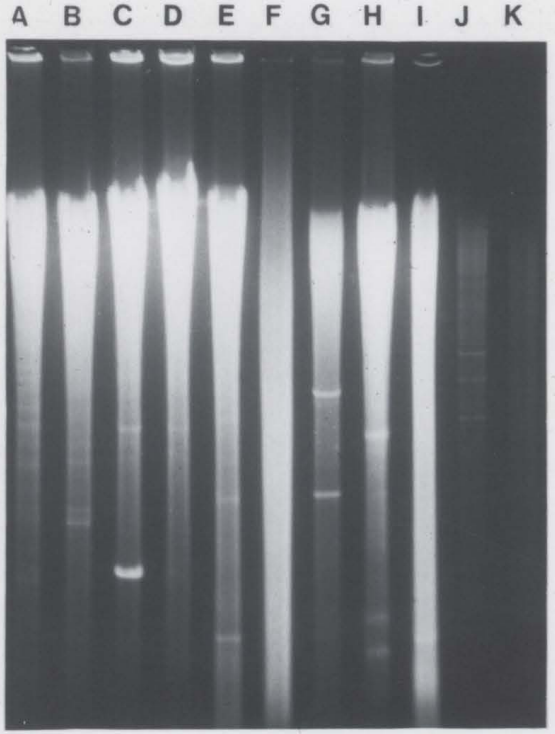
3.3. Evolutionary conservation of genomic troponin C sequences

Chicken embryonic hearts were used as a source of cardiac myocytes for studying troponin C gene expression. Chick embryos were easy to obtain and a good yield of myocyte cells was achieved (section **3.1**). It was, therefore, important to test whether the non-homologous, quail troponin C cDNA could be used efficiently to detect chicken troponin C gene transcripts. I also compared hybridisation of DNA from different species with the cCIII clone to carry out a preliminary study of the evolutionary conservation of genomic troponin C sequences. Avian, mammalian, piscine, yeast and mycoplasma DNA were digested with Eco RI restriction enzyme, electrophoresed on an agarose gel and transferred to a *Zetabind* membrane. The membrane was hybridised with [^{32}P]-labelled cCIII probe and the resulting autoradiograph is shown in figure 3.4(II). The quail troponin C cDNA probe hybridised to all the bird DNAs examined, although the hybridisation signals with pigeon and herring gull DNAs were very weak. In each case, two bands were observed. However, there were no hybridisation signals from the mammalian, piscine, yeast or mycoplasma DNAs. The faint band of 4.6 kbp seen in lane J with yeast DNA is an artifact produced by hybridisation of [^{32}P]-labelled pBR322 DNA with yeast DNA. This was verified by removing the probe from the blot and re-hybridising with [^{32}P]-labelled pBR322 DNA. This gave the same band pattern on the yeast DNA as produced by cCIII. Hence, although troponin C has

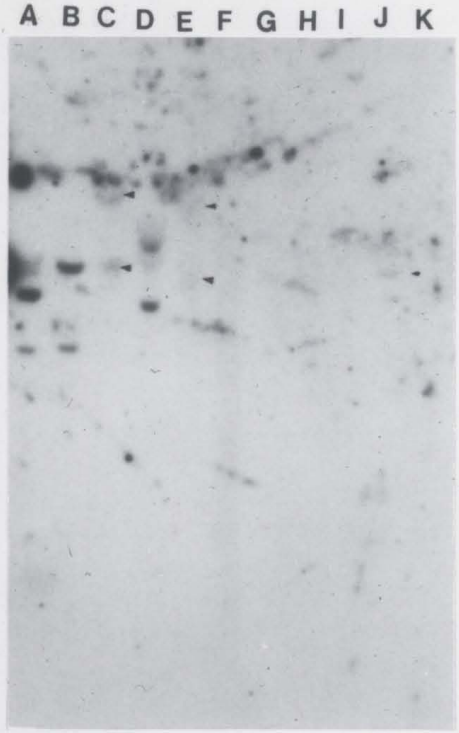
Fig. 3.4. Evolutionary conservation of genomic troponin C sequences.

Genomic DNAs from various sources were digested with Eco RI and resolved on an 0.8% agarose gel. The DNA was transferred to a *Zetabind* membrane and hybridised to 2 x 10^7 cpm of [^{32}P]-labelled probe (specific activity, 2 x 10^8 cpm/μg) as described under 'Materials and Methods'. Lanes A - K : digested DNAs from (A) Quail, (B) chicken, (C) pigeon, (D) duck, (E) herring gull, (F) herring sperm, (G) rat cell line L6, (H) hamster, (I) human, (J) yeast, (K) mycoplasma. In lanes A - I 15 μg of DNA, in lane J, 1 μg, and in lane K, 0.4 μg of DNAs were run. I. Photograph of ethidium bromide stained agarose gel. II. The corresponding autoradiograph following 2 days' exposure.

I



II



Evolutionary conservation of genomic troponin C sequences.

considerable amino acid sequence homology amongst different classes, especially between birds and mammals, it seems that this homology does not extend considerably to the gene level.

3.4. Comparison of mRNA accumulation and myocyte protein synthesis during cell culture

There is very little information available on the regulation of cardiac troponin C synthesis in myocyte cells in tissue culture. The regulation of slow (cardiac) troponin C synthesis in cardiac myocytes in culture was examined in this study. These studies were important for two reasons. The first was to find out whether the level of troponin C mRNA paralleled the level of troponin C polypeptide synthesis. This information would highlight any possible role of post-transcriptional control in the regulation of troponin C gene expression. Secondly, in order to examine whether the troponin C gene of cardiac myocytes is preferentially associated with the nuclear matrix, it is important to know the time of maximal transcription.

3.4.1. Myocyte protein synthesis

Initial experiments were carried out to determine the synthesis of slow troponin C by cardiac myocytes in a tissue culture environment. For this analysis, cardiac myocyte cell cultures were labelled for 4 hours with [^{35}S]-methionine at different times after plating. Total cellular extracts were

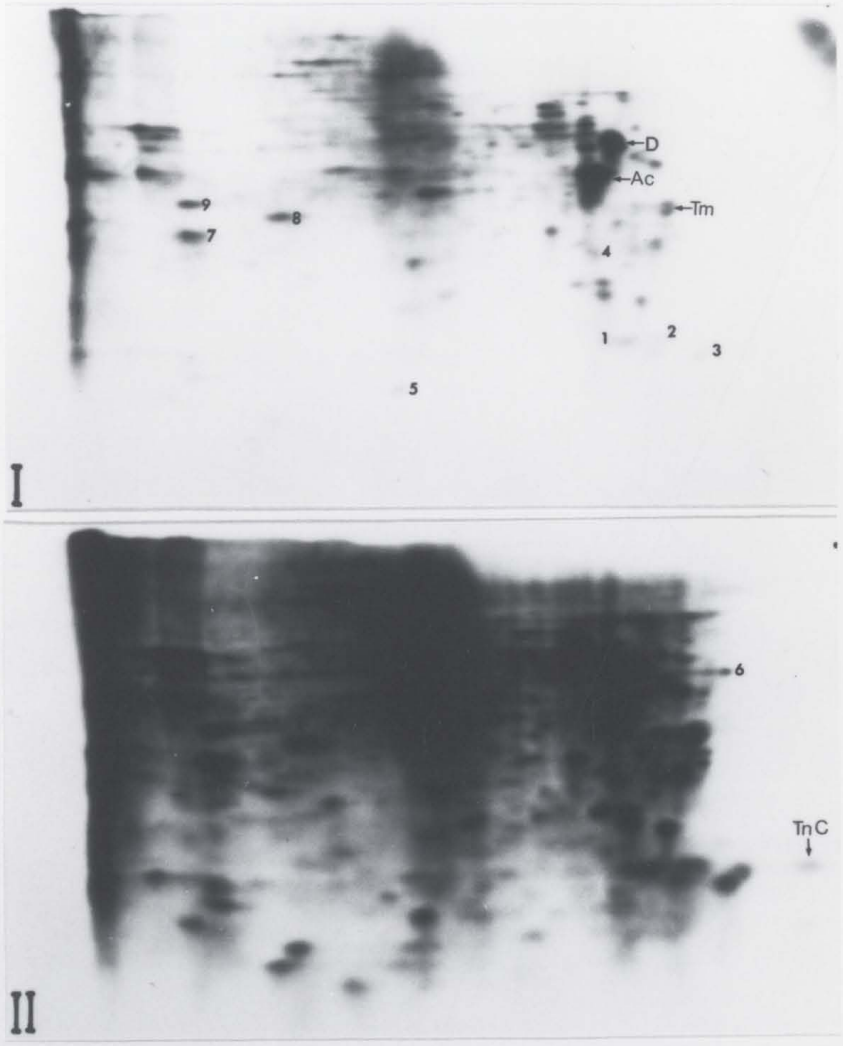
analysed by electrophoresis on two-dimensional gels (Fig. 3.5). Identification of troponin C was based on its isoelectric point, molecular weight and co-migration with bovine cardiac troponin C. Other peptides, eg. actin and tropomyosin were recognizable from previous reports (eg. Caravatti et al, 1982).

Long exposures of autoradiographs of a series of two-dimensional gels are shown in figure 3.6. The troponin C protein synthesis was highest at 36 hour after plating (Fig. 3.6A) and it decreased to a very low level at 4 days after plating (Fig. 3.6B). At 7 and 14 days after plating weak spots for troponin C were observed. In another set of experiments, troponin C synthesis was examined 20 and 48 hours post-culture. The synthesis level was high in 20 hour cultured cells but the maximum synthesis was observed in 48 hours post-culture (results not shown). The gels used to measure troponin C synthesis were reexposed for shorter time periods so that actin and tropomyosin synthesis could also be monitored (Fig. 3.7). It is evident that actin and tropomyosin synthesis levels were at a minimum at 36 hour after plating. However, unlike troponin C, the actin and tropomyosin synthesis levels increased by 7 days after plating.

To examine the regulation of actin, tropomyosin and troponin C in myocyte cultures at various times after plating, the radioactivity present in those spots corresponding to these proteins on the two dimensional gels was determined. Simple quantitation of the their synthesis, based on directly comparing the radioactivity of spots excised from the gel would have produced erroneous

Fig. 3.5. Fluorographs of 2-dimensional gels of total cellular extracts from labelled chicken myocyte cell culture.(a).

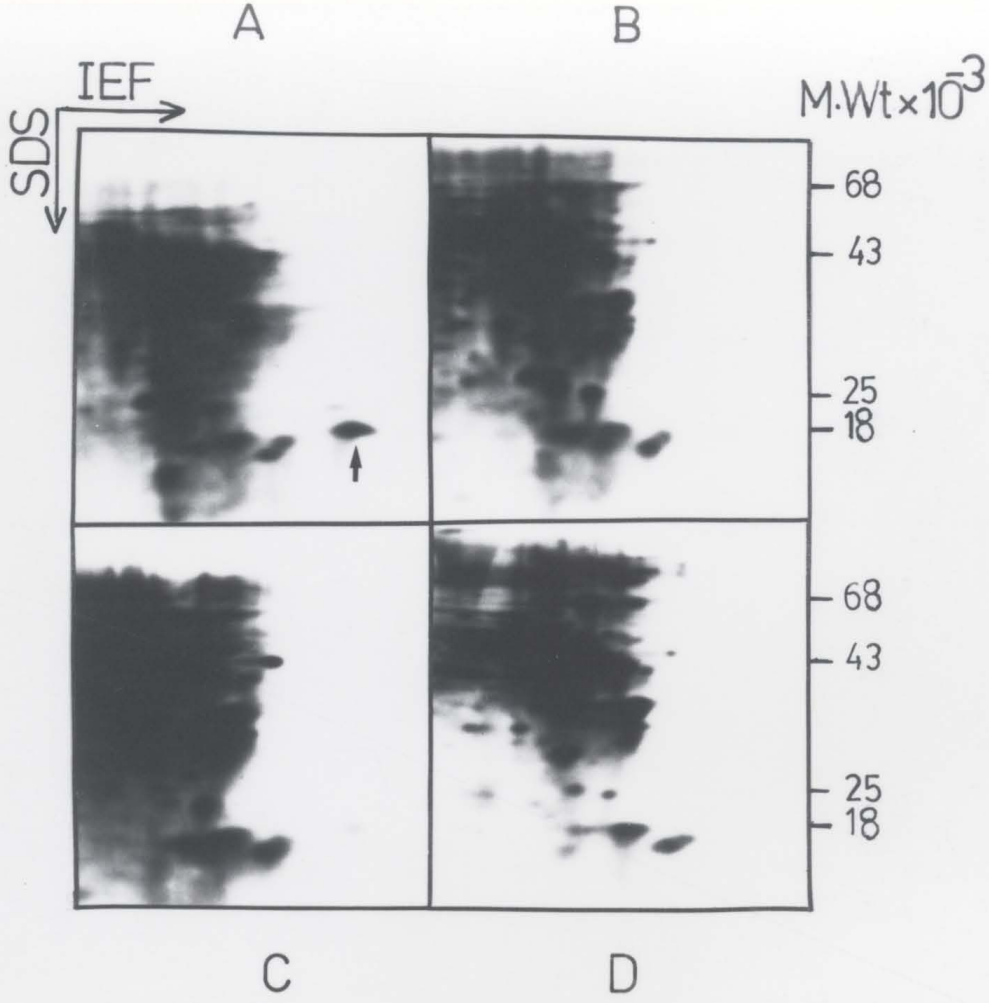
[^{35}S]-methionine labelling, preparation of total cellular extracts and 2-dimensional gel electrophoresis were performed as described in 'Materials and Methods'. 5 x 10^5 acid-precipitable counts were loaded on the isoelectric-focussing gels. I and II are the autoradiographs from the same gel with 24 h and 4 days' exposure, respectively. Ac, actin; D, desmin; Tm, tropomyosin; Tn C, troponin C; Number 1-9 indicate the spots used as internal markers for quantitation of results.



Fluorographs of 2-dimensional gels of total cellular extracts from labelled chicken myocyte cell cultures.

Fig. 3.6. Fluorographs of 2-dimensional gels of total cellular extracts from labelled chicken myocyte cell cultures.(b).

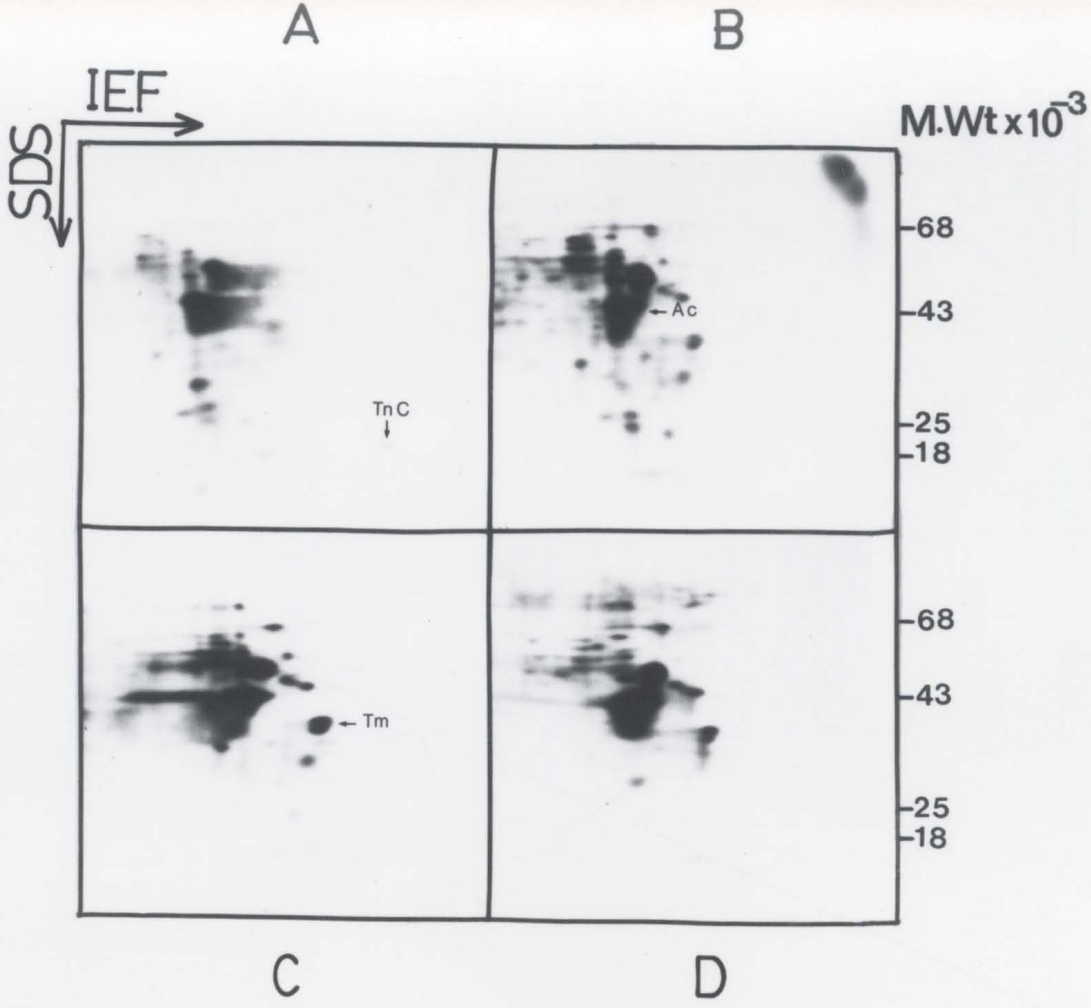
[^{35}S]-methionine labelling, preparation of total cellular extracts and 2-dimensional gel electrophoresis were performed as described in 'Materials and Methods'. For each analysis, 5 x 10^5 acid-precipitable counts were applied on isoelectric-focussing gels. Extracts from (A) 36 hour, (B) 4 day, (C) 7 day and (D) 14 day old myocyte cultures were analysed. An arrow indicates the position of troponin C. A, B and C were exposed for 4 days and D was exposed for 7 days. Only the right hand portions of the two dimensional gels are shown here.



Fluorographs of 2-dimensional gels of total cellular extracts from labelled chicken myocyte cell cultures.

Fig. 3.7. Fluorographs of 2-dimensional gels of total cellular extracts from labelled chicken myocyte cell cultures.(c).

Same as the legend to figure 5 except shorter exposure. A, B and C were exposed for 24 hours and D was exposed for 2 days. Ac, actin; Tm, tropomyosin and Tn C, troponin C.



Fluorographs of 2-dimensional gels of total cellular extracts from labelled chicken myocyte cell cultures.

results because even though equal number of acid-precipitable counts were applied to each gel, not all counts entered the gel. Therefore, it was decided to calculate the synthesis of contractile muscle proteins relative to other proteins whose levels did not significantly vary in myocyte culture during the course of the experiment. Nine random protein spots which could be easily excised without contamination from neighbouring spots, were chosen as internal markers (Fig. 3.5), and the radioactivity of those spots was measured in each gel (Table 3.2). It was expected that most, if not all of these 9 proteins would represent housekeeping proteins. Ratios of radioactivity incorporated into actin, troponin C and tropomyosin to that incorporated into each of the control spots was calculated (Table 3.3 a, b and c respectively). The normalized ratios of troponin C relative to spots 1-9 at various days of plating is shown in figure 3.8. The relative levels of troponin C to all nine spots followed similar courses; the highest troponin C synthesis measured was at 36 hour after plating, the lowest at 4 days and this was followed by an increase by 7 days. However, in 14 day old cultures, a complex pattern of the relative level of troponin C synthesis was observed.

The change in troponin C synthesis during myocyte culture was strikingly different from that observed for the other two contractile proteins actin and tropomyosin. The results present in Tables 3.3b and 3.3c show that in contrast to troponin C, both actin and tropomyosin syntheses increase for at least up to 7 days in culture. A representative of the normalized ratios of actin and

Table 3.2

Radioactivity incorporated into 2-dimensional gel polypeptide spots following different days in culture

Spot	Spot Counts (cpm)			
	Days in culture			
	1.5	4	7	14
1	89.6	150.4	114.4	59.5
2	117.0	38.7	121.4	24.4
3	43.0	60.7	68.4	48.1
4	46.4	44.7	38.5	68.6
5	154.7	78.8	102.0	35.9
6	27.7	30.5	63.2	*
7	445.2	280.5	253.3	315.1
8	579.3	225.3	135.5	346.1
9	258.3	195.2	374.4	178.7
Actin	2178.5	3130.5	12122.0	7834.2
Troponin C	230.1	20.6	148.6	62.3
Tropomyosin	177.3	469.9	1891.5	634.7
Blank	49.3	45.0	52.7	50.4

Actin, tropomyosin, troponin C and the nine control spots (Fig. 3.5) from 2-D gels of labelled cellular extracts of chicken myocyte cells of different ages were excised from gels and the radioactivity incorporated was measured in omnifluor-toluene scintillant. Blank represents background counts in each gel (it is an average of three values). In all the values shown (except blank), the background counts have already been substracted. * - not determined since the spot was not clear in the autoradiograph.

Table 3.3a

Relative levels of troponin C protein synthesis

Relative Levels				
Reference spot	Days in Culture			
	1.5	4	7	14
1	2.57	0.14	1.29	1.05
2	1.95	0.53	1.22	2.55
3	5.31	0.34	2.17	1.29
4	4.96	0.46	3.86	0.90
5	1.49	0.26	1.46	1.73
6	8.30	0.68	2.35	*
7	0.52	0.07	0.59	0.20
8	0.40	0.09	1.09	0.18
9	0.89	0.11	0.40	0.35

The ratios of radioactivity in the troponin C protein spot relative to spots 1 - 9 (from table 3.2) are shown.

Fig. 3.8. The relative level of troponin C synthesis in myocyte cell cultures of different ages.

The troponin C spots from 2-D gels were excised and the amount of radioactivity incorporated was measured. To correct for variations in the rate of protein synthesis among different cultures, the radioactivity of specific internal control spots from the same gel (Fig. 3.5.) was determined and the ratio of troponin C radioactivity to that of other spots was calculated. The data were normalized, with the lowest value in each set was considered as a one.●-●, ○-○ ,▲-▲, △-△, ⬟-⬟, ⬡-⬡,■-■, □-□, and ◨-◨ , : ratios of troponin C synthesis to those of spots 1 - 9.

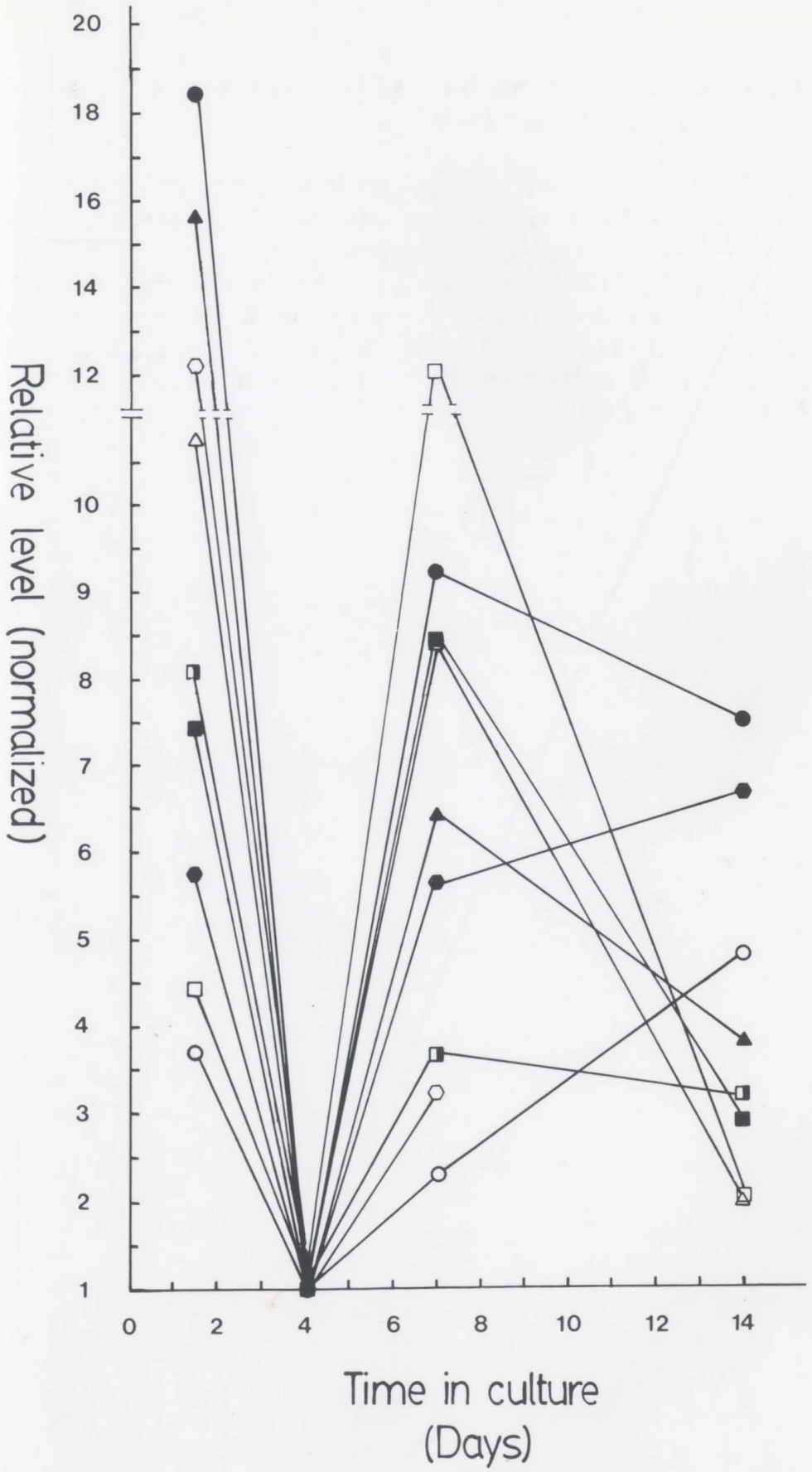

Relative level (normalized)
20
18
16
14
12
10
9
8
7
6
5
4
3
2
1
0
2
4
6
8
10
12
14
Time in culture
(Days)

Table 3.3b

Relative levels of actin protein synthesis

Relative Levels				
Reference spot	Days in Culture			
	1.5	4	7	14
1	24.31	20.80	105.96	132.30
2	18.62	80.89	99.85	321.07
3	50.30	51.57	177.22	162.87
4	46.95	70.03	314.86	114.20
5	140.80	39.73	118.84	218.20
6	78.60	102.60	191.80	*
7	4.89	11.16	47.86	24.86
8	3.76	13.90	89.46	22.60
9	8.43	16.04	32.38	43.84

The ratios of radioactivity in the actin protein spot relative to spots 1 - 9 (from table 3.2) are shown.

Table 3.3c

Relative levels of tropomyosin protein synthesis

Relative Levels				
Reference spot	Days in Culture			
	1.5	4	7	14
1	1.98	3.12	16.53	9.13
2	1.52	12.14	15.58	26.01
3	4.09	7.74	27.65	13.20
4	3.82	10.51	49.13	9.25
5	1.15	5.96	18.54	16.68
6	6.40	15.41	29.93	*
7	0.40	1.68	7.47	2.01
8	0.31	2.09	13.96	1.83
9	0.69	2.41	5.05	3.55

The ratios of radioactivity in the tropomyosin protein spot relative to spots 1 - 9 (from table 3.2) are shown.

tropomyosin syntheses relative to spots 1-9 at various days of plating is shown in figures 3.9 and 3.10. Compared to troponin C synthesis, regulation of actin and tropomyosin syntheses was quite different. The minimum synthesis observed for these proteins was at 36 hours after plating and the synthesis increased steadily (3.5-25 fold for actin and 7-45 fold for tropomyosin) up to 7 day after plating.

To further elucidate the regulation of contractile muscle proteins, the rate of synthesis of actin and tropomyosin relative to troponin C synthesis was calculated. The ratio of actin to tropomyosin synthesis was also compared (Fig. 3.11). The actin to tropomyosin synthesis ratio produced an almost linear plot over the entire 14 day time period. This suggests that actin and tropomyosin are co-regulated. The actin to troponin C ratio was at a minimum in 36 hour old cultures, increased about 16 fold in 4 day old cultures and decreased slightly in 7 day old cells. The plot of tropomyosin synthesis relative to troponin C synthesis with various days of plating followed a similar pattern, again suggesting that actin and tropomyosin are co-regulated while troponin C is regulated differently.

3.4.2. Myocyte mRNA synthesis

In order to determine if troponin C synthesis is regulated at the level of mRNA translation, the cytoplasmic troponin C mRNA levels in cardiac myocytes were measured at different days after plating. Initially, the troponin C

Fig. 3.9. The relative level of actin synthesis in myocyte cell cultures of different ages.

The same as figure 3.8., except the radioactivity in the actin spots was measured. Only plots of spots 2 (●-●), 8 (■-■) and 9 (▲-▲) are shown.

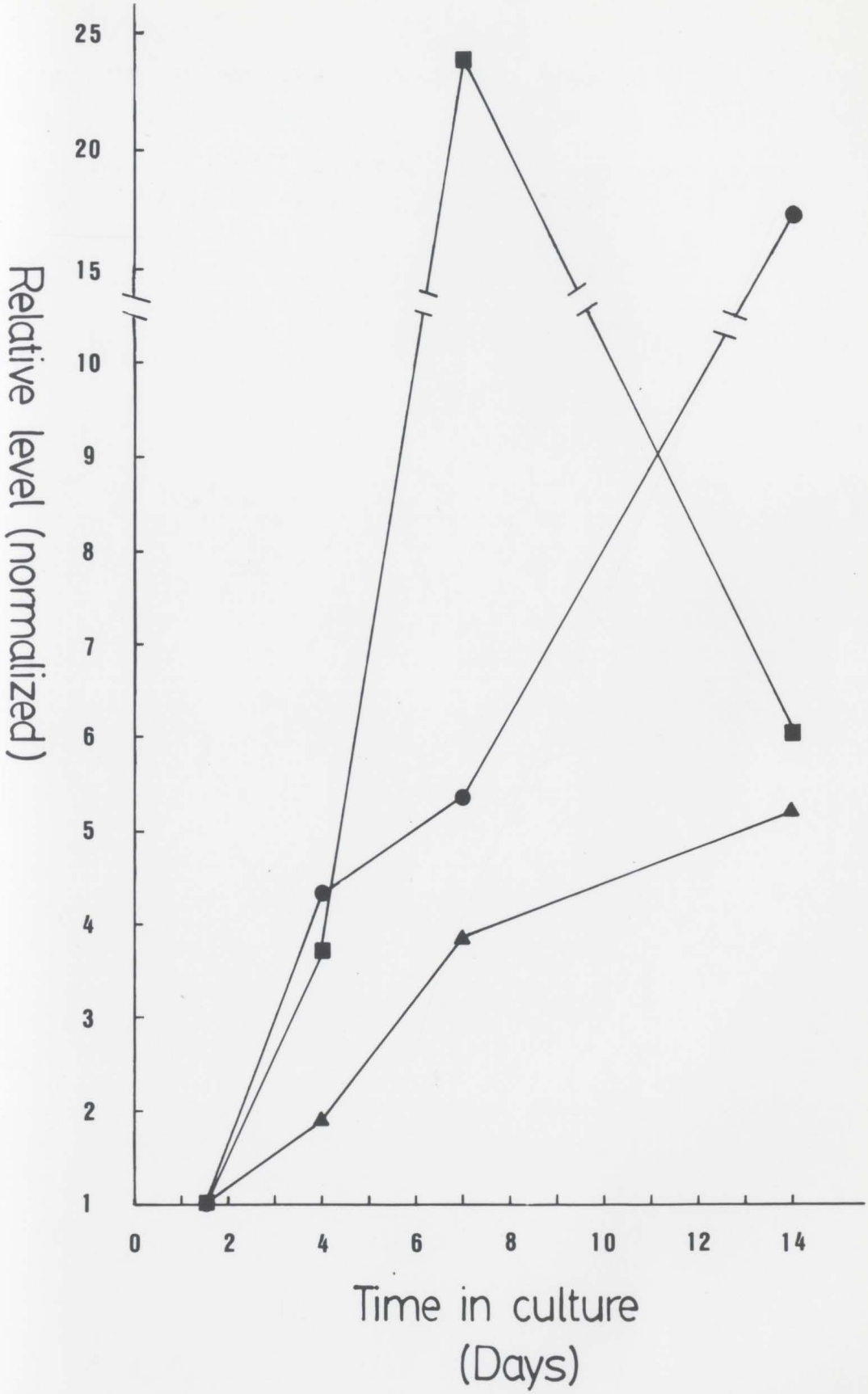

Relative level (normalized)
25
20
15
10
9
8
7
6
5
4
3
2
1
0
2
4
6
8
10
12
14
Time in culture
(Days)

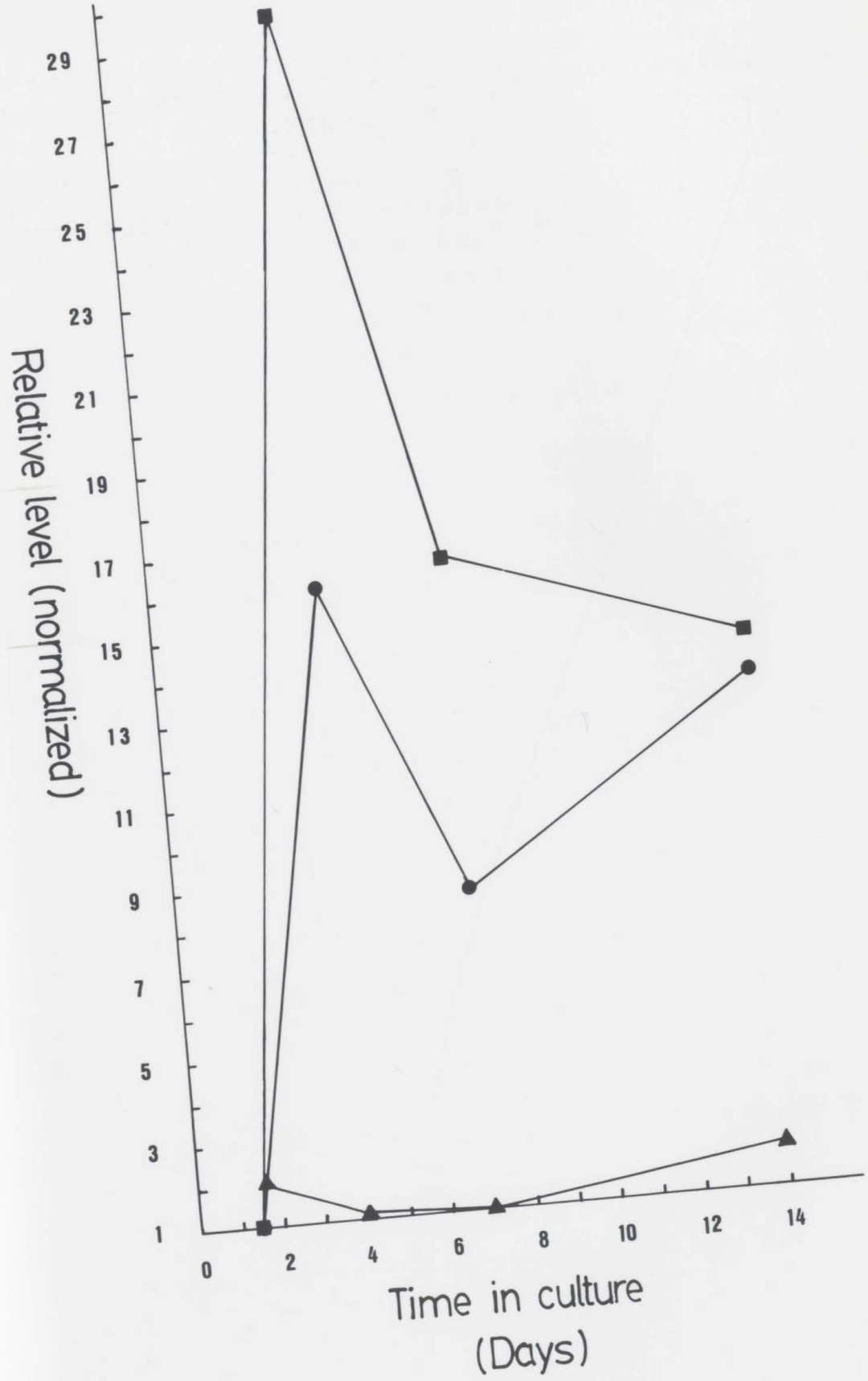
Fig. 3.10. The relative level of tropomyosin synthesis in myocyte cell cultures of different ages.

The same as figure 3.9, except radioactivity in the tropomyosin spots was measured.

45
35
25
15
10
9
8
7
6
5
4
3
2
1
Relative level (normalized)
0
2
4
6
8
10
12
14
Time in culture
(Days)

Fig. 3.11. Actin and tropomyosin protein synthesis relative to troponin C synthesis and actin synthesis relative to tropomyosin synthesis in myocyte cultures of different ages.

The radioactivity in the actin, troponin C and tropomyosin spots was measured and the ratios were calculated. Ratios of actin and tropomyosin to troponin C (●-● and ■-■ respectively) and actin to tropomyosin (▲-▲) are plotted. All ratios were normalized.


Relative level (normalized)
29
27
25
23
21
19
17
15
13
11
9
7
5
3
1
0
2
4
6
8
10
12
14
Time in culture
(Days)

mRNA levels were determined using the cCIII probe (Plasmid containing cDNA insert from quail slow troponin C gene). For this purpose equal amounts of poly(A)$^+$ mRNA, extracted from cells 36 h, 4, 7 and 14 days in culture, was slot-blotted onto nitrocellulose filters and hybridised with [^{32}P]-labelled cCIII probe. The results are shown in figure 3.12.

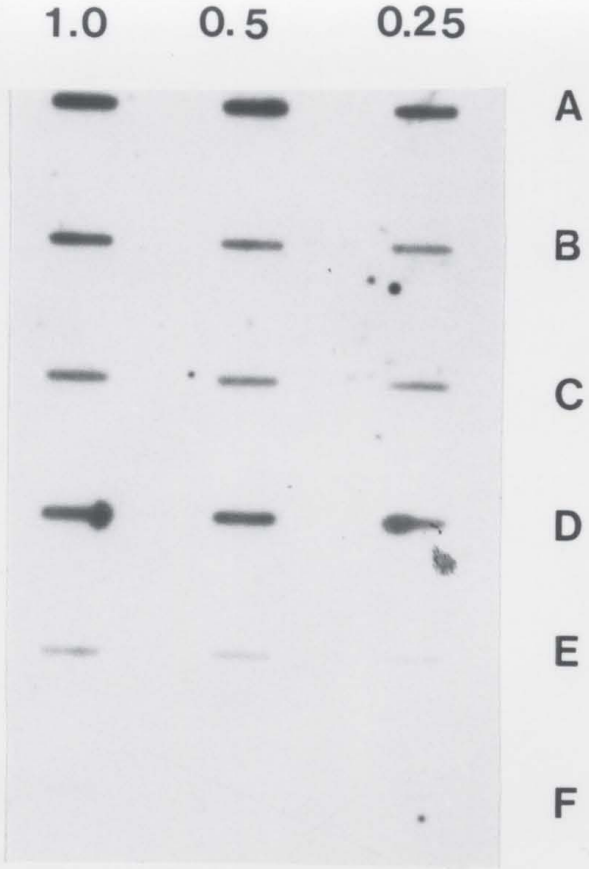
The concentration of poly(A)$^+$ mRNA used was within the linear response of the assay system used in these studies (results not shown). The maximum poly(A)$^+$ troponin C mRNA level was present in the 36 h old culture (Column A, Fig. 3.12). The level then dropped at days 4 and 7 and an approximate two fold increase (relative to days 4 and 7) was observed at day 14. Hepatocyte cells also showed the presence of a very low level of troponin C mRNA whereas equal amount of purified globin mRNA from rabbit reticulocytes (Amersham Chemicals) failed to produce any signal. Therefore, the level of non-specific hybridisation was below the level of detection under these conditions of hybridisation and washing.

3.4.3. Comparison of myocyte mRNA and protein synthesis levels

There was a significant amount of troponin C mRNA in cells from 4, 7 and 14 day old cultures. The level of troponin C mRNA in 36 hour old culture was 4.9, 5.2 and 2.0 fold higher than that present in 4, 7 and 14 day old cultures respectively. These results show that there was a large drop in the level of troponin C mRNA synthesis between 36 hour and 4 day old cultures. How-

Fig. 3.12. Slot blot hybridisation of poly(A)$^+$ RNA with cCIII.

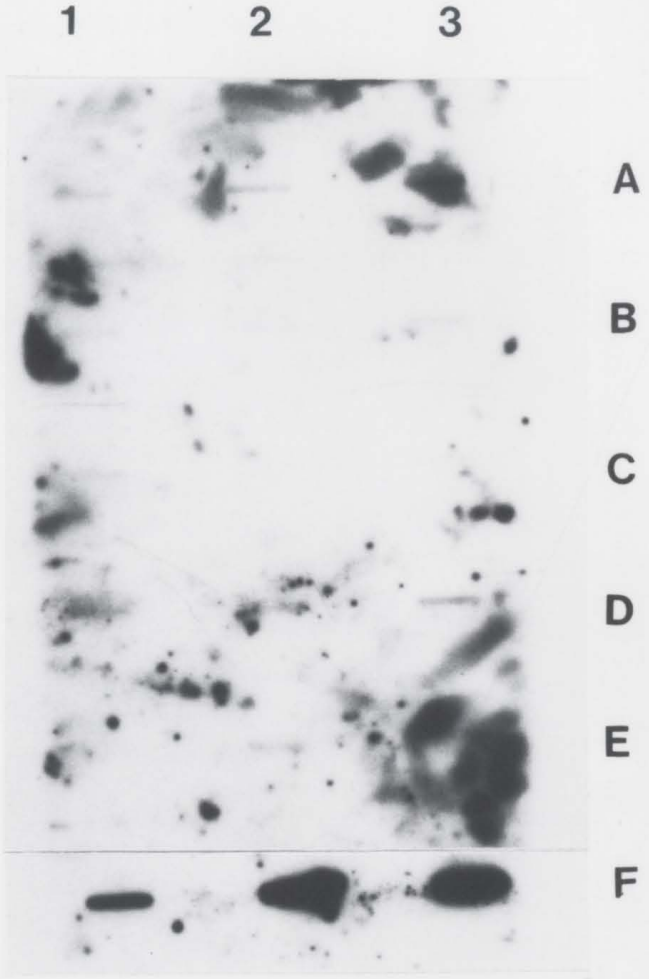
Extraction of poly(A)$^+$ RNA, slot blotting and hybridisation were performed as described in 'Materials and Methods'. 2 x 10^7 cpm of [^{32}P]-labelled cCIII probe (specific activty, 2 x 10^8 cpm/μg) was used for hybridisation. 0.25, 0.5 and 1.0 μg of poly(A)$^+$ RNA from (A) 36 hour, (B) 4 day, (C) 7 day and (D) 14 day old myocyte cultures and (E) 4 day old hepatocyte cell cultures was analysed. Rabbit β-blobin mRNA was used as a negative control (lane F). Autoradiograph following 1 day's exposure.



Slot blot hybridisation of poly(A)$^+$ RNA with cC111.

Fig. 3.13. Slot blot hybridisation of poly(A)$^-$ RNA with cCIII.

Extraction of poly(A)$^-$ RNA, slot blotting and hybridisation were performed as described in 'Materials and Methods'. 1 x 10^7 cpm of [^{32}P]-labelled cCIII (specific activity, 2 x 10^8 cpm/μg) was used for hybridisation. In lanes A - E, 10, 20 and 40 μg (1, 2, and 3 respectively) of poly(A)$^-$ RNA from (A) 36 h, (B) 4 day, (C) 7 day and (D) 14 day old myocyte cultures and in lane (E) 4 day old hepatocyte cultures and in lane (F) 25, 50 and 100 pg of cCIII plasmid was spotted as a positive control. Autoradiograph following 1 day's exposure.



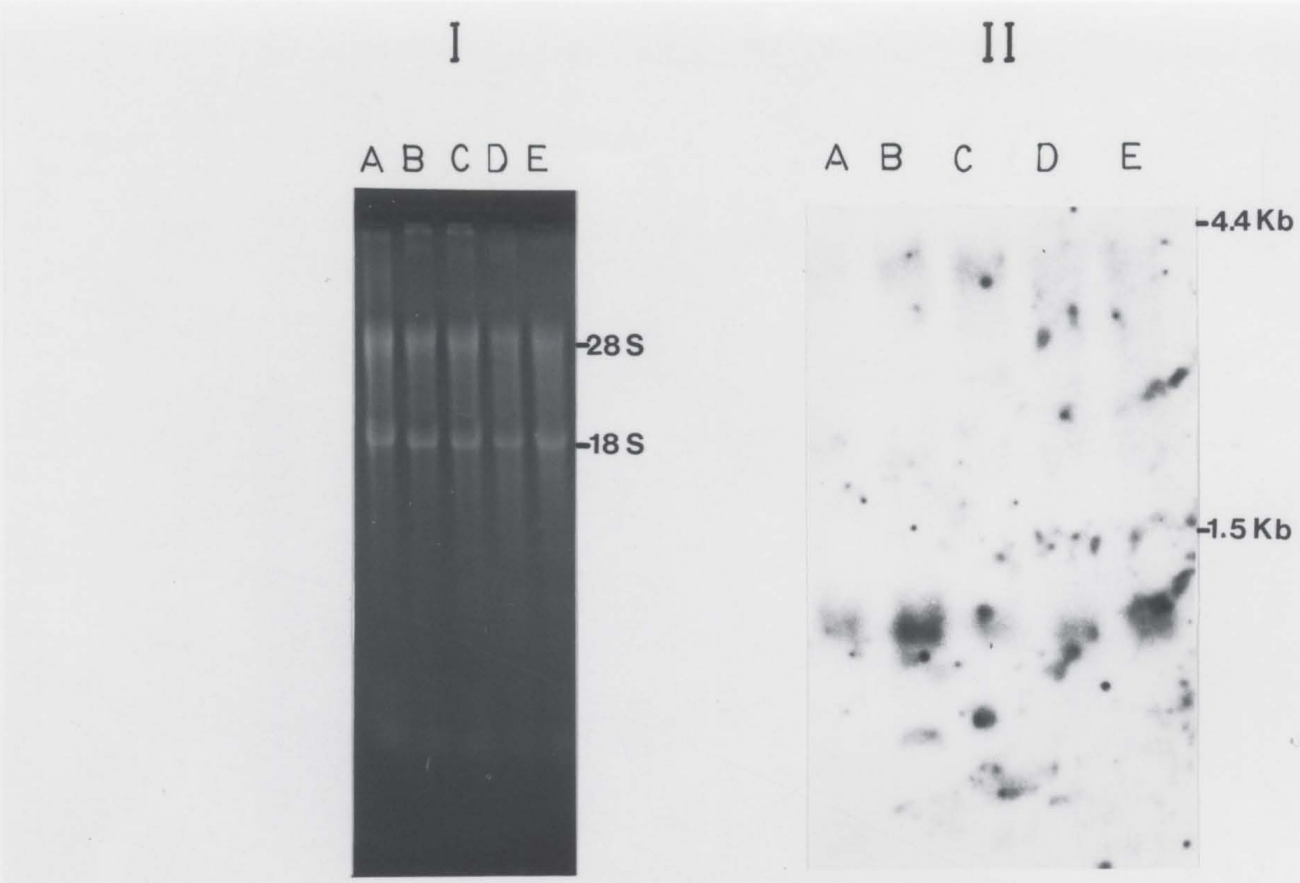
Slot blot hybridisation of poly(A)$^-$ RNA with cC111.

ever, there was a dramatic decrease in troponin C polypeptide synthesis between 36 hour and 4 days in culture compared to the decrease in mRNA level. Furthermore, even when the level of mRNA between day 4 and day 7 remained unchanged, the level of polypeptide synthesis altered during this period. These results indicate the possibility of repression of troponin C mRNA at the level of translation. In order to examine if there was a significant level of troponin C poly(A)$^-$ mRNA, I also examined the cytoplasmic poly(A)$^-$ fraction by a similar method. The results are shown in figure 3.13. These results indicate that the contribution of a poly(A)$^-$ population to the total levels of troponin C mRNA was negligible.

To test further whether the troponin C mRNA observed in 4 day and 7 day old cultures was processed in a manner different from that present in 1 or 2 day old cultures, the size and quantity of troponin C mRNA was analysed using denaturing agarose gel electrophoresis. The results of Northern blot analysis on the total cytoplasmic RNA are shown in figure 3.14. No detectable difference in the size of troponin C mRNA from cells of various ages was observed. The estimated size of troponin C mRNA was found to be approximately 1 kb in each case. In each of these lanes a high molecular weight band (approximately 4 kb) was also observed. It is not certain whether this band is an artifact of hybridisation of plasmid DNA to 28 S rRNA. By scanning the autoradiograph (Fig. 3.14) the relative levels of troponin C mRNA were calculated and plotted against time in culture (Fig. 3.15). The level of troponin C

Fig. 3.14. Northern blot analysis of troponin C coding RNA in chicken myocyte cell cultures.

Samples of total cytoplasmic RNA (25 μg/lane) was fractionated on a 1.5% formaldehyde-agarose gel. The RNA was transferred to nitrocellulose paper and hybridised with 1 x 10^7 cpm of [^{32}P]-labelled cCIII probe (specific activity 1.2 x 10^8 cpm/μg) as described under 'Materials and Methods'. In lanes A - E, RNA from (A) 1 day, (B) 2 day, (C) 4 day, (D) 7 day and (E) 14 day old cultures were analysed. I. Photograph of acridine orange stained gel, II. Autoradiograph following 1 days' exposure.



Northern blot analysis of troponin C coding RNAs in chicken myocyte cell cultures.

mRNA in the 2 day old culture was approximately 4 fold higher than that present in the 4 and 7 day old cultures and 2 times higher than that present in the 14 day old culture. These ratios are similar to those observed using the slot blot hybridisation of poly $(A)^+$ RNA with cC111 probe (Fig. 3.12). Again these results showed that the decrease in the level of troponin C polypeptide synthesis exceeded the drop in the level of troponin C mRNA (Table 3.3a), suggesting some form of repression of troponin C mRNA translation.

In addition to the troponin C mRNA, the changes in the level of α-actin mRNA was examined in cardiac myocytes at various times after plating. The results of the hybridisation of ^{32}P-labelled DNA of cC118 (plasmid containing quail skeletal α-actin cDNA insert) with total cytoplasmic RNA are shown in figure 3.16. The level of α-actin mRNA behaved in a manner similar to the troponin C mRNA. The highest α-actin mRNA level measured was at day 2 which decreased dramatically afterwards. The lowest actin polypeptide synthesis measured was, however, at day 1 and the synthesis continued to increase until at least day 7 (Table 3.2). This difference may be due to a switch to the synthesis of a different actin isoform. Since the actin probe used was only 600 nucleotides long (Hasting and Emerson, 1982), it is possible that it failed to bind to other cardiac specific actin mRNAs. From the Northern blot results it is clear that only the α-actin signal was detected.

Fig. 3.15. The relative levels of hybridisation of troponin C probe to the RNA from myocytes cells of different ages.

The relative levels of hybridisation of troponin C probe to the polyA$^+$ (●-●)and total cytoplasmic RNA (■-■) were obtained by scanning the autoradiographs (in figures 3.12 and 3.14) and calculating the area under the peaks.

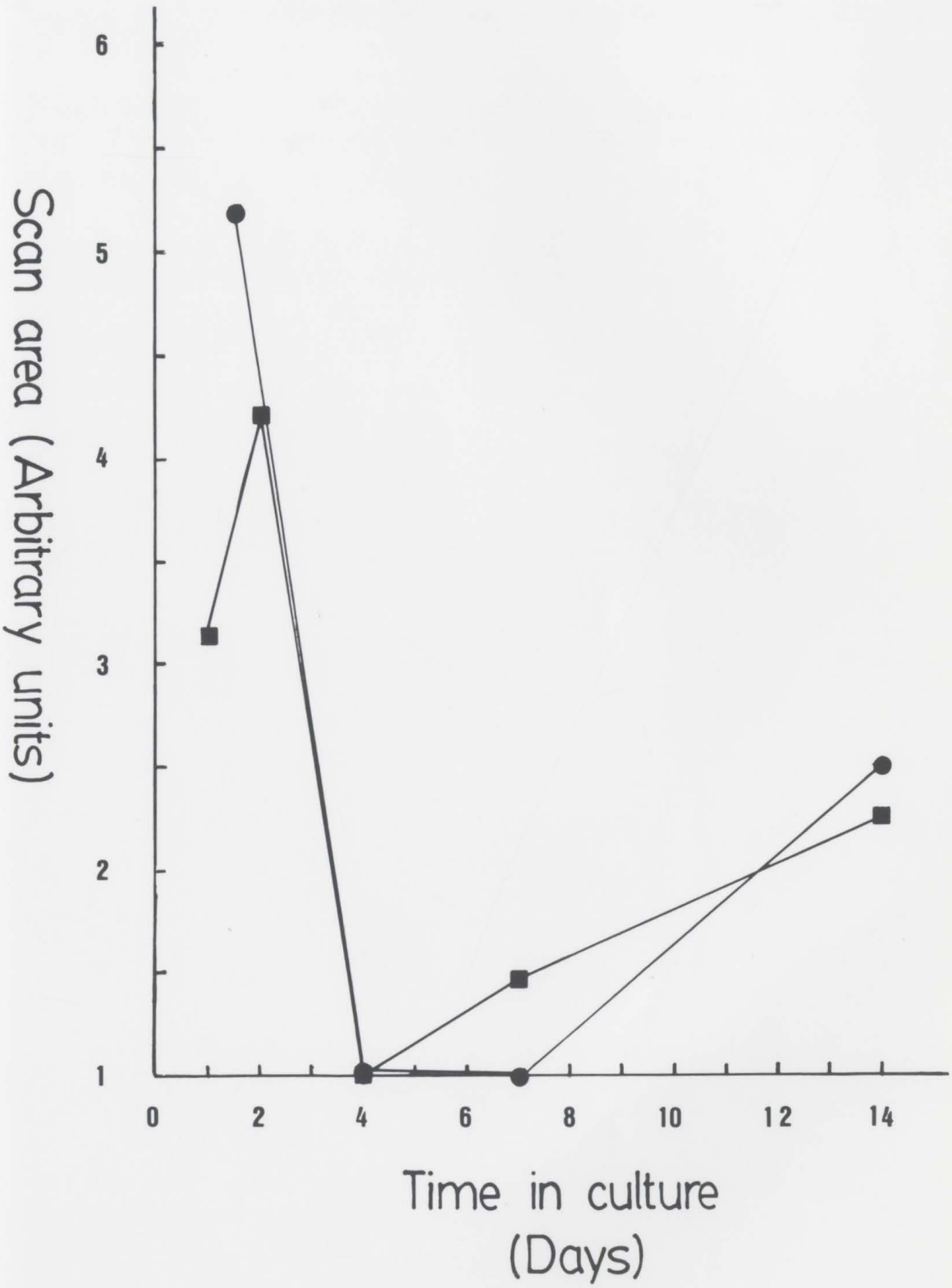
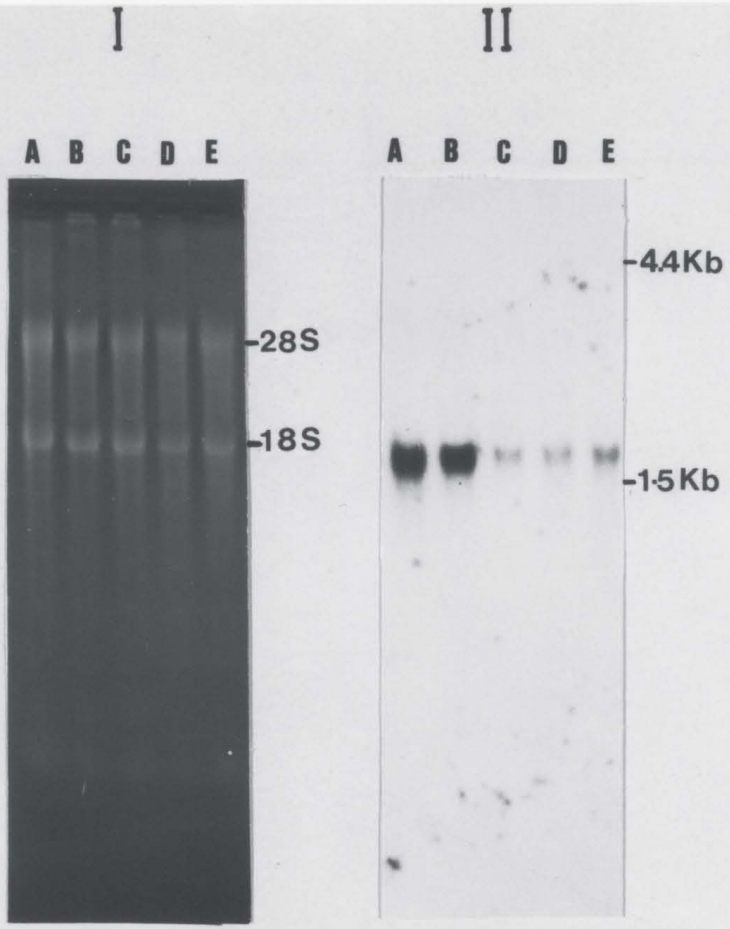

Scan area (Arbitrary units)
6
5
4
3
2
1
0
2
4
6
8
10
12
14
Time in culture
(Days)

Fig. 3.16. Northern blot analysis of α-actin coding RNAs in chicken myocyte cell cultures.

Total cytoplasmic RNA was fractionated and transferred to nitrocellulose membrane as described in the legend to figure 10. The hybridisation was carried out using 1 x 10^7 cpm of [^{32}P]-labelled cC118 probe (specific activity 1.9 x 10^8 cpm/μg). Lanes A - E : RNA from 1 day (A), 2 day (B), 4 day (C), 7 day (D), and 14 day (E) old cultures. I. Photograph of acridine orange stained gel, II. Autoradiograph following 24 hours' exposure.



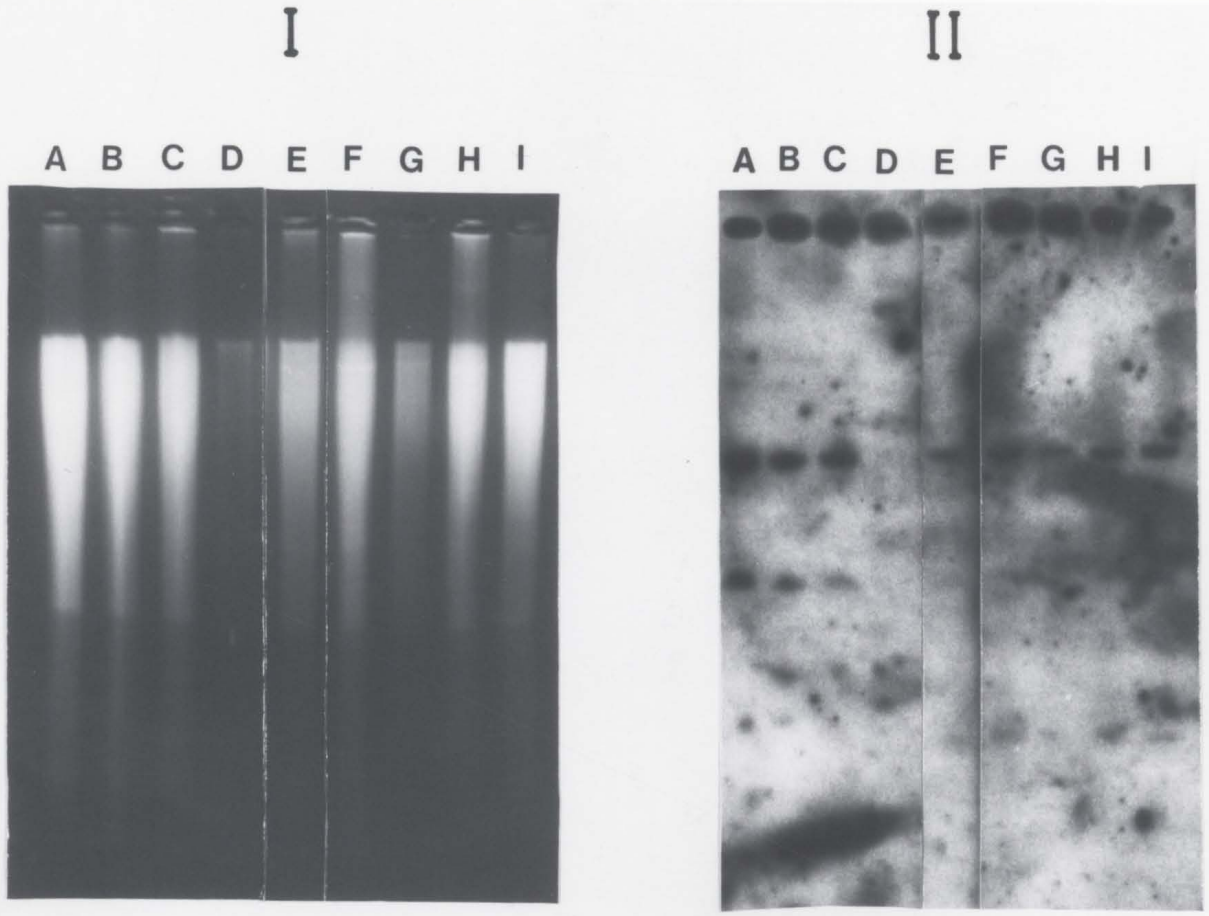
Northern blot analysis of α-actin coding RNAs in chicken myocyte cell cultures.

3.5. Association of troponin C gene with the nuclear matrix

The mechanism of tissue specific gene expression is still unclear. To gain more insight into how genes for muscle specific proteins (like troponin C) are expressed in muscle cells, troponin C gene expression in cardiac muscle cells was studied. In recent years studies from a number of laboratories (See section **1.5**) have shown a close relationship between gene expression and the association with the nuclear matrix structure. To examine whether the transcriptionally active troponin C gene of cardiac myocytes is preferentially associated with the nuclear matrix structure, nuclear matrix DNA from cardiac myocyte cultures of different ages was prepared. Hepatocyte cultures were used as a negative control. Nuclear matrix DNA was prepared by solubilising the DNA which was not anchored to the nuclear matrix by digesting with Eco RI restriction enzyme. The pelleted DNA and solublised DNAs were further digested to completion with the same enzyme to measure the level of the troponin C gene in these fractions by Southern blot hybridisation using nick-translated cCIII DNA. The results are shown in figure 3.17. As previously described, two large fragments of 4.8 and 2.3 kbp hybridised to the cCIII probe in all cases, although the 2.3 kbp band was very faint. The nuclear matrix DNA from the transcriptionally active 2 day old myocyte cultures did not show a preferential enrichment of the troponin C gene (Fig. 3.17.II lane D). Similar results were obtained when 4 and 7 days old cultures were analysed. Lane D shows 2, very

Fig. 3.17. Hybridisation of cCIII probe to the nuclear matrix and control DNAs.

A, B and C - Eco RI digested total DNA from chick myocytes, 15, 10 and 5 μg respectively.
D - 2 μg of pellet DNA from a 2 day old cardiac myocyte culture.
E - 10 μg of 30% pellet DNA from a 4 day old cardiac myocyte culture.
F - 10 μg of 34% pellet DNA from a 7 day old cardiac myocyte culture.
G - 10 μg of supernatant DNA from a 7 day old culture.
H - 10 μg of 15% pellet DNA from a chicken hepatocyte culture.
I - 10 μg of supernatant DNA from hepatocytes.
Pellet DNA refers to the DNA which remained insoluble after Eco RI restriction enzyme digestion of the DNA in the nuclear cages (nuclei devoid of histones). 5, 15, 30 and 34% pellet indicates the percentage of total nuclear DNA remaining insoluble (in the pellet fraction) following restriction enzyme digestion. The DNA from both pellet and supernatant were deproteinised and digested with Eco RI to completion before gel electrophoresis. Agarose gel electrophoresis, transfer of DNA to *Zetabind* membrane and hybridisation with 1 x 10^7 cpm of [^{32}P]-labelled cCIII DNA probe (specific activity 2 x 10^8 cpm/μg) was as described under 'Materials and Methods'. I. Photograph of ethidium bromide stained gel, II. Autoradiograph after 20 days' exposure.



Hybridisation of cC111 probe to the nuclear matrix and control DNAs.

faint bands of troponin C gene from 2 μg DNA (the bands did not reproduce in the photograph shown). Since this 2 μg DNA was derived from nearly 40 μg of total DNA (95% of the nuclear DNA was in soluble fraction), it was expected that the intensity of signal in both bands would be much stronger than that present in 5, 10 or 15 μg of total DNA (lane A, B and C) if the gene was attached to the nuclear matrix. However, only faint bands were noticed. Similarly, 10 μg of 30 and 34% pellet DNA from 4 and 7 day old cultures was equivalent to nearly 30 μg of total DNA. The intensity of the bands (lanes E and F) was lower than those with 5 μg of total DNA (lane C). Furthermore, equal amounts of supernatant DNAs from a 7 day culture produced 2 bands of low intensities (lane G). These results indicate that there is no preferential enrichment of the troponin C gene in the insoluble (or pellet) nuclear matrix associated fraction. This was more evident when nuclear matrix DNA from myocytes was compared with that from the hepatocyte cultures. Since extremely low troponin C transcript was found in the RNA preparation from liver (Fig. 3.12), the troponin C gene was considered to be inactive in liver cells. The pelleted nuclear matrix DNA from these cells showed similar intensity of both bands as nuclear matrix DNA from cardiac myocytes.

CHAPTER 4

DISCUSSION

4.1. Isolation of chicken myocyte cells

The chick myocyte cell cultures were comprised almost exclusively of myocyte cells, with less than 5% of the cells being of fibroblast origin. One cannot be certain about the absolute purity of myocyte cells since it is possible that myocyte cell aggregates may obscure the presence of fibroblasts. The physiological effect of 5-bromo 2'deoxyuridine on myocyte cells was uncertain, although it must be emphasized that to reduce undesirable effects the prolonged use of 5-bromo 2'deoxyuridine was avoided. After 24 hours of culture, the medium was replaced with the medium without 5-bromo 2'deoxyuridine.

To date, there have been no reports on the use of 14 day old chick embryonic hearts for high yields of myocyte cells. Most of the previous studies used 5-8 day old embryonic hearts (Chacko and Joseph, 1974; Clarke, 1976) because of the ease in isolating cells from young tissue, but for those studies there was no need to isolate a large number of pure myocyte cells. In the present study I found that 14 day old embryonic hearts were most suited to yield large numbers of viable myocyte cells. Under the culture conditions employed here, the myocyte cells remain viable for over three weeks and they form colonies by enlargement and possibly proliferation.

4.2. Troponin C gene copy number, restriction analysis and evolutionary conservation

The dot blot analysis indicated that the chicken cardiac troponin C gene was present in a low copy number, and there were 2.6 copies of this gene per haploid genome. This analysis, however, may not be very accurate and the real number could be from 1 to 5 genes per haploid genome. Since the restriction analysis also gave simple restriction patterns, often a single band, it is quite likely that the chicken slow troponin C gene is present as a single copy.

The presence of a single copy gene for cardiac troponin C would be an interesting observation for a muscle protein gene. Most of the contractile muscle proteins so far investigated are either members of multigene families eg. actin and myosin heavy chain (Buckingham & Minty, 1983) or their multiple isoforms are produced by differential RNA splicing eg. troponin T (Breitbart *et al.* 1985).

Troponin C belongs to a family of calcium-binding proteins. The other members of this family are calmodulin, parvalbumins and myosin light chains (Kendrik-Jones & Jakes, 1976; Means & Dedman, 1980). These proteins share a considerable degree of homology of their amino-acid sequences. However, I have always found a simple pattern of restriction fragments hybridisable to the troponin C probe. This result implies that under the hybridisation conditions used in these studies, the cCIII probe did not cross-hybridise with the other

genes of this superfamily.

We were interested to find out whether the conservation of troponin C at the protein level was reflected at the level of the structural gene(s). The results indicate that the conservation of protein observed among birds and mammals (Holroyde, Robertson, Johnson, Solaro & Potter, 1980) did not exist at the gene level. On the contrary, the quail troponin C cDNA did not share demonstratable homology with mammals, fishes, yeast or mycoplasma. Also, amongst birds, the extent of homology varied considerably. Compared to chicken and duck, pigeon and herring gull DNAs hybridised very poorly with quail cDNA. This agrees with the evolutionary relationships since quail and chicken are members of the same order (Order Galliformes) and the members of the order Anseriformes (which includes duck) and Galliformes have the same evolutionary lineage. On the other hand, Columbiformes (eg. pigeon) and Charadriiformes (herring gull) are distantly related to Galliformes which may explain the relatively poor hybridisation of the troponin C probe from quail with pigeon and herring gull DNA. Using quantitative micro-complement fixation analysis to study protein evolution in birds, Prager and Wilson (1976) also found that duck and quail are immunologically more closely related to chicken than to gull and pigeon.

In the absence of complete sequence data of the slow and fast form of troponin C genes from different species, the above studies offer only preliminary knowledge on the homology of slow troponin C in different species. For

further studies on the evolutionary conservation of the troponin C gene, the hybridisation experiments would have to be performed under conditions of various stringencies. The use of a large genomic probe would also be required to obtain a clearer picture on the evolutionary conservation of the troponin C gene.

4.3. Regulation of troponin C gene expression

The observed behaviour of troponin C protein synthesis in the cardiac myocyte cultures is quite surprising. The increase in actin and tropomyosin protein synthesis was as expected and corresponded well with an observation by Holland (1979) who used a relatively impure population of cardiac myocytes from chick embryos. Holland reported that in a cell culture of embryonic chick heart, the synthesis of a number of muscle specific proteins, eg., sarcoplasmic reticulum ATPase and myosin heavy chain were increased up to 120 hours after plating. Unfortunately, there is no other data available on the level of troponin C synthesis in chick myocyte cells. The present study is, therefore, the first to show non-coordinate regulation of contractile muscle protein synthesis in cardiac myocyte cells.

A number of studies on differentiating skeletal muscle cells in culture have unequivocally shown that the synthesis of all contractile muscle proteins is co-regulated (Devlin & Emerson, 1978). However, when one examines the other differences between cardiac and skeletal muscle differentiation, one realizes

that this break down of co-ordinate regulation in cardiac muscle cells is quite likely. One of the major differences in these two types of muscle cells is the regulation of DNA synthesis. In skeletal muscle, biochemical differentiation, i.e., the increased synthesis of contractile muscle proteins and cessation of DNA synthesis (or withdrawal from the cell cycle) is tightly coupled (Devlin, Merrifield & Konigsberg, 1982). On the other hand, Holland (1979) has shown that the cessation of DNA synthesis of embryonic chick heart cells in culture is not associated with the activation of the synthesis of muscle-specific proteins. It is, therefore, not very surprising to also find differences in the way individual muscle protein synthesis is controlled. However, it is not certain whether these results are an artifact of an *in vivo* culture environment. It is possible that the turn over rate of troponin C is highest 4 days post-culture, and is not related to the synthesis rate of troponin C. Further studies using heart muscle from developing chick embryos are necessary to resolve this issue.

In 14 day old cultures, a complex pattern of the relative level of troponin C synthesis was observed (Fig. 3.8). While this anomalous behaviour in 14 day old cultures could be due to a number of factors, two obvious possibilities are:

(i) Condition of culture - Even though the myocyte cells continue to beat after 14 days of plating, it is likely that cell aging would induce metabolic changes causing changes in the rate of synthesis of the 9 control proteins.

(ii) Presence of fibroblasts - As mentioned in Section **3.1**, myocyte cells form

clumps, and the presence of any remaining fibroblasts would be obscured by clumps. If the number of fibroblasts in a culture increases considerably, it would also produce irregular results.

The analysis of troponin C mRNA levels in cardiac myocyte cultures showed that it followed the same pattern as that of the troponin C polypeptide synthesis. The highest mRNA level was in the 2 day old cultures and the level declined dramatically in 4 day old cultures. However, the extent of change in mRNA level was less than that of the polypeptide synthesis. Compared to 2 day old cultures, there was 25% troponin C protein synthesis in 4 day old cultures. Following this drop, the troponin C protein synthesis in 7 day old culture was increased to a value of approximately 60% of the 2 day old culture. In contrast, the mRNA level was approximately 20, 20 and 50% of the 2 day maximum level in 4, 7 and 14 day old cultures respectively. These results suggest that there is some form of translational control of troponin C synthesis, most probably a repression of troponin C mRNA in 4 day old cells.

Events happening in 4 day old cultures are of particular interest. Troponin C mRNA measurements showed similar levels of mRNA in 4 and 7 day old cultures. However, analysis of troponin C polypeptide synthesis showed that troponin C synthesis was higher in seven day old cultures than that in 4 day old cultures. This implies that the troponin C synthesis was specifically repressed in 4 day old cultures and this repression of troponin C mRNA was

In contrast to troponin C, actin polypeptide synthesis and its mRNA levels appeared to have followed a different pattern. In spite of the observed increase in actin synthesis in 4, 7 and 14 day old cultures compared to 2 day old cultures, the mRNA level decreased considerably. This implies that there was a change in the efficiency of actin mRNA translation. An alternate possibility is that a new cardiac specific isoform appeared in day 4 which did not hybridise with the skeletal muscle α-actin probe used in these studies (Hastings & Emerson, 1982a). This seems a plausable explanation, since Minty *et al.* (1982) demonstrated weak hybridisation of a 1100 bp skeletal muscle actin cDNA with cardiac-specific actin mRNA. The clone used in my studies was only 600 bp long and of skeletal origin, and therefore, might not have hybridised with the cardiac actin mRNA. This probe also failed to hybridise with the rat skeletal muscle RNA and rat genomic DNA (unpublished observations). In the absence of any other data on the actin mRNA level in cardiac myocyte cultures, I believe that the second explanation for the observed decrease in actin mRNA is more likely.

4.4 Nuclear matrix and the troponin C gene

To understand the mechanism of tissue-specific expression of the troponin C gene, its association with the nuclear matrix was examined. In a number of reports, it has been claimed that actively transcribing genes are associated with the nuclear matrix structure (Berezney, 1984 and the references therein).

Only a small number of genes have been examined in these studies. In the present study, I examined whether the cardiac troponin C gene followed a similar mechanism of tissue-specific gene expression as globin, ovalbumin and heat shock genes.

Nuclear matrices were prepared by treating whole cells with a non-ionic detergent and 2 M NaCl. Nuclear matrices prepared by this method (originally developed by Cook *et al.*,1982) were more satisfactory than those prepared by a method described by Robinson, Nelkin and Vogelstein (1982). For instance, Cook's procedure required minimal handling and the histone-free DNA was protected from breakage by the nuclear cages, whereas, the method by Robinson *et al.* required treatment of isolated nuclei with a non-ionic detergent and then with 2 M NaCl. Further, the nuclear matrices so prepared by Robinson's technique were always tightly clumped and the clumps could not be disrupted without destroying the matrices.

The results presented here show that the cardiac troponin C gene was not associated with the nuclear matrix in either cardiac myocytes or hepatocytes. I found no enrichment in the nuclear matrix fraction. It could be argued that the association of troponin C gene with the nuclear matrix was not observed in our experimental system due to the following reasons:

(i) The troponin C gene is repressed in cardiac myocytes. This is unlikely, as I found that the mRNA level increases from 24 hour to 48 hour old cul-

tures (Fig. 3.16). However, as there was a significant decrease in the troponin C mRNA level in 4 day old cultures, it is possible that the preparation of nuclear matrix DNA from 48 hour old cultures actually represented the troponin C gene in a repressed state. Further studies on the transcription of the troponin C gene in cardiac myocytes would be necessary to resolve this issue.

(ii) It is possible that the choice of the particular restriction enzyme (Eco RI) might have influenced the result. Eco RI produced 2 fragments of 4.8 and 2.3 kbp, which are significantly larger than the 1 kb size of troponin C mRNA. Quail DNA also produced 2 bands of similar sizes. Since there is no Eco RI site in the 420 bp cDNA used here, this result indicates that there is possibly an intron(s) at the 3' end of the troponin C gene. It was previously reported for the ovalbumin gene that the entire transcribed region shows preferential attachment to the nuclear matrix (Ciejek *et al.* 1983). Therefore, it is unlikely that the absence of preferrential attachment of the troponin C gene to the nuclear matrix was due to limitations imposed by the restriction enzyme or the particular probe. However, recently, Cockerill and Garrard (1986) have mapped the matrix associated region within the kappa immunoglobulin gene. It is an approximately 600 bp A-T rich region and is located about 200 bp upstream of the tissue-specific enhancer which is 5' to the gene. Also, Mirkovitch *et al* (1984) found the matrix associated site for the hsp 70 heat shock genes to be

upstream of the 5' regulatory elements. Hence, further studies with a full size genomic probe are required to resolve the question of preferential attachment of the troponin C gene to the nuclear matrix.

SUMMARY

This is the first comprehensive report on troponin C protein synthesis, its mRNA levels and its gene organisation. The main objective of this project was to examine how expression of the troponin C gene is regulated in cardiac myocyte cells. The following conclusions can be drawn from the studies described in this thesis:

1. In chicken, the slow troponin C gene is possibly present as a single copy .
2. Although troponin C is a conserved protein, this conservation does not extend to the gene level, as observed by Southern blot hybridisations.
3. The correlation between troponin C synthesis and its cytoplasmic mRNA level indicate that troponin C is regulated mainly at the transcription level. However, there are indications of translational control of troponin C synthesis in 4 day old myocyte cultures.
4. In chicken cardiac myocyte cultures contractile muscle proteins are not co-ordinately regulated since actin and tropomyosin protein synthesis follow a very different pattern from that of troponin C synthesis.
5. The 3' end of the troponin C gene is not preferentially enriched in the nuclear matrix fractions of actively transcribing myocyte cells.

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